An Investigation of the Genetic Control of Biofilm Formation in Bacteria (*E. coli* K-12 MG1655)

By

Mohd Adnan, M. Sc., MSB

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Collaborating Establishment: Department of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal, 4710-057

BISMILLAHIR-RAHMANIR-RAHIM

“In the name of God, the Most Gracious, the Most Merciful”

Dedication

This thesis is dedicated to my father Ehsanul Haque and my mother Jahan Ara, for without their constant support, endless love and encouragement this thesis would never have been completed.

Contents

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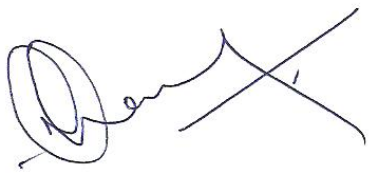
Signature of Candidate 

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Abstract

The ability to adapt to changing environments is essential for survival. Bacteria have developed sophisticated means by which they sense and respond to stresses imposed by changes in the environment. *Escherichia coli (E. coli)* have served as a model organism for studies in molecular genetics and physiology since the 1960s. I have undertaken this study and address three outstanding questions. Firstly, the involvement of morphogene *bolA* and RNA polymerase sigma factor (*rpoS)* in biofilm formation. Secondly, the effect on respiratory activity of *E. coli* in presence and absence of these two genes and thirdly, the adherence pattern and formation of biofilm by *E. coli* on stainless steel, polypropylene and silicon surfaces under various stress-induced conditions.

Bacterial biofilms are structural assemblages of microbial cells that encase themselves in a protective self produced matrix and irreversibly attach to surface. Their intense resistance to antibiotic and various environmental stresses has implicated them as playing a possible role in the pathogenesis of many chronic diseases. Although, the role of *rpoS* and *bolA* genes in long term stationary phase growth conditions and their response to it is now well-known, their objective presence and importance in short term response to different environmental cues which may lead to biofilm formation remains unknown.

The *rpoS* gene encodes a stationary phase specific sigma factor of RNA polymerase and is a key regulator of *E. coli* stationary phase responses. It has been experiential under laboratory conditions that gene expression is induced by stressful environmental conditions and certain metabolic intermediates. Various

stress environments were employed both in planktonic and biofilm phases to examine the sudden response of *rpoS* against different environmental conditions. However, it was observed that sudden *rpoS* response varies from stress to stress conditions. The gene *bolA* has been shown to trigger the formation of round cells when over expressed in stationary phase. From this research, it is concluded that *bolA* is not only confined to stationary phase, it also involves in biofilm formation under stress environments and essential for normal cell morphology. It also plays a major role in respiration and attachment of *E. coli* under diverse environmental stress surroundings.

The main objective of this study was to understand the impact of heat, cold, acid and hydrogen peroxide on *E. coli* K-12 MG1655 and its stress response in presence and absence of *rpoS* and *bolA* genes. *E. coli* cells were exposed to sub-lethal levels of each stress for 15 minutes in both planktonic and biofilm phases and post-stress response i.e. gene expression level was evaluated. A real-time reverse transcription polymerase chain reaction (RT-PCR) assay, using the Applied Biosystems 7500™ real-time cycler, was developed for the purpose of this investigation of *rpoS* and *bolA* genes transcription. The assay was used specifically to quantify *rpoS* and *bolA* mRNA levels; however the method can readily be applied to the study of other *E. coli* genes. The method was uniquely applied to the investigation of these two genes throughout the growth cycle of *E. coli* in planktonic and biofilm phase in LB broth, in order to ascertain the patterns of expression for these genes. Scanning electron microscopy (SEM) was used for direct examining the cell attachment and biofilm formation on various surfaces under different stress conditions.

In summary, this thesis embodies research investigating the role of *rpoS* and *bolA* genes in *E. coli* K-12 MG1655 biofilm formation and provides further evidence, that bacterial biofilms play a major role in resistance to various environmental cues.

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Abbreviations

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
A_{420}	absorbance at 420 nm
A_{600}	absorbance at 600 nm
AGE	agarose gel electrophoresis
bp	base pair
cDNA	complementary DNA
c.f.u.	colony forming units
C_T	threshold cycle
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
et al.	*et alia*
EtOH	ethanol
g	gram
gDNA	genomic DNA
h	hour(s)
kb	kilobase
l	litre
LB	Luria-Bertani
M	Molar
$MgCl_2$	Magnesium Chloride
µg	microgram

mg	milligram
min	minute(s)
µl	microlitre
ml	millilitre
µM	micomolar
mM	millimolar
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger RNA
NA	nutrient agar
NB	nutrient broth
ng	nanogram
nm	nanometre
PCR	polymerase chain reaction
qPCR	quantitative PCR
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	reverse transcription
RT PCR	Real-Time PCR
σ	sigma factor
sec	second(s)
TE	tris EDTA
T_m	melting temperature
UV	ultra violet

Chapter 1

Introduction

This chapter reviews current research on the features of biofilm formation and new methodologies for biofilms control with a special focus on genomic study of biofilms.

1.1 Background

Most bacteria live attached to surfaces as sessile communities often referred to as biofilms. Biofilms are ubiquitous, and exist wherever surface contact is available in naturally occurring fluids. These biofilms cause serious health problems in medical, ecological and industrial settings including living tissues, indwelling medical devices, industrial portable water system piping, or natural aquatic systems (Hall-Stoodley et al. 2004).

Biofilms are pervasive and problematic because they are more resistant to antibiotics, hydrodynamic shear forces, UV light, and chemical biocides; increased rates of genetic exchange, altered biodegradability and increased secondary metabolite production, than their planktonic counterparts. Food and food processing sources can also be contaminated by biofilm of various bacteria (Harvey et al. 2007). It is still difficult to understand mechanisms of biofilm formation as biofilms in the environment and industrial settings are heterogeneous and being composed of complex microbial communities.

It has been estimated that 65% of infections are biofilm associated (Costerton et al. 1999;Mah and O'Toole 2001). Reduced susceptibility of the biofilm bacteria to antimicrobial agents is a vital problem in the treatment of chronic infections (Costerton et al. 1999;Mah and O'Toole 2001). The single species biofilms exist in a variety of infections and on the surface of indwelling medical implants. The mechanism of biofilm formation can be better understood at the molecular level by studying single species biofilms under controlled conditions.

Recently, research on genetic control of biofilm formation has gained importance. Various intrinsic properties within bacterial biofilms indicate that gene expression is

different to that in their planktonic counterparts and numerous genes have been proposed to be important in biofilm formation (Beloin et al. 2006;Richmond et al. 1999). Vast arrays of genes are implicated in biofilm formation. Two of the possibly important genes are *rpoS* (RNA polymerase sigma factor) and morphogene *bolA*. *RpoS* is a sigma subunit of RNA polymerase in *E. coli* that is induced and can replace vegetative sigma factor *rpoD* to some extent, under several stress conditions. As a consequence, transcription of numerous σ^S – dependent genes is activated.

Morphogene *bolA* was first described to be involved in adaptation to the stationary growth phase (Santos et al. 1999). However its function is still not fully understood. Its expression might be induced by different forms of stresses which results in the high level of expression of *bolA* mRNA and the formation of biofilms. It also has a major effect on the bacterial envelope and therefore, may be implicated in cellular protection under adverse growth conditions. Even though the significance of the *rpoS* gene in biofilm development has been suggested, the role of *rpoS* and *bolA* gene in the formation of biofilm and its expression under different types of stresses has not been investigated.

1.2 Literature Review

1.2.1 Historical perspectives

At the end of the 19th century the role of microbes in diseases was documented. It was recognised that bacteria grow attached to surfaces and embedded in a self produced extracellular matrix, referred to as biofilms (Latasa et al. 2006). Research on biofilms has come a long way since the first characterization by Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces over three centuries ago in his seminal studies of dental plaque (which he

called ‘scurf’) and was accredited with the discovery of microbial biofilms (Sauer 2003). It was observed that bacterial growth and activity were substantially enhanced by the incorporation of any surface to which these organisms could attach (Heukelekian and Heller 1940;Zobell 1943). However the detail examination needed the high resolution photo microscopy which can give apparent results at much higher magnifications.

Scanning (SEM) and transmission electron microscopy (TEM) have been used to examine biofilms (Jones et al. 1969). Two major developments in the last decade have dramatically changed our understanding of biofilms, firstly the utilization of the confocal laser scanning microscopy for the characterization of biofilm structure and secondly the investigation of the genes involved in biofilm formation. A variety of sophisticated molecular and microscopic approaches have been used to interpret biofilm development. It has been discovered that different, unrelated bacteria produce the same exopolysaccharides (cellulose, poly-β-1,6-N-acetylglucosamine) to build the biofilm matrix and the same secondary messenger, c-di-GMP (cyclic diguanosine monophosphate), an important bacterial signalling molecule, to regulate the production of biofilm matrix (Latasa et al. 2006).

1.3 Biofilm Lifecycle

In 1974 Dr. Bill Costerton coined the term “biofilm” which is now defined as; “a community of microorganisms encased within a secreted exopolysaccharide (EPS) matrix and attached to a surface”. Biofilm formation is considered to be an alternative “way of life” for microbial cells as opposed to the historically alleged notion that cells grow and exist only in a planktonic or single cell state

(Costerton 1995). Five main steps in biofilm lifecycle have been recognised through proteomic studies (Fig . 1.1) (Sauer et al. 2002):

1. **Reversible attachment**

During this stage microbial cells become reversibly associated with a surface and exhibit species specific behaviour such as rolling, creeping, aggregate formation (Stoodley et al. 2002).

2. **Irreversible attachment**

This stage employs molecularly mediated binding between microbes and the surface, many of which are regulated at the transcriptional level. This permits the rapid transition between planktonic and sessile forms depending on environmental factors. One such example is the polysaccharide intercellular adhesion (PIA) that mediates the cell-cell interactions in some staphylococcal biofilms (Jefferson 2004;Timmerman et al. 1991). At the conclusion of this stage, biofilm attachment is considered irreversible making these structures extremely difficult to remove without chemical intervention and considerable mechanical force.

3/4. Aggregation and Maturation

During these stages, the surface bound organisms begin to replicate which increases the overall density and complexity of the biofilms. Genetic and proteomic studies have shown that in this stage, biofilm bacteria have radically different levels of genetic and protein expression compared to their planktonic counterparts (Stoodley et al. 2002).

5. **Detachment**

When biofilms reach their critical mass as determined by numerous conditions, such as the availability and perfusion limit of nutrients and wastes, the peripheral layer of growth begins to re-differentiate into planktonic organisms (McDougald et al. 2012).

There is recent evidence to suggest that all these stages of biofilm formation and development may be regulated by genes that respond to population density (Davies et al. 1998).

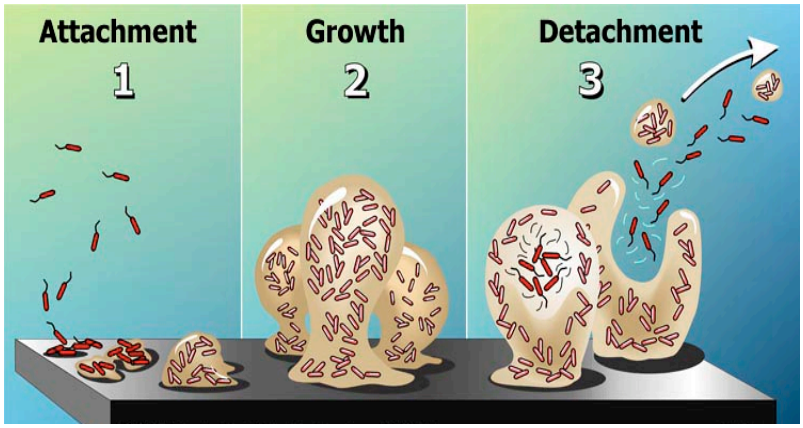


Figure 1.1: Biofilm life cycle in three steps (Adapted from Centre for Biofilm Engineering image library, Montana State University).

1.3.1 Ultrastucture of biofilm and function

In the past, microbiologists believed that biofilms contained disorderly clumps of bacteria situated in no particular structure or pattern. New techniques to magnify biofilms without destroying the gel-like structures have enabled researchers to determine the complex arrangement of biofilms (Flemming and Wingender 2010). The structures which make up a biofilm are mainly composed of microbial cells, EPS and canals through which nutrients circulate (Leriche et al. 2000). Confocal scanning laser microscope (CSLM) has revealed the three-dimensional structure of biofilms (Costerton 1995;Hall-Stoodley and Stoodley 2002). Biofilms formed by single species or mixed species demonstrate similar structural characteristics (Costerton 1995;Danese et al. 2000b). The microcolonies, which constitute the biofilm, are mainly composed of single species population or multimember

communities of bacteria, depending on the environmental parameters under which they are formed. Certain conditions, such as surface and interface properties, nutrient availability, composition of microbial community and hydrodynamics can have an effect on the structure of biofilm (Purevdorj et al. 2002).

Biofilms have also been examined under a variety of hydrodynamic situations such as laminar and turbulent flows and it was shown that biofilm structures are distorted in response to flow conditions. In Biofilms grown under laminar flow, aggregates detached by interstitial voids (Purevdorj et al. 2002). Biofilms grown in the turbulent flow cells were also unstable, but "streamers" that stretched out and oscillated in the bulk fluid were observed (Fig. 1.2). Now it can be simply said that biofilm development is polymorphic and structurally adapted to changes in nutrient availability.

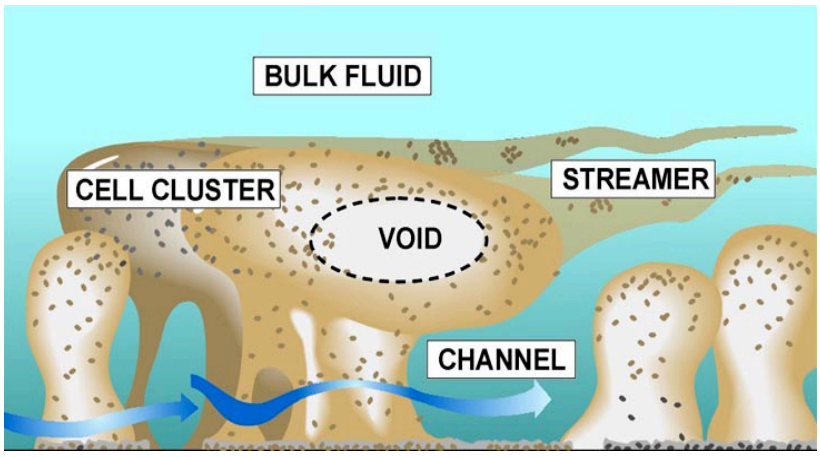


Figure 1.2: Heterogeneous structure of biofilm which includes cell cluster, void, channel and streamer (Adapted from Centre for Biofilm Engineering image library, Montana State University).

Structural organization is a property of biofilm communities that differentiates this unusual mode of growth from conventional forms. Interstitial voids or channels are

an integral part of the biofilm structure. The channels seem to be the linkage of the system as they supply a means of nutrient transportation as well as exchanging metabolic products. For example, in situ measurements of dissolved oxygen using microelectrodes, revealed that oxygen was available in the biofilms which shows that channels transfer the oxygenated bulk fluid throughout the biofilm (Costerton 1995;Lewandowski et al. 1993). Also in situ measurements of toluene degradation in a multispecies biofilm indicated that toluene was available to cells in deep within the biofilm. It showed that the channel were a vital part of the biofilm structure and function and therefore there should be a molecular mechanism for the formation as well as the maintenance of these structures (Moller et al. 1996). This is a key area for future investigations.

Biofilms provide an ideal home for microbes to exchange their extra chromosomal DNA (plasmids) by the mechanism called conjugation. Since the majority of bacteria in natural settings reside within biofilms, it follows that conjugation is a possible mechanism by which bacteria in biofilm transfer genes within or between populations. Biofilm formation has been related with conjugative plasmids (Ghigo 2001). The presence of plasmids might induce biofilm formation and the high cell densities in biofilms support higher rates of horizontal transfer of plasmid DNA. The main reason for enhanced conjugation is that the biofilm environment provides least shear and closer cell-cell contact (Ghigo 2001;Jefferson 2004).

1.3.2 Detailed mechanism of biofilm development

The development of the three-dimensional structures inherent within biofilms is a vigorous process and involves a coordinated series of molecular events that include mechanisms for adhesion, aggregation and community expansion as described above

(O'Toole et al. 2000). Adhesion is the first essential step in bacterial colonization on a surface. Different structures such as flagella, fimbriae, outer membrane proteins (OMPs), curli (a proteinaceous surface structure) and EPS are involved in biofilm formation (Danese et al. 2000a). Most bacteria are able to express several adhesins that confer specific recognition and attachment to a various range of molecules on target surfaces, ranging from surface components of tissue or cell surfaces to surfaces of abiotic materials, such as glass and plastic (Prigent-Combaret et al. 2000;Timmerman et al. 1991).

In general bacterial adhesins are organised as thin, thread like organelles referred to as fimbriae, example type IV pili in *Pseudomonas aeruginosa*, thin aggregative fimbriae (SEF17) in *Salmonella enteritidis*, type I pili and curli in *E .coli*, the autolysin At1E and SSP adhesions in *Staphylococcus epidermidis*, (Di Martino et al. 2003;Pratt and Kolter 1998;Prigent-Combaret et al. 2000;Timmerman et al. 1991). These structures have distinct roles in different species and under different environmental conditions (Richter et al. 1999).

Flagellar motility is imperative for bacteria to overcome the forces which drive back bacteria from reaching many abiotic surfaces. Once reaches the surface, the non flagellar appendages other than those involved in transfer of viral or bacterial nucleic acids (called pili), OMPs and curli are then required or activated to attain stable cell to cell and cell to surface attachment (Pratt and Kolter 1998). The expression of various bacterial adhesins is phase variable (i.e. reversible switching between expressing and non expressing states) and can also be affected by environmental conditions. Motile bacteria can swim along a chemical concentration gradient towards a higher concentration of a nutrient. The movement of organisms in

response to a chemical (nutrient) gradient is called Chemotaxis. *Pseudomonas aeruginosa* is one of the motile bacteria which uses a flagellum to shift toward higher nutrient concentrations. Motility and chemotaxis allow movement across the target surface to sites of increased nutrient availability (Hall-Stoodley and Stoodley 2002;Sauer et al. 2002).

The attachment of bacteria to surface often results in the propagation into more complex microcolony structures and this process is facilitated by autoaggregation factors. For example, in *E. coli,* a number of factors including curli, Antigen 43 (Ag43), and fimbriae have been implicated in autoaggregation and microcolony formation (Hasman et al. 2000). Cell-to-cell signalling mechanisms that observe population density play an important role in prevailing community structure (Prigent-Combaret et al. 2000;Prigent-Combaret et al. 2001). A metabolic interaction between different organisms helps in microcolony expansion by permitting organisms to co-exist in a co-operative symbiotic manner. It was found that transfer constitutive IncF plasmids induce *E. coli* biofilm expansion which results in a structure resembling those reported for *Pseudomonas aeruginosa* (Reisner et al. 2006a;Reisner et al. 2006b).

The formation of the characterized and defined biofilm architecture is an important step in biofilm development (Flemming and Wingender 2010). Several techniques have been utilized to analyze the architecture of biofilm, e.g. a mutant of *P. aeruginosa* unable to synthesize the key quorum-sensing molecules acylhomoserine lactones (acyl-HSLs), was used to develop a biofilm and the architecture was shown to be drastically altered (Gonz+ílez Barrios et al. 2006;Kjelleberg and Molin 2002). Quorum sensing circuits control a variety of physiological functions in bacteria these

include motility, conjugation, competence, sporulation, virulence and biofilm formation. In addition to this some bacteria respond to multiple autoinducer signals, this allows them to differentiate between species in their natural niche (Gonz+ílez Barrios et al. 2006;Kjelleberg and Molin 2002).

The environmental factors that control the transition from planktonic state to biofilm state vary greatly among organisms, for example some strains of *E. coli* K-12 cannot form biofilms in minimal medium unless supplemented with amino acids (Pratt and Kolter 1998), whilst *P. aeruginosa* can form biofilms under the majority of conditions that support growth. *E. coli* O157:H7 has been reported to build a biofilm only under low-nutrient conditions (Dewanti and Wong 1995). Environmental signals that can initiate attachment are osmolarity, iron availability, pH, temperature and oxygen tension (Pratt and Kolter 1998). Although the information about the environmental signals triggering biofilm development may vary from organism to organism, it is understandable that environmental parameters have a profound impact on the transition of bacteria from planktonic form of growth to biofilm growth.After attachment to surface, bacteria undergo further adaptation to form a biofilm and various features emerge to create a defensive environment and cause biofilm to be a tenacious clinical problem (Table 1.1). Biofilm bacteria may also develop other properties, including increased resistance to UV light, altered biodegradative capability, increased rates of genetic exchange, and increased secondary metabolite production (Moller et al. 1996;Zobell 1943).

Table 1.1 Effects associated to extracellular polysaccharide (EPS) matrix formation in biofilms (Laspidou and Rittmann 2002).

Function	Relevance
Adhesion to surfaces	Initial step in the colonization of inert and tissue surface, accumulation of bacteria on nutrient-rich surfaces in oligotrophic environments
Aggregation of bacterial cells, formation of biofilms	Bridging between cells and inorganic particles trapped from the environment, immobilization of mixed bacterial populations, basis for development of high cell densities, generation of a medium for communication processes, cause for biofouling and biocorrosion events
Cell-cell recognition	Symbiotic relationships with plants or animals, initiation of pathogenic processes
Enzymatic activities	Digestion of exogenous macromolecules for nutrient acquisition, release of biofilm cells by degradation of structural EPS of the biofilm
Interaction of polysaccharides with enzymes	Accumulation/retention and stabilization of secreted enzymes
Protective barrier	Resistance to non-specific and specific host defences, resistance to biocides
Sorption of exogenous organic compounds	Scavenging and accumulation of nutrients from the environment
Sorption of inorganic ions	Accumulation of toxic metal ions, promotion of polysaccharide gel formation and mineral formation
Structural elements of biofilms	Mediation of mechanical stability of biofilms, determination of the shape of EPS structure

1.4 Significance of Biofilms

Every material that comes into contact with naturally occurring fluids is vulnerable to bacterial colonization. Environmental microbiologists have long known that composite bacterial communities are accountable for driving the biogeochemical cycle that maintains the biosphere (Makin and Beveridge 1996). Industrial pipelines, nuclear power stations, air conditions systems, water distribution systems and the hospital, are all susceptible to colonization by microorganisms growing in biofilms (Table 1.2) (Hall-Stoodley et al. 2004). The majority of them persist attached to surface within a arrangement and not as free floating organisms (Costerton 1995). Biofilms comprise of single or multiple microbial species and can form on a range of surfaces. Although mixed-species biofilms predominate in most environments; but single species biofilms exist in a variety of infections and on the surface of medical implants (Costerton et al. 1999).

Bacteria seem to instigate biofilm development in response to specific environmental cues. Although these conditions vary widely, the Gram-negative organisms with some exclusion undergo a shift from free living, planktonic cells to sessile form in response to a nutrient rich medium. These biofilms continues to extend as long as fresh nutrients are provided, but when they are nutrient deprived, they detach from the surface and return to planktonic mode of growth (Kolter et al. 1993). Most likely, this starvation response allows the cells to search for a fresh source of nutrients and this adaptation that bacteria undergo when nutrient become inadequate is well studied. It is remarkable that most microorganisms seems able to make the transition to life on a surface, irrespective of their physiological capabilities (Costerton 1995).

Table 1.2: Below table shows the detrimental effects of biofilm in various industrial and medical settings.

System	Effects
Food processing	Contamination
Secondary oil recovery	Plugging of water injection wells corrosion
Cooling water towers and heat exchangers	Energy losses due to increased fluid frictional and heat transfer resistances
Drinking water distribution	Increased suspended solids; coliform contamination
Process equipment	Biodeterioration
Medical implants, catheters	Persistent infections
Metalworking	Degradation of metal working fluid
Swimming pools	Health risks; cosmetic degradation
Clean surfaces (health care, consumer)	Health risks; cosmetic degradation
Ship hulls	Increased frictional drag
Dental plaque	Caries; periodontal disease
Paper manufacture	Degradation of product quality

1.4.1 Bacterial biofilm associated infections

It was recognised early on, that biofilm bacteria can withstand disinfection processes and can be up to 1000 fold more resistant to antibiotic treatment than planktonic bacteria, but the mechanism by which the biofilm bacteria attain this resistance is still unknown (Gilbert et al. 1997). There are multiple mechanisms of resistance within biofilm by microorganisms which are likely to be considered include (Fig. 1.3):

1. Phenotypic changes in bacteria.
2. Inactivation of the antibiotics by extracellular polymers or modifying enzymes.
3. Nutrient limitation resulting in slowed growth rate.

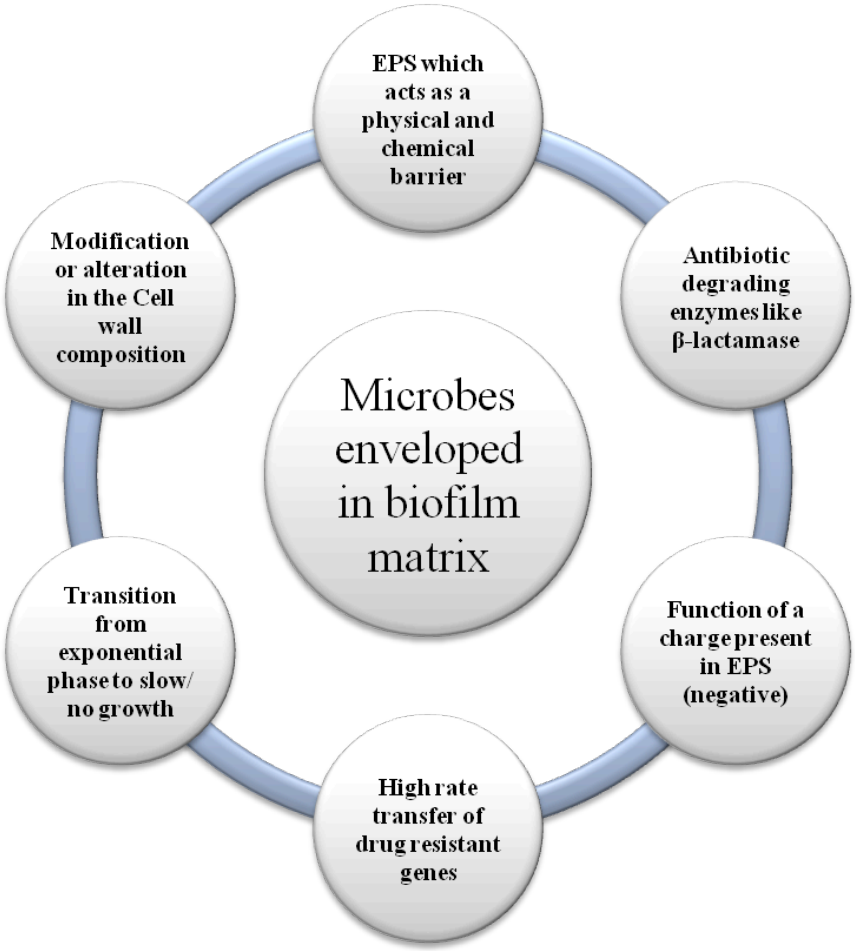


Figure 1.3: Diagrammatic representation of multiple mechanisms of resistance within biofilm by microorganisms.

Biofilm infections are hardly ever resolved by the host's immune system. Biofilm bacteria stimulate the production of antibodies by releasing the antigens, yet bacteria residing in biofilms are resistant to these resistance mechanisms (Costerton et al. 1999). In fact, this immune response may even cause harm to the surrounding tissues. Therefore, an improved understanding of biofilm formation is essential to develop novel strategies for dealing with these infections. The role of biofilms in the contamination of medical implants has been well known. Early electron microscopy studies of medical implants shown signs of bacteria residing in biofilms on those abiotic surfaces (Curtis Nickel et al. 1989) and contact lenses (Gorlin et al. 1996).

The function of biofilms in non-implant diseases is less well recognized. One example of a disease where biofilms plays an imperative role is the occurrence of lung infections by *Pseudomonas aeruginosa* in patients of cystic fibrosis (CF). An additional example of a possible biofilm mediated infection is chronic ear infection otitis media. These infections are frequently caused by biofilm forming bacteria (Dingman et al. 1998). Periodontitis is another instance of a biofilm mediated infection that results in chronic inflammation of the tissue supporting the gums and can ultimately lead to tooth loss. One of the most studied biofilm communities is dental plaque (Lamont and Jenkinson 1998). This system is predominantly complex because it consists of hundreds of bacterial species, and new species are still being isolated, including known bacterial pathogens not typically associated with the oral activity (Kolenbrander et al. 1993).

1.4.2 Mechanisms of biofilm resistance to antimicrobial agents

There is no answer to the question of why and how bacteria growing in a biofilm develop increased resistance to antimicrobial agents. The key components of biofilms is the surrounding extrapolymeric substance and the best studied of these components is EPS. Bacteria experience a certain degree of protection and homeostasis when resides within a biofilm. Exopolysaccharide synthesis is often associated with the formation of complex three-dimensional structure and depth and probably enhances resistance against anti bacterial agents. Most bacteria are capable of producing polysaccharides, either as extracellular excretions into the surrounding environment or as wall polysaccharides (capsules). EPS plays a variety of roles in the structure and function of different biofilm communities. EPS plays a essential role in both structure and formation of sludge granules, and is clearly an important part of the structural organization of biofilms

(Veiga et al. 1997). EPS has also been shown to adsorb dissolved organic compounds, such as diclofop methyl (an herbicide) and other xenobiotics, from the bulk fluid, thereby providing a method by which the bacterial community can concentrate essential nutrients and growth components (Wolfaardt et al. 1998). The matrix of EPS also has the potential to physically block the entry of certain antimicrobial agents into the biofilms.

1.4.3 Resistance and the extracellular polymeric matrix

The extracellular polymeric matrix, prevents the access of antimicrobial agents to the cells embedded in the biofilm community (Mah and O'Toole 2001). The presence of a charged, hydrated exopolymer matrix around individual cells and micro-colonies adversely affects the entry of antimicrobial agents. By a combination of ionic interaction and molecular sieving events, restricted dispersion from the surrounding medium may occur for appropriate classes of molecules (Costerton et al. 1987). The constituents of the biofilm matrix can act as an ion exchange resin and actively remove strongly charged molecules (Gilbert et al. 1997). Total diffusion failure will only occur when the reaction sites are sufficient to diminish the bulk concentration of the antimicrobial agent, or replacement of the matrix proceeds at a faster rate than does adsorption/reaction and diffusion (Allison et al. 1990;Cloete 2003;Gilbert et al. 1997). Studies on diffusion limitation have generally focused on antibiotics rather than biocides and upon medically relevant biofilm populations rather than biofilms in industrial situations (Stewart 1996).

In addition to the possibility of the biofilm matrix to react directly and chemically quench reactive moieties, retention of enzymes with the capability

to inactivate antimicrobial agents within the biofilm matrix will increase its barrier properties with respect to the diffusion of appropriate substrates (Heinzel 1998).

1.4.4 Resistance associated with growth rate and nutrient availability

Starvation in a bacterial cell for a particular nutrient results in slow growth. Transition from the exponential to stationary phase of growth is generally accompanied by an increase in resistance to antimicrobial agents (Lewis 2001). Because cells growing in biofilms are likely to experience some kind of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents (Cloete 2003;Mah and O'Toole 2001). When comparison was made between the resistance of planktonic and biofilm cells at different stages during exponential growth up to the entry into stationary phase, it was found that resistance increased when the planktonic and biofilm cells approached to stationary phase. The maximal resistance by both form of cells occurred in stationary phase where the biofilm cells were nearly 15 times more resistant than the planktonic cells. These results suggested that some determinant other than growth rate is responsible for a certain level of resistance and slow growth adds extra protection (Fux et al. 2005).

Oxygen gradients within the biofilm may also directly influence the activity of some antibacterial agents (Gilbert et al. 1997). Another phenomenon associated with biofilm is the existence of physiological gradients across biofilms on growth and metabolism of cells at the periphery to consume nutrients before they permeate to the more deeply placed cells. The peripheral cells will have

growth rates and nutrient profiles that are similar to those of planktonic cells, allowing for the existence of heterogeneity within biofilm. Advances in technology have resulted in the ability to visualize the heterogeneity within a biofilm (Fux et al. 2005).

The environmental heterogeneity that exists within a biofilm might promote the formation of a heterogeneous population of cells, such different levels of resistance can be expressed throughout the community (Fux et al. 2005). So, a major contributor towards the inefficacy of antimicrobial treatments when applied to biofilms must, therefore, be associated with physiological heterogeneity (Allison et al. 1990;Mah and O'Toole 2001).

1.4.5 Resistance associated with the adoption of resistance phenotypes

Bacteria can sense the closeness of a surface. This up-regulates the production of EPS and rapidly adjusts their susceptibility to antimicrobial agents after binding. In some instances, a 3 to 5 fold decrease in susceptibility occurs instantly on attachment. This occurs in the presence of antimicrobial agents above the minimum inhibitory concentration for planktonic cells (Fux et al. 2005;Gilbert et al. 1997). Immediately after bacterial attachment and before biofilm formation the extent of the decrease in susceptibility observed, which is generally far less than that observed in mature biofilms and is insufficient to account for the reported levels of resistance in biofilm communities (Gilbert et al. 1997).

The microorganisms undergo physiological changes that act to protect the cell from various environmental factors. Therefore, the cells are protected from the

unfavourable conditions such as heat shock, cold shock, changes in pH and many chemical agents. Nevertheless, the physiological changes begin when cells attach to a surface, by expressing a biofilm phenotype that can confer resistance face to stress environmental conditions (Mah and O'Toole 2001). This type of resistant phenotype might be induced by certain types of stress, nutrient limitation, high cell density, efflux of the treatment agent or a combination of these phenomena (Mah and O'Toole 2001).

It has been suggested that regulation of EPS, under the control of signal molecules such as *N*-acyl homoserine lactones, is responsible for the early transcriptional events associated with biofilm formation (Davies et al. 1998). Such global regulators are responsive to increases in cell density, beyond critical threshold values, and may be general regulators of biofilm specific physiology (Davies et al. 1998). In biofilms, signal molecules would become concentrated within the geometric centre of biofilm, thereby increasing EPS production. This would alter the distribution and density of cells throughout the matrix and confer some level of structural organization upon the community to provide customized microniche at various points within the biofilm (Gilbert et al. 1997).

Sub-lethal concentrations of antimicrobial agents might act as inducers/transcriptional activators of more tolerant phenotypes, such as those expressing the multidrug resistance operons and efflux pumps in *E. coli* (Ma et al. 1993). A new hypothesis for the substantial recalcitrance of biofilm relates to the potential of damaged bacterial cells to undergo apoptosis or programmed cell death. Death of cells, following treatment with antimicrobial agents results

not from direct action of the agent but from a programmed suicide mechanism and cellular lysis (Lewis 2001). Following the absence of an adverse condition, the damaged persistent cells would grow rapidly in the presence of nutrients released from their lysed community partners and the community would become restored. These cells would survive treatment phases and proliferate in the post-treatment phase, thereby stimulating considerable recalcitrance upon the biofilm community.

1.5 Genomics Study of *E. coli* Biofilms

Gene expression profiling has eased the efforts to understand the genetically programmed process of biofilm formation. It is noticeable that the adaptation of bacterial cells from planktonic to sessile form involves a highly complex regulatory process. This affects the expression of diverse groups of genes. For example changes in the expression levels of about 38% of *E. coli* genes can occur during the conversion from the planktonic to biofilm form of growth (Tenorio et al. 2003).

Gene expression in *E. coli* biofilms has been studied during sessile growth and compared to that occurring during planktonic growth (Ulett et al. 2006). It has emerged that the genes encoding proteins involved particularly in adhesion (type 1 fimbriae) and autoaggregation (Antigen 43) were highly expressed in the adhered population in a way that was consistent with current models of sessile community development.

Several novel gene clusters were induced upon the transition of bacterial cells from planktonic growth to biofilm growth. These included genes which express under oxygen-limiting conditions, genes encoding putative transport proteins, putative oxido-reductases and genes associated with enhanced heavy metal resistance. It is

possible that genes which are induced by stresses relevant to biofilm growth such as nutrient limitation and oxygen, may be the main factors that trigger enhanced resistance mechanisms in sessile communities to antibiotics and hydrodynamic shear forces (Ulett et al. 2006).

It is still difficult to know whether the transcription factors which regulate gene during the planktonic phase are the same transcription factors which regulate genes in biofilm mode (Danese et al. 2001;Lazazzera 2005). Increased expression of some genes has been shown in biofilms. Particular genes involved in adhesion, autoaggregation, several encoding structural proteins like *OmpC, OmpF, OmpT, lpxC, slp. OmpC and slp* are associated with the initial steps of biofilm formation by *E. coli* on abiotic surfaces (Sauer 2003;Whiteley et al. 2001).

1.9% of the *E. coli* K-12 genome is significantly differentially expressed in the biofilm phase when compared to the exponential phase. The genes induced in these conditions correspond to stress response as well as energy production, envelope biogenesis and unknown functions. This provides the evidence that the expression of stress envelope response genes, such as the *psp* operon or elements of the *cpx* and *rpoE* pathway, is a general feature of *E. coli* mature biofilms (Beloin and Ghigo 2005).

Using gene disruption of 54 of the most commonly occurring biofilm-induced genes, it has been shown that 20 of these genes were required for the formation of a mature biofilm (Beloin and Ghigo 2005). 11 genes of previously unknown function were found in this group. These results constituted a broad analysis of the global transcriptionally response triggered in mature *E. coli* biofilms and provided insights into its physiological signature (Beloin and Ghigo 2005).

Little is known about the genes involved in maintaining biofilms when compared to the genes involved in cell attachment and biofilm development. However, this is important as several infections are strongly associated with the antibiotic resistance of mature biofilms and major changes in the pattern of gene expression occur during biofilm development (Potera 1999). Different cell functions are more obviously expressed in sessile bacteria including the colanic acid exopolysaccharide (*wca* locus, formerly called *cps*), tripeptidase T (*pepT*), the OmpC porin, the high-affinity transport system of glycine betaine (encoded by the *proU* operon) and the nickel high-affinity transport system (*nikA*). Such a genetic reprogramming of gene expression in biofilms seems to result from changes in multiple environmental physico-chemical conditions (Prigent-Combaret et al. 1999).

The application of DNA microarray technology has been used to determine the genes which are controlled by a particular transcription factor or by any environmental signal. One major problem in DNA microarray analysis is the fact that the biofilm might comprise of mixed genera (Lazazzera 2005). Biofilm formation seems to be a programmed developmental process, similar to sporulation in *B.subtilis* and the formation of fruiting bodies in *Myxococcus xanthus*. If we take the example of development of sporulation in *B.subtilis*, by the means of global gene expression profiling it is very clear that it needs a unique set of genes and transcription factors to control these genes however DNA microarray analysis does not appear to identify unique set of genes and transcription factors which influence biofilm formation (Danese et al. 2001;Sauer 2003). Researchers have used DNA microarrays to study the gene expression in *E. coli* biofilms and have compared them with the expression in planktonic cells of the stationary phase, the results show of an overall change of more than 600 genes, which indicates around 9% of the whole genome being

activated and 4.5% repressed in the biofilm cells (Sauer 2003). Specifically in *E. coli* and other enteric bacteria, σ^S (*rpoS*) is known to be the master regulator of the general stress response in planktonic phase (Sauer 2003). However its role in biofilm under exponential phase has not been studied.

1.5.1 Alternative sigma factor *rpoS*

RpoS (RNA polymerase sigma factor) has been biochemically confirmed to be an alternate sigma transcription factor (Loewen et al. 1998). Its synthesis is controlled by an indeterminate mechanism. σ^S controls many important genes that are expressed in response to environmental stresses specially nutrient deficiency. Differential expression of subfamilies of genes within the regulon is affected by additional regulatory factors, working both individually and in combination to modulate activity of different σ^S-dependent promoters (Hengge-Aronis 1996;Loewen et al. 1998).

Under stress conditions, microorganisms have their own signal transduction systems to sense the stresses and to control their coordinated genetic response (Sauer 2003). A common regulatory mechanism involves sigma factors (*rpoS*) which is a small protein that binds to the RNA polymerase. The core RNA polymerase has 5 subunits (α_1, α_2, β, β' and ω), but to bind to promoter specific regions, the core enzyme needs another subunit called sigma factor (σ). Its presence allows the complete holoenzyme to bind to the specific promoter region and initiate the transcription of particular genes (Hengge-Aronis 1996). This reduces the attachment of RNA polymerase to non specific regions. *RpoS*, the σ^S subunit of RNA polymerase, is one of the most important proteins which play a key role in biofilm formation.

It has been known that in *E. coli*, the transcription factor σ^S, encoded by *rpoS* controls the expression of many genes involved in cellular responses to a various number of stresses, including starvation, osmotic stress, acid/heat/cold shock and oxidative DNA damage. It is also considered as a master regulator of general stress response in *E. coli* (Hengge-Aronis 1996). Further it was shown that deletion of *rpoS* makes *E. coli* unable to establish a sessile community. A diagram of general stress response in *E. coli* is described below (Fig. 1.4).

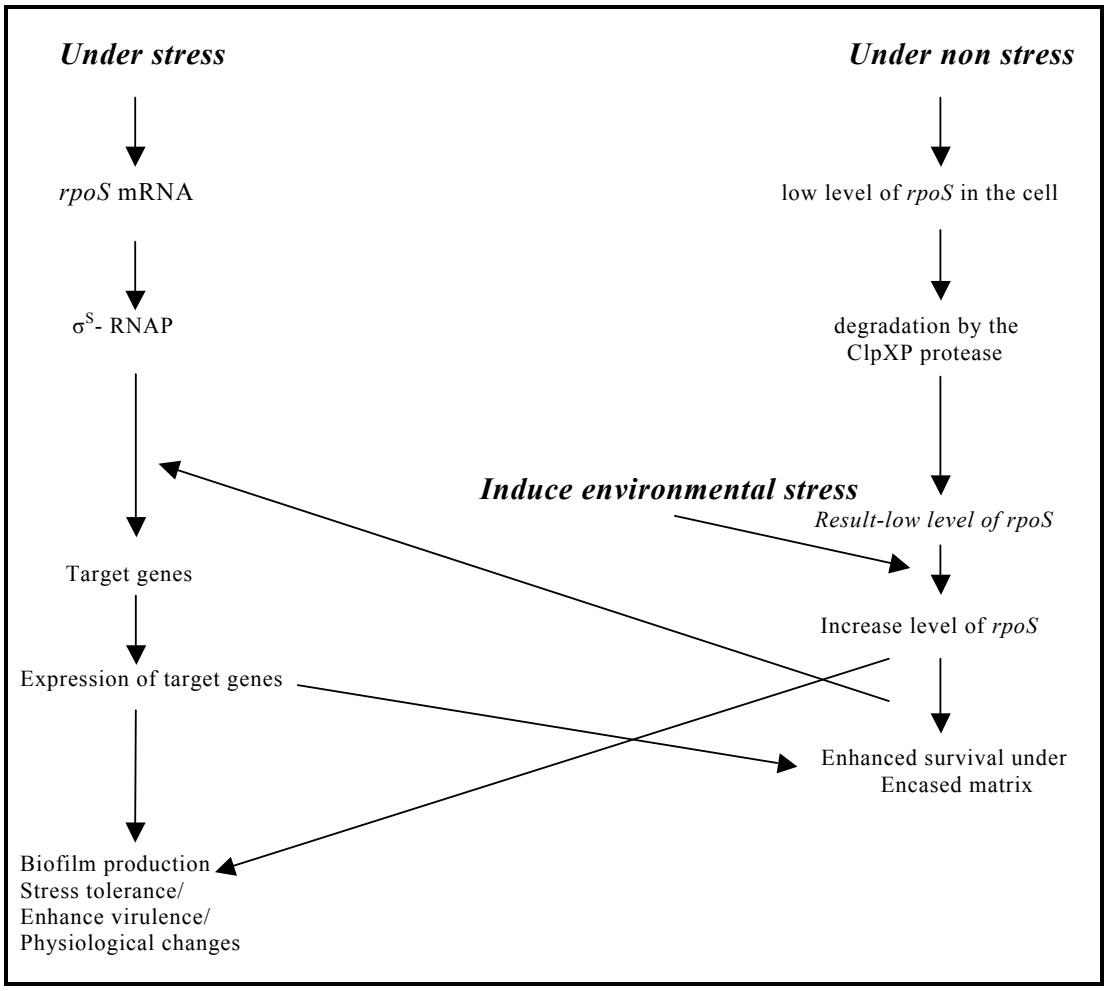


Figure 1.4: Schematic model of stress and non-stress responses (*rpoS* response) of *E. coli* under different environmental conditions. Note that, in stress and non-stress conditions, induction of external environmental parameters results in expression of target genes, which lead to biofilm production, enhance virulence and other physiological changes.

Under stress conditions various sigma factors work differently, resulting in the expression of specialty regulons defined as a system in which two or more structural genes are subject to coordinated regulation by a common regulator molecule. Thus gene expression is altered by different sigma factors (Sauer 2003). Under non stress

conditions, the level of *rpoS* in the cell are low because the mRNA of *rpoS* forms a stable secondary structure which leads to a poor translation in normal conditions (Fig. 1.4) (Jishage et al. 1996;Schweder et al. 1996).

The sigma factor (*rpoS* Protein) also gets degraded continuously in normal conditions by the ClpXP protease in *E. coli*, as a result low level of σ^S than σ^{70} in non stressed cells (Jishage et al. 1996;Schweder et al. 1996). *E. coli* sigma factors are listed with their functions (Table 1.3):

Table 1.3: Sigma factors of *E. coli,* their relevant genes and gene function.

Sigma factor	Gene	Function
σ^{70}	*rpoD*	Housekeeping functions
$\sigma^{54}(\sigma^N)$	*glnF, nrtA, rpoN*	Nitrogen-regulated genes
σ^{32}	*htpT, rpoH*	Heat-shock genes
$\sigma^{24}(\sigma^E)$	*rpoE*	Heat-shock genes
σ^{28}	*flbB+flaI, rpoF*	Flagella synthesis/chemotaxis
$\sigma^{38}(\sigma^S)$	*rpoS, katF*	Starvation/general stress response

Genome-wide expression profiling, indicated that up to 10% of the *E. coli* genes were under direct or indirect control of σ^S and that σ^S should be considered a second vegetative sigma factor with a major impact not only on stress tolerance but also on the entire cell physiology under non optimal growth conditions (Weber et al. 2005). Biofilm formation in *E. coli* is a programmed development that involves slow growth and stress conditions where different growth phase regulated genes and several molecular signals are involved.

Table 1.4: Number of genes regulated by different sigma factors in *E. coli* K-12 MG1655.

Gene name	Number of genes regulated
rpoD (Sigma)	969
rpoE (Sigma)	92
rpoH (Sigma)	34
rpoN (Sigma)	104
rpoS (Sigma)	114

An important distinguishing feature of *rpoS* is that its control of gene expression largely depends on growth conditions (Weber et al. 2005). Many genes are only controlled by *rpoS* under specific conditions which may be due to the modulating effect of other regulators such as Crl and ppGpp (Dong and Schellhorn 2009;Weber et al. 2005). Previous work on the recognition of the *rpoS* regulons has focused on growth in rich medium and has shown that growth of *E. coli* differs significantly between different growth conditions. In Luria-Bertani (LB) rich media, there are few fermentable sugars available and cells utilize amino acids as the major carbon source (Dong and Schellhorn 2009;Weber et al. 2005).

In glucose minimal media, however, glucose is the carbon source and all essential cellular building blocks, such as nucleotides and amino acids, are synthesized from glucose and inorganic phosphate and nitrogen sources (Tao et al. 1999). As a result of the increased anabolic demand on the cell, the growth rate is reduced. Comparison of transcriptome expression in rich and minimal media reveals considerable alterations in gene expression (Dong and Schellhorn 2009;Tao et al. 1999). *RpoS* is likely to be an important factor contributing to these changes, as *rpoS* expression is sensitive to growth conditions. More than 100 genes are regulated by *rpoS*, but the

number varies from one growth condition to other (Dong and Schellhorn 2009). Appendix 2, lists genes that are *rpoS*-regulated in Luria-Bertani media with their functions.

Gene expression is a complicated process that often involves multiple regulators which may function synergistically or independently to modulate expression in response to specific environmental signals (Dong and Schellhorn 2009), but our knowledge about these regulators is still limited at the genome scale. *RpoS* mediates the expression of intermediate regulators, which in turn control the expression of different sets of genes. *RpoS* may also have a self regulatory circuit by controlling the expression of different genes that regulate *rpoS*, either positively (e.g., hfq) or negatively (e.g., clpXP). Many genes are regulated by more than one regulator within the *rpoS* regulon (e.g., gadAB controlled by gadE and gadX) (Dong and Schellhorn 2009). These regulators may work communally, separately, or may have an opposing effect on gene expression. For example, CsrA, the carbon storage regulator, down-regulates the expression of glgAC for glycogen synthesis but positively regulates flhCD for flagella formation and *eno* for glycolysis. In contrast, *rpoS* positively regulated the expression of csrA, eno, and glgAC, but had an opposing effect on the expression of flhCD. This antagonistic effect represents an important regulatory mechanism for balanced gene expression (Dong and Schellhorn 2009;Tao et al. 1999;Weber et al. 2005). Thus, large set of genes were controlled by *rpoS* in various growth conditions and many of these were not formerly known to be *rpoS*-controlled. This project was designed to investigate the role of *rpoS* in biofilms under exponential phase in various stress-induced environments.

1.5.2 Morphogene *bolA*

Stress response genes are induced whenever a cell needs to adapt and survive under unfavourable growth conditions (Vieira et al. 2004), morphogene *bolA* in *E. coli* is one of the examples of these genes. It was first reported to be involved in adaptation to the stationary form of growth (Aldea et al. 1989;Lange and Hengge-Aronis 1991). However, its function is still not fully understood and is not only confined to stationary phase, but its expression might be induced by different forms of stresses such as heat shock, acidic stress, cold shock etc. which results in high level of expression of *bolA* mRNA and may lead to the formation of biofilms (Aldea et al. 1989;Lange and Hengge-Aronis 1991). The high level of expression of *bolA* mRNA is mainly due to the specific transcription of the *bolA*1p promoter by the σ^S factor. It also has a major effect on the bacterial envelope and, therefore, is probably involved in cellular protection under adverse growth conditions (Santos et al. 1999).

BolA gene was also shown to regulate the transcript levels of D,D-carboxypeptidases PBP5 (encoded by dacA gene), PBP6 (dacC) and β-lactamase AmpC (ampC), all of which are involved in murein metabolism (Aldea et al. 1989;Santos et al. 1999). In order to survive within stressed environments, over expression of *bolA* leads to the round cell morphology in order to render the cell shorter and rounder, causing a decrease in surface to volume ratio and a reduction in the surface area exposed to the damaging or unfavourable environment. *BolA* seems to be involved in switching between cell elongation and septation systems during the cell division cycle. Normally the expression of *bolA* is growth rate regulated, being induced during the transition into stationary phase from exponential phase (Vieira et al. 2004). Expression of *bolA* is governed by two promoters, P1 and P2 (Fig. 1.5). The main promoter, P1, is proximal to the structural gene, and is a gearbox promoter under the

control of σ^S. P2 is located further upstream from the structural gene; it is under the control of σ^D and transcribes *bolA* constitutively. Increased expression and morphological changes due to sudden carbon starvation and osmotic shock still occurs when σ^S is not present which shows that expression of *bolA* is not confined to stationary phase, but it can also play an important role in general stress response (Aldea et al. 1989;Lange and Hengge-Aronis 1991).

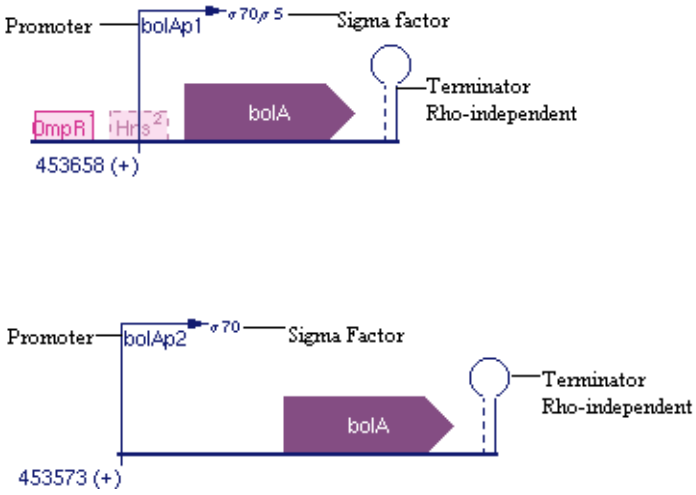


Figure 1.5: Diagrammatic representation of transcription units of *bolA* (Adapted from EcoCyc database of *E. coli* K-12 MG1655).

1.6 New Methodologies for Biofilm Control

The biotechnology sector has just started dealing with the biofilm associated problems by developing antimicrobial agents with novel mechanism of action. Some studies look for prevention of biofilm formation, while others aim to develop antimicrobial agents to treat existing biofilms, and many others are trying to disrupt the polymeric ties that attach the biofilms together (Schachter 2003).

1.6.1 Biofilm control with enzymes

The use of enzyme-based detergents as biocleaners, also known as "green chemicals", can provide a possible option to overcome biofilm associated problems in the food industry. Enzymes can be used to degrade biofilms, due to the heterogeneity of the EPS in the biofilm, mixture of different enzymes may be necessary for a sufficient degradation of bacterial biofilms. Detergents and enzymes have also been used as synergists to improve disinfectant effectiveness (Johansen et al. 1997). The specific mode of action makes it a complex technique, adding to the difficulty, is finding enzymes that are effective against all different types of biofilms. Formulations containing several different enzymes seem to be fundamental for a successful biofilm control strategy. Basically, proteases and polysaccharide hydrolysing enzymes may be useful (Johansen et al. 1997).

1.6.2 Biofilm control with phages

When phages come in contact with biofilms, further interactions occur, depending on the susceptibility of the biofilm bacteria to phage and to the availability of receptor sites (Hughes et al. 1998). If the phage also possesses polysaccharide-degrading enzymes, or if considerable cell lysis is affected by the phage, the integrity of the biofilm may rapidly be destroyed. Hughes et al. (1998) working in the control of biofilms of *Enterobacter agglomerans* by the use of phages, found that the cells were readily lysed and the biofilm degraded by the addition of bacteriophage if certain criteria were met. The bacteria had to be susceptible to the phage, and the phage polysaccharide depolymerise had to be able to degrade the biofilm EPS. The phage then lysed the biofilm cells; the

polymerase enzyme degraded the EPS and caused the biofilm slough off. If only one of these criteria was met, there was still a substantial degree of biofilm degradation. Alternatively, coexistence between phage and host bacteria within the biofilm may be developed (Hughes et al. 1998).

However, phage have been proposed as a means of destroying or controlling biofilms, the technology for this has not yet been successfully developed and relatively little information is available on the action of bacteriophage on biofilms (Hughes et al. 1998).

1.6.3 Biofilm control by means of interspecies interactions – bioregulation

The existence of multiple interactions or the simple production of a metabolite can interfere with the development of what seems to be structurally organized communities existent within a biofilm. Competition for substrate is considered to be one of the major evolutionary driving forces in the bacterial world, and numerous experimental data obtained in the laboratory under well-controlled conditions show how different microorganisms may effectively out compete others because of better utilization of a given energy source (Christensen et al. 2002). Furthermore, many bacteria are capable of synthesizing and excreting surfactants. In a competitive environment this phenomenon could play a significant role. It has been suggested that biosurfactants might be involved in the transfer of exopolymer from one bacterial species to another, taking place more efficiently within the matrix of a biofilm where the cells are in closeness to each other.

Nevertheless, in a mixed species biofilm, this cell feature promoted by a bacterial specie could have antimicrobial properties to the others species. The production of biosurfactants can impair the formation of biofilms. Surfactin from *Bacillus subtilis* disperses biofilms without affecting cell growth and prevents biofilm formation by microorganisms such as *Salmonella enterica*, *E. coli*, and *Proteus mirabilis* (Mireles, II et al. 2001). Also, lactic acid bacteria and their products have been well documented for their antimicrobial activity against the growth of *Listeria monocytogenes* (Harvey et al. 2007). The discovery that wide spectrums of bacteria use quorum sensing to perform biofilm formation and differentiation makes it an attractive target for biofilm control.

1.7 Research Aims and Objectives

The objective of this research is to understand the involvement of morphogene *bolA* and RNA polymerase sigma factor (*rpoS*) in biofilm formation under various stress-induced environments. Therefore this project is designed with specific aims to:

1. Study the expression of *rpoS* and *bolA* genes in *E. coli* K-12 MG1655 under sudden change (15 minute stress) from optimum growth conditions to heat, cold, acid and hydrogen peroxide stress.
2. Study the expression of *bolA* gene under various stress environments in absence of *rpoS*. Post stress response was measured and evaluated using Applied Biosystems 7500^{TM} real time cycler both in planktonic and biofilm phases.

3. Study the respiratory activity of *E. coli* K-12 MG1655 in presence/absence of *rpoS* and *bolA* genes under various stress environments, using biological oxygen monitor (BOM).
4. Study the adherence pattern of *E. coli* K-12 MG1655 wild type and mutant strains on different surfaces under various stress-induced conditions, using scanning electron microscopy (SEM).

Chapter 2

Materials and Methods

This chapter describes the general methodologies used during this study and details of the experiments carried out.

2.1 Bacterial Strains

Escherichia coli K-12 MG1655 wild type (WT) and mutant strains (Δ) have been used in this study and were kindly provided by National Institute of Genetics, Japan. The mutants were *E. coli* K-12 MG1655 *rpoS* mutant (Δ*rpoS*) and *E. coli* K-12 MG1655 *bolA* mutant (Δ*bolA*).

2.2 Microbiological Media

Microbiological media and reagents were prepared using deionised water and sterilised by autoclaving at 121 °C, 15 psi for 15 min. Agar medium was subsequently cooled to 55 °C, prior to the addition of any supplements and poured into sterile plastic petridishes (Bibby Sterillin Ltd.) in approx. 30 ml volumes. All chemicals were purchased from Sigma-Aldrich Company Ltd. (UK).

2.3 Inoculum Preparation

A bacterial suspension was prepared by gently removing bacteria from the solid media using a sterile nichrome loop to inoculate the bacteria into a 500 ml flask containing 200 ml of sterile nutrient medium. This bacterial suspension was incubated at 37 ºC with agitation at 120 rpm for 18 h, in order to have bacteria in the exponential phase of growth.

2.4 Maintenance of Bacterial Cultures

E. coli strains were maintained by sub culturing them every 3-4 weeks onto Luria-Bertani agar (10g Tryptone, 5g yeast extract, 5g sodium chloride and 10g agar) purchased from Sigma-Aldrich Company Ltd. (UK) with a final pH 7.2. Cultures were put onto slopes of the agar and after incubation overnight at 37

ºC, were stored at 4 ºC. Luria-Bertani agar is the preferred and recommended medium for molecular genetics studies with *E. coli* K-12 strains and is used for routine cultivation. Stored cultures were recovered after approximately 18 h of incubation at 37 °C in 10 ml of fresh medium. The cell densities of the cultures were determined by measuring the absorbance of 1 ml sample using a Pharmacia LKB Novaspec II spectrophotometer at 600 nm.

Cells were also stored/preserved for long term using a MAST CRYOBANK™ (Mast Group Ltd., UK). MAST CRYOBANK™ is based on a cryovial system comprising chemically treated ceramic beads covered with a special cryogenic preserving solution. It is the most convenient, reliable and versatile system for storing and preserving bacteria over long periods at -20 or -70 °C.

2.5 Measurement of Growth Rates

For measurement of growth rates, cells from a fresh overnight culture, or a freshly streaked colony, were resuspended in 10 ml of the same medium in a 125 ml Erlenmeyer flask and incubated at 37 °C. At various times after inoculation, samples were withdrawn from the culture and optical densities at 600 nm were measured with spectrophotometer. Optical density of 0.4 at 600 nm corresponds to early mid-exponential growth phase. Flasks containing cultures reaching their mid-exponential phase were immediately transferred to stress assay. In some experiments, growth rate was monitored by plating appropriate dilutions of the withdrawn culture sample and calculating the number of colony forming units (CFU) per ml of the culture.

2.6 Stress Response Experiment

2.6.1 Heat shock, Cold shock, pH stress and H_2O_2 stress

0.1 ml of *E. coli* K-12 MG1655 cultures (WT, Δ*bolA* and Δ*rpoS*) were withdrawn at 2 min intervals and plated out directly to determine the viable cell numbers. Percent survival was defined as the percentage change in the CFU counts per ml obtained after incubation onto LB medium for 15 minutes following a sudden shift from optimal growth conditions, i.e. heat shock temperatures, 42 °C and 46 °C, cold shock temperatures, 5 °C and 20 °C, pH stress levels , pH 5, 6, 8 and 9 and different concentrations of H_2O_2, 3 mM, 4 mM and 5 mM, This was done in order to check the rapid change in expression level of *rpoS* and *bolA* genes.

2.7 Preliminary Study and Confirmation of *rpoS* Mutant Status

To ensure that the *E. coli* K-12 strain used in these studies possessed a functional *rpoS* sigma factor. *RpoS*$^+$ Strains were screened for their ability to synthesize glycogen (Notley-McRobb et al. 2002), as glycogen synthesis is under the direct control of *rpoS* (Hengge-Aronis and Fischer, 1992). Among all tested strains, pronounced glycogen production was exhibited by *rpoS*$^+$ strain, and therefore it was chosen for further experimentation. As shown in the figure in chapter 3, the glycogen synthesis in the *E. coli rpoS* mutant (white colonies indicate the absence of glycogen), while the wild type strain was shown to be glycogen-positive (dark colonies).

2.8 Glycogen Assay

To confirm the *rpoS* mutant status, both *E. coli* wild-type and *rpoS* mutant strains were streaked on LB agar plates and incubated overnight at 37 ºC. After

incubation, plates were left at 4 ºC for 24 h before they were flooded with concentrated iodine solution. Glycogen-deficient *rpoS* mutants gave a negative staining reaction (white colonies), whereas the wild-type glycogen-excess strains generated a positive staining reaction (dark brown colonies).

2.9 Biofilm Plate and Assay Formation (Crystal Violet Staining)

Biofilm cells (adhered cells on the surface) used in this study were cultured and cultivated using 6 well PVC microtiter plate (Orange Scientific). 3 ml of LB media was inoculated with 300 µl of an overnight culture at OD600 was inoculated. After the incubation period (cultures reaching their mid-exponential phase) the liquid media with free floating cells was discarded and the wells were washed three times with distilled water to remove any planktonic cells. Surface and wall of wells were then immediately scraped using a scraper (Orange Scientific) into 3 ml of distilled water to remove the adhered cells from the surface and then transferred to a stress assay.

A biofilm formation assay was performed using a microtiter plate. 20 µl aliquots of an overnight culture with OD600 of 1.0 were inoculated into 200 µl medium in a PVC microtiter plate. After 72 h incubation, the medium was removed from wells which were then washed five times with sterile distilled water and unattached cells were removed. Plates were air-dried for 45 min and each well with attached cells were stained with 1% crystal violet (CV) solution in water for 45 min. After staining, plates were washed with sterile distilled water five times. At this point, biofilms were visible as purple rings formed on the side of each well. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. 100 µl from

each well was transferred to a new microtiter plate and the level (OD) of the crystal violet present in the destaining solution was measured at 595 nm.

2.10 XTT Reduction Assay

2.10.1 Biofilm cells

The quantification of biofilm cellular activity was assessed through the XTT reduction assay as previously described (Logu et al., 2003), with some modifications. Accordingly, the coupons containing the biofilm were washed twice with 4 ml of 0.9% NaCl and transferred to a new microtitre plate with each well containing 1 ml of XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-Htetrazoliumhydroxide} solution at 200 mg/l, plus PMS (phenazine methosulfate) at 20 mg/l. The microtiter plates were incubated under agitation (120 rpm) for 3 h at 37 ºC, in the dark. Each solution was then centrifuged for 5 min at 9500 g and the absorbance read at 490 nm.

2.10.2 Planktonic cells

Cells were inoculated in 15 ml of TSB and grown for 24 h (± 1) at 37 ºC in an orbital shaker at 130 rpm. 100 µl of each cell suspension were then transferred to 60 ml of fresh TSB and incubated for 18 h at 37 ºC at 130 rpm to reach the late exponential phase. After incubation, cells were harvested by centrifugation at 10500 g for 5 min at 4 ºC and washed twice with 0.9% NaCl. 1 ml of the suspension obtained was centrifuged at 9500 g and the pellet was resuspended in 900 µl of sterile ultra pure water. To each sample, 100 µl aliquot of XTT at 200 mg/l and PMS at 20 mg/l was added. The suspensions were then incubated in the dark for 3 h at 37 ºC at 120 rpm. Following that each solution was

centrifuged for 5 min at 9500 g and colorimetric changes were measured using microtiter plate reader at 490 nm.

2.11 Catalase Activity

Cultures were tested qualitatively for catalase activity by applying 6% (wt/vol) H_2O_2 directly onto colonies on Luria agar plates. Vigorous bubbling indicated wild-type *rpoS* activity and positive reaction to hydrogen peroxide.

2.12 Experimental Replication

Data from all experiments, including control treatments for both the planktonic and biofilm phase, represent the averages of at least three independent experiments.

2.13 Extraction of Genomic DNA

To isolate genomic DNA (gDNA) from *E. coli* K-12 MG1655 Puregene® DNA Purification kit was used and DNA was extracted according to manufacturer's instructions.

2.13.1 Cell lysis

500 µl of cell suspension containing 0.5-1.5 billion cells was added to a 1.5 ml microfuge tube on ice, than centrifuged at 13,000 x g for 5 second to pellet cells. Carefully removed the supernatant. To the residual supernatant 300 µl cell lysis solution was added and pipette up and down until cells were suspended and suspension was incubated at 80 °C for 5 min to lyse cells.

2.13.2 RNase treatment

1.5 µl RNase A solution was added to the cell lysate and mixed it by inverting the tube 25 times and incubated at 37 °C for 30 min.

2.13.3 Protein precipitation

Cool the sample to room temperature and 100 µl protein precipitation solution added to the cell lysate. Vortex at high speed for 20 sec to mix the protein precipitation solution and the sample was centrifuged at 13000 x g for 3 min, the precipitated protein formed a tight pellet, sometimes the pellet was not tight than vortex it again for 20 sec to mix the protein precipitation solution followed by incubation on ice for 5 min.

2.13.4 DNA precipitation

The supernatant containing the DNA was poured, leaving behind the precipitated protein pellet, into a clean 1.5 ml microfuge tube containing 300 µl 100% Isopropanol (2-propanol) and mixed by inverting it gently 50 times. After centrifuging at 13,000 x g for 1 min, DNA was visible as a small white pellet. The supernatant was discarded and 300 µl of 70% ethanol was added and tube was inverted several times to wash the DNA. Again the sample was centrifuged at 13,000 x g for 1 min and ethanol was poured off carefully.

2.13.5 DNA hydration

50 µl of DNA hydration solution was added to the DNA pellet and the sample was rehydrated by incubating it for 1h at 65 °C. DNA was stored at 4 °C for short term storage or at -20 °C for long term storage.

2.14 RNA Isolation and Purification

The RNeasy® Protect^{TM} Bacteria Mini Kit (Qiagen Ltd., UK) was subsequently used for routine isolation and purification of RNA from *E. coli* K-12 MG1655

and it was proved to be most reliable by consistently giving the highest RNA yields. Isolations were performed according to the manufacturer's instructions in each case.

2.14.1 RNA isolation using the RNeasy® Protect™ Bacteria Mini Kit

RNA was routinely isolated using the RNeasy® Protect™ Bacteria Mini Kit (Qiagen Ltd., UK), which comprises two steps: immediate stabilization of bacterial RNA and subsequent isolation and purification of total RNA. Firstly RNA was stabilized in vivo by the addition of 5-7.5 x 10^8 cells (approx 1 ml of a culture of *E. coli* K-12 MG1655 at an A_{600} of 0.8) to two volumes of RNAprotect® Bacteria reagent (Qiagen Ltd., UK), followed by vortexing for 5 seconds and kept standing at room temperature (15-25 °C) for 5 min. The stabilized cells were collected by centrifugation at 5000 x g for 10 min and the supernatant decanted off. Any residual supernatant was removed by gently dabbing the inverted tube onto a paper towel. Cell pellets were stored for up to 2 weeks at -20 °C. Secondly, the pellet containing the stabilized cells was re-suspended thoroughly in 200 µl TE buffer (10 mM Tris-HCL, 1.0 mM EDTA. pH 8.0) containing 1mg/ml lysozyme. The resulting homogeneous cell suspension was incubated for 10 min at room temperature (15-25 °C), with vortexing for 10 sec every 2 min. Lysis was completed by the addition of 700 µl buffer RLT (Qiagen Ltd., UK) and vigorous vortexing for 15 sec. 700 µl of 96% ethanol was then added and the sample was mixed by successive pipetting. 700 µl of ethanol lysate was then transferred to an RNeasy® spin column assembled inside a 2 ml collection tube, and drawn through the silica matrix by centrifugation (8000 x g for 15 sec). The flow-through was discarded.

Buffer RW1 (Qiagen Ltd.), was then added (700 µl) to the RNeasy Mini spin column and centrifuged for 15 sec at 8000 x g to wash the spin column membrane. The flow-through and the collection tube were discarded. The spin column was then transferred to a new 2 ml collection tube. 500 µl buffer RPE (Qiagen Ltd.) was added and centrifuged again at 8000 x g for 15 sec. The flow-through was discarded and the spin column washed with 500 µl RPE buffer. The spin columns were centrifuged for 2 min at 8000 x g and transferred to a new microfuge tube. To remove any residual ethanol solution the column was dried by further centrifugation at 8000 x g for 1 min over a new 2 ml collection tube. The column was then transferred to a new 1.5 ml microfuge tube and 50 µl RNase-free water was added directly to the spin column membrane followed by centrifugation for 1 min at 8000 x g to elute the RNA. The eluted total RNA was stored at -80 °C.

2.14.2 RNA cleanup

Following DNase treatment, it was necessary to re-purify the RNA before reverse transcription. This was done by using the RNeasy® mini kit according to the manufacturer's instructions (Qiagen Ltd., UK). The DNase reaction mixture was made up to 100 µl with nuclease-free water and vortexed vigorously with 350 µl buffer RLT (Qiagen Ltd., UK). Following the addition of ethanol (250 µl), the sample was pipetted up and down successfully and 700 µl was then transferred to an RNeasy spin column assembled in a 2 ml collection tube. The assembly was centrifuged at 8000 x g for 15 sec to permit binding of the RNA to the column matrix. The RNA was then washed with RPE buffer as described in section 2.14.1 and subsequently eluted in 50 µl RNase-free water. Purified RNA was stored at -80 °C.

2.15 Analysis of DNA and RNA Samples

2.15.1 Quantification of nucleic acids

The concentration of nucleic acid samples was determined by spectrophotometric analysis using Pharmacia LKB Novaspec II spectrophotometer. At 260 nm, absorbance of 1 is equal to 40 µg of RNA/ml and 50 µg of double stranded DNA/ml, and their absorbance measured against a blank containing only nuclease-free water. Concentrations in µg/ml were calculated by multiplying the absorbance reading by the dilution factor and either 40 µg for RNA or 50 µg for DNA. Three replicates were measured to ensure accurate quantification. The purity of nucleic acid samples was estimated using the ratio of absorbance at 260 nm and 280 nm. A ratio (A_{260}/A_{280}) of 1.9-2.1 indicates pure nucleic acid samples.

2.15.2 Analysis of DNA integrity

The integrity and size distribution of nucleic acid samples was routinely determined by horizontal agarose gel electrophoresis (Sambrook *et al*., 1989). Depending on the size of DNA fragments being analysed 0.8-2% (w/v) SeaKem® agarose (Biowhittaker Molecular Applications) gels in 1x TAE buffer (40 mM Tris-acetate/1mM EDTA) were used. Electrophoresis was then carried out using a 200 ml capacity electrophoresis apparatus (EmbiTec RunOne™ Electrophoresis Cell) run at 100 V for 0.5-1 h. Samples were mixed with the appropriate volume of 10 x Reddy Run® gel loading buffer (Thermo Scientific®, UK) and loaded routinely up to 6 µl in each lane.

For size estimation the following markers were used 50 µg/ml Reddy Run® Super ladder-low 100 bp (Thermo Scientific®, UK). Nucleic acids were

visualised after suspending the agarose gels in a solution of ethidium bromide (0.5 µg/ml) for 20 min (Sambrook *et al*., 1989), and examined under 302 nm UV light by transillumination using the UV Products (UVP) BioDoc-It™ system.

2.15.3 Analysis of RNA integrity

The integrity of total RNA samples was determined by using denaturing (formaldehyde) agarose gel electrophoresis. RNA samples, used for RT-PCR analysis, were routinely checked using this method for the presence of two clear, sharp bands of 16S and 23S *E. coli* ribosomal RNA, which are indicative of intact RNA. Firstly 1.5% (w/v) SeaKem® agarose (Biowhittaker Molecular Applications) gels were prepared in MOPS buffer (20mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7) with the addition of 18 ml of 12.3 M (37% v/v) formaldehyde per 100 ml gel. After thorough mixing the gel was cast in a 10 cm x 15 cm mould and allowed to set for 45 min. RNA in 5 µl volume was mixed with 20 µl sample buffer (13% v/v 10x MOPS buffer, 65% v/v formamide, 22% v/v formaldehyde (38% v/v stock) and 5 µl dye solution, and prepared by heating to 65 °C for 5 min in a hot block (Techne DRI-BLOCK® DB-2A) to denature the RNA, followed by rapid cooling on ice. The samples were then loaded (25 µl) onto gels, which were subjected to 100 V (60 mA) for 1 h. For size estimation the following markers were used 0.5-10 Kb RNA ladder (Invitrogen™, UK). After electrophoresis excess formaldehyde was removed from the gel by gentle agitation in deionised water for 1 h.

The gel was then stained for 15 min in ethidium bromide (0.5 µg/ml) and destained for about 1 h in 1 mM $MgSO_4$, to reduce background fluorescence,

which is prominent in formaldehyde gels. The RNA was then visualised by UV illumination using the UV Products (UVP) BioDoc-It^{TM} system.

2.16 Primer Designing

2.16.1 Primer specificity

Non-specific amplification is one of the greatest challenges for the successful deployment of real-time PCR methods. In addition to the sequence of interest, primers might bind to other sequences. The design of PCR primers for this purpose should therefore take into account the potential contribution of all possible off-target template sequences, in order to prevent mispriming.

2.16.2 Primer length

PCR primers are typically 16–28 nucleotides long. If the length is too short, it is difficult to design gene-specific primers and choose an optimal annealing temperature. On the other hand, very long oligos unnecessarily increase oligo synthesis cost and are more likely to form secondary structures that result in decreased PCR efficiency or promote primer dimer formation.

2.16.3 Primer GC content

In most PCR applications the primer GC content lies between 35% and 65%. If the GC content is too high, mispriming frequently results. This is because even a short stretch of oligo sequence may form a stably annealed duplex with non-target templates. On the other hand, very low GC content may result in poor primer binding, leading to decreased PCR efficiency.

2.16.4 Primer melting temperature

The melting temperature (Tm) is the most important factor in determining the optimal PCR annealing temperature. An ideal PCR reaction should have forward and reverse primers with similar Tm values. Tm is not only determined by primer sequence, but also by other parameters, such as salt concentration and primer concentration. Currently, the following method for Tm calculation is adopted by most primer design programs.

The '4 + 2' rule

Tm = 4 * (*G* + *C*) + 2 * (*A* + *T*).

This is a simple equation solely based on primer GC content. Tm is calculated by counting the total number of G/C and A/T. Each G/C contributes 4 ºC and each A/T contributes 2 ºC to Tm.

2.16.5 Amplicon size

PCR efficiency can be affected by amplicon size. Very long amplicons leads to decreased PCR efficiency. Since PCR efficiency is one of the most important factors for accurate expression quantification, the amplicon should be smaller than 250 bp; typically, the size range is 100–250 bp.

2.16.6 Primer design tool

Primer designing was done using Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

2.16.7 Experimental design

Primers are synthetic oligonucleotides with a specified sequence and are desalted (5 OD units, 50 nmole) through normal phase chromatography column.

They were synthesised by Invitrogen™ life technologies, UK. All primers were re-hydrated in nuclease-free water and dispensed into 10 µM aliquots of working stock solution prior to storage at -20 °C. Primers were designed using Primer 3 software with the following design parameters: 20 ± 2 bp primer length; 45-55% GC content; 55-65 °C primer melting temperature (Tm); and avoiding the GC-rich 3' end. Potential hair pin formation, (self complementarity of primers) was checked using a oligonucleotides properties calculator. Primer sequences for *rpoS*, *bolA* and 16S rRNA is listed below (Table 2.1):

Table 2.1: List of primer sequences for *rpoS*, *bolA* and 16S rRNA (housekeeping gene).

Primer	Sequence	Length (bp)	GC%	PHPF	Annealing temperature
16S rRNA (Forward)	AGGCCTTCGGGTTGTAAAGT	20	50%	No	**55 °C**
16S rRNA (Reverse)	CGGGGATTTCACATCTGACT	20	50%	No	**55 °C**
***RpoS* (Forward)**	GATGACGTCAGCCGTATGCTT	21	52%	No	**59 °C**
***RpoS* (Reverse)**	GAGGCCAATTTCACGACCTAC	21	52%	No	**59 °C**
***BolA* (Forward)**	CCGTATTCCTCGAAGTAGTGG	21	52%	No	**59 °C**
***BolA* (Reverse)**	GCAACCCTTCCCACTCCTTAA	21	52%	No	**59 °C**

2.17 Optimization of PCR Primers

The optimization of the primer concentration is essential. Each set of primers works best at a different concentration. Primer concentration is usually determined to be optimal when the specific amplification relative to primer-dimers is maximal, in a positive versus negative control experiment. One major limitation in primer optimization is, however, the availability of a good positive template for optimization. Again primer optimization differs from one type of assay to another.

2.17.1 Optimisation of cycling parameters

Cycling parameters were based on manufacturer's guidelines and optimised empirically. Essentially the annealing temperature was the only parameter that required notable optimisation, and was based upon the melting temperature of the primers.

2.17.2 Optimization of the magnesium chloride concentration

Magnesium chloride is usually used in PCR reactions. Concentration should be optimised according to template and primer combination. For standard PCR, a magnesium chloride dilution series with concentration ranging from 1.5 mM to 3 mM, in 0.5 mM steps. was set up in 0.5 ml, thin walled PCR tubes, A master mix containing all the reagents, except $MgCl_2$ and template DNA, was then was then dispensed into the tubes. Following the addition of template DNA, the samples were amplified as described and analysed by agarose gel electrophoresis.

2.17.3 Optimization of the primer concentration

Where it was necessary to optimise the primer concentration, a dilution series was prepared from 0.3 µM to 1.0 µM, in 0.1 µM steps with equimolar amounts of forward and reverse primer in 0.5 ml, thin walled, PCR tubes. A master mix containing all the reagents except the primers and template DNA was then dispensed into the tubes. Following the addition of template DNA the samples were amplified as described and analysed by agarose gel electrophoresis.

2.18 Polymerase Chain Reaction Amplification

PCR is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA. To perform a PCR, the DNA to be amplified is denatured by heating the samples. In the presence of DNA polymerase and excess of deoxyribonucleoside triphosphates (dNTP´s), oligonucleotides that hybridize specifically to the target sequence, can prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete short product which accumulates exponentially with each successive round of amplification. This can lead to many million fold amplification of the discrete fragment over the course of 20 to 30 cycles. There are three nucleic acid segments: the segment of double-stranded DNA to be amplified and two single-stranded oligonucleotide primers flanking it. Additionally, there is a protein component (DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTP´s). The primers are added in excess compared to the DNA to be amplified. They hybridize to opposite strands of the DNA and are oriented with their 3` ends facing each other so that synthesis by DNA polymerase, which catalyzes strands 5`- 3`, extends across the segment of

DNA between them. One round of synthesis results in new strands of indeterminate length which, like the parental strands, can hybridize to the primers upon denaturation and annealing. These products accumulate only arithmetically with each subsequent cycle of denaturation, annealing to primers, and synthesis. The second cycle of denaturation, annealing, and synthesis produces two single-stranded products that together compose a discrete double-stranded product which is exactly the length between the primer ends. Each strand of this discrete product is complementary to one of the two primers and can therefore participate as a template in subsequent cycles. The amount of this product doubles with every subsequent cycle of synthesis, denaturation, and annealing.

2.18.1 Analysis of PCR Results

2.18.1.1 Cycling parameters of PCR

Samples were analysed after the amplification using 1.1 x ReddyMix™ PCR Master Mix (Thermo Scientific™, UK) in a 12.5 µl reaction with 1.5 mM $MgCl_2$ concentration. The contents of the total reaction are described below:

Table 2.2: Total contents with volume for the PCR amplification reaction.

PCR contents	Volume (μl)
Master Mix	10 µl
Primer Forward (0.3 µM)	0.375 µl
Primer Reverse (0.3 µM)	0.375 µl
DNA Template	0.5 µl
H_2O	1.25 µl
Total	**12.5 μl**

2.18.1.2 Electrophoresis of PCR products

Electrophoresis was then carried out using a 200 ml capacity electrophoresis apparatus (EmbiTec RunOne™ Electrophoresis Cell) run at 100 V for 20 min. Samples were then loaded up to 6 µl in each lane.

For size estimation the following markers were used 50 µg/ml Reddy Run® Super ladder-low 100 bp (Thermo Scientific®, UK). Nucleic acid bands were visualised after suspending the agarose gels in a solution of ethidium bromide (0.5 µg/ml) for 20 min (Sambrook *et al*., 1989), and examined under 302 nm UV light by transillumination using the UV Products (UVP) BioDoc-It™ system.

2.19 Real-Time PCR

Real-time reverse transcription PCR (real-time RT-PCR), is a sensitive method for quantifying mRNA in biological samples. RNA quantification begins with cDNA (complementary DNA) preparation using reverse transcriptase.

RT-PCR comprises of three fundamental steps:

1. the reverse transcriptase RT-dependent conversion of RNA into cDNA,
2. amplification of cDNA using the PCR and
3. detection and quantification of amplification products in real time.

Target sequences must be amplified by successive cycles of the Polymerase chain reaction (PCR) before quantification can take place.

PCR requires a DNA template, therefore, the first step is to reverse transcribe the mRNA template into cDNA (complementary DNA). This reaction is carried by reverse transcriptases, enzymes with DNA polymerase activity which can use RNA as a template. A primer is required to initiate the Polymerase chain reaction, which are short oligonucleotides (usually between 18 and 22 bp) which hybridise to sequence specific sites on the template strand. With the help of primer design, the selective amplification of specific targets can be achieved.

2.19.1 Detection chemistry used in real-time PCR

The basic methodology which is commonly used in the detection of RNA or DNA targets by real-time PCR is the utilization of fluorescent dyes. During each succeeding PCR cycle, a low initial fluorescent signal is increased proportionally in tandem with the exponential increase in the DNA product(s) formed. The simplest assay system involves the incorporation of a free dye into

the newly formed double-stranded DNA product. The most commonly used dye for this purpose in real-time PCR is SYBR® Green I (Molecular Probes/Invitrogen). The background fluorescence from SYBR® Green I when in solution as a free dye and stimulated by light of the appropriate wavelength is very low. The same is true for single-stranded nucleic acids at the concentrations used for real-time PCR. In contrast, as the double-stranded DNA product is formed, SYBR® Green I binds to the minor groove of the double-stranded DNA. The DNA-dye complex results in a dramatic increase in fluorescence output, when properly illuminated, of roughly 2,000 times the initial, unbound fluorescent signal (Fig. 2.1).

The popularity of SYBR® Green I assays with real-time PCR users is due to three factors: 1) low cost for the dye; 2) ease of assay development, only a pair of primers is required; and 3) the same detection mechanism can be used for every assay. The down side is that every double-stranded molecule made in the reaction such as primer dimmers or inappropriate PCR products will generate a signal, this fact puts a high premium on good primer design and careful quality control during assay development.

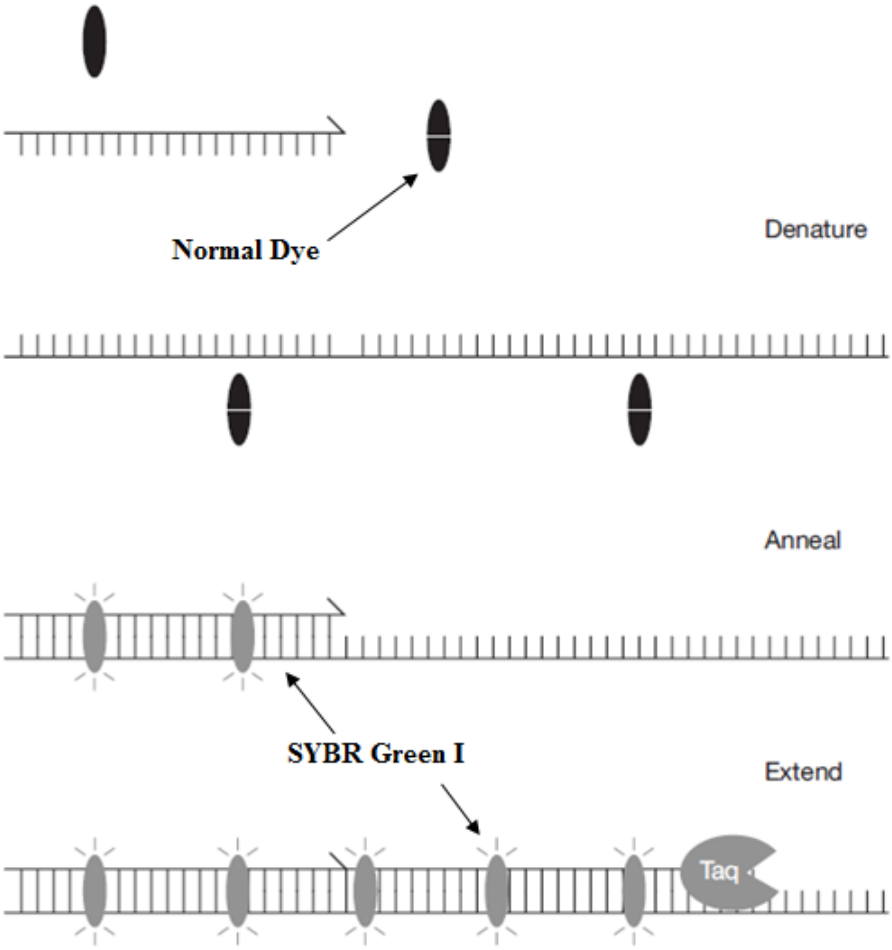


Figure 2.1: Diagrammatic representation of SYBR® Green I dye resulting in increased fluorescent signal during the reaction. Free dye has very low fluorescence and will not bind to single stranded or denatured DNA. During primer annealing, a double-stranded structure is formed and SYBR® Green I dye is bound resulting in a dramatic increase in fluorescent signal. During primer extension by *Taq* DNA polymerase, the fluorescent signal increases proportionally to the number of SYBR® Green I dye molecules bound per double-stranded molecule.

2.20 cDNA Synthesis for Real-Time Two Step RT-PCR

For the quantification of cDNA targets with the QuantiTect® SYBR® Green PCR kit, RNA must first be transcribed into cDNA. A portion of the reverse transcription reaction is then transferred to another tube, where real-time PCR takes place. This entire process is known as real-time two step RT-PCR, since reverse transcription and real-time PCR are carried out in separate tubes.

2.20.1 Reverse transcription

Messenger RNA was reverse transcribed into cDNA using the QuantiTect® Reverse Transcription kit (Qiagen Ltd., UK) which includes gDNA wipe-out buffer which was effective for the elimination of genomic DNA contamination, Quantiscript® Reverse Transcriptase enzyme made up of a Quantiscript® Reverse Transcriptase and an RNase inhibitor. Quantiscript® Reverse Transcriptase is a unique mix of Omniscript® and Sensiscript® Reverse Transcriptases, which are recombinant heterodimeric enzymes expressed in *E. coli*, 5x Quantiscript RT buffer, RT primer mix and RNase-free water. The protocol comprises two steps: Firstly genomic DNA elimination reaction which was prepared on ice according to manufacturer's instructions using 7x gDNA wipeout buffer, 1 µg template RNA and RNase-free water. The total volume per reaction was 14 µl, followed by incubation for 2 min at 42 °C and was placed immediately on ice.

After genomic DNA elimination, the RNA sample was ready for reverse transcription using a master mix prepared from Quantiscript Reverse Transcriptase, Quantiscript RT buffer, and RT Primer mix. The entire reaction

took place at 42 °C followed by inactivation at 95 °C. Reverse Transcription reactions was stored at -20 °C for long term storage.

2.20.2 Preparation of standard curve

The standard curve is a very useful tool for determining the qualities of an assay. Using a defined template, such as a plasmid containing a relevant portion of the gene of interest, PCR product, synthetic oligonucleotide or transcribed RNA to perform a standard curve, will allow determination of the PCR efficiency of the assay along with the sensitivity and dynamic range independently of any variables associated with the sample preparation and/or reverse transcription.

Using a standard curve for quantifying mRNA or DNA is referred to as absolute quantification. The standard curve allows the amount of unknown samples to be computed on a per cell or unit mass basis. But, no matter how accurately the concentration of the standard material has been determined, the final result is relative to a defined unit of interest (Pfaffl, 2004). Most real-time instruments have software that will calculate the amount of unknown values in the same units designated in the standard curve.

2.20.3 Protocol for generating standard curve (According to Applied Biosystems™)

Step 1

Identify the genome size of the organism of interest.

Step 2

Identify the mass of DNA per genome

Calculate the mass of the genome by inserting the genome-size value in the formula below:

$$m = [\,n\,]\left[\,1.096e\text{-}21\ \frac{g}{bp}\,\right]$$

Where,

n = genome size (bp)

m = mass

e-21 = $\times 10^{-21}$

Step 3:

Divide the mass of the genome by the copy number of the gene of interest per haploid genome.

Step 4:

Calculate the mass of gDNA containing the copy #s of interest.

Copy # of interest × mass of haploid genome = mass of gDNA needed

Step 5

Calculate the concentrations of gDNA needed to achieve the copy number of interest. Divide the mass needed (calculated in Step 4) by the volume to be pipetted into each reaction.

Step 6

Prepare a serial dilution of the gDNA.

For the dilutions we have used the formula, $C_1V_1 = C_2V_2$

2.20.4 Real-Time PCR (experimental design)

Transcribed cDNA was then transferred for real-time PCR analysis using a QuantiTect® SYBR Green PCR kit with Applied Biosystems® 7500 Real-Time cycler. The components in the kit were 2x QuantiTect® SYBR Green PCR Master Mix and RNase-free water.

The components of 2x QuantiTect® SYBR Green PCR Master Mix includes HotStar Taq DNA Polymerase, which is a modified form of a recombinant 94-kDa DNA polymerase, originally isolated from Thermus aquaticus, cloned into *E. coli*, QuantiTect® SYBR® Green PCR buffer which contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 5 mM $MgCl_2$, pH 8.7 and promotes a high ratio of specific primer binding during the annealing step of each PCR cycle, dNTP mix, fluorescent dye SYBR Green 1 which binds to all double stranded DNA molecules followed by emitting a fluorescent signal on binding and lastly PCR grade ultrapure RNase-free water.

The protocol was followed according to manufacturer's instructions starting with preparation of reaction mix containing 2x QuantiTect® SYBR Green PCR Master Mix, final concentration 1x followed by 0.3 µM forward and reverse primer and RNase-free water.

2.20.5 Real-Time PCR procedure

1. 2x QuantiTect SYBR Green PCR Master Mix, template DNA, cDNA, primers, and RNase-free water were thawed and solutions were mixed individually.
2. Reaction mix was prepared (Table 2.3).

Table 2.3: Reaction setup for Real-Time PCR experiment

Component	Volume/reaction	Final concentration
2x QuantiTect SYBR Green PCR Master Mix	12.5 µl	1x
Primer F	0.75 µl	0.3 µM
Primer R	0.75 µl	0.3 µM
Template DNA or cDNA (added at step 4)	0.50 µl	≤ 500 ng/reaction
RNase-free water	10.5 µl	
Total reaction volume	25 µl	

3. Reaction mix was mixed thoroughly, and appropriate volumes were dispensed into PCR plates.
4. Template cDNA (≤ 500 ng/reaction) was added to the individual wells of PCR plate containing the reaction mix.
5. Real-time cycler was programmed (Table 2.4). Data acquisition was performed during the extension step.

Table 2.4: ABI 7500 Real-Time PCR cycling conditions

Stages	Time	Temperature	
Initiation	2 min	50 °C	
Initial Denaturation	10 min	95 °C	
Denaturation	15 sec	95 °C	40 cycles
Annealing	1 min	60 °C	
Extension	30 sec	72 °C	

6. Prepared PCR plate was placed in the real-time cycler and the PCR program was started. The threshold value of 0.02 was set to analyze the data.

2.21 Development of a Reverse Transcription-Polymerase Chain Reaction Assay for the Absolute Quantification

2.21.1 Introduction

In this section, the development of a reverse transcription-polymerase chain reaction (RT-PCR) assay is described for the direct analysis of *bolA* and *rpoS* gene transcription. The aim was to monitor the transcription of these genes under various stress conditions in order to confirm the hypothesis that expression of these genes are important under various stress induced conditions. A two-step RT-PCR approach was adopted for this purpose. The products were quantified in real-time using the ABI 7500 software. Confirmation of PCR products by incorporating a melting curve analysis step ensured that only target mRNA was amplified and detected.

Absolute quantification was employed for determination of transcription levels over relative quantification because of the nature of the experiments. The selected house-keeping gene (16S rRNA) is co-amplified with the target genes and used as a benchmark against which mRNA levels of the target template can be judged. The house-keeping gene must be expressed at a steady level under all conditions tested. Because the intention of this investigation was to study the expression of *rpoS* and *bolA* under various stress conditions both in planktonic and biofilm phase, no satisfactory benchmark exists except 16S rRNA, which expresses at a steady level in almost all stress induce environments except hydrogen peroxide stress. The advantage of absolute quantification is the quality of results, which provide information on actual levels of a given mRNA, in this case *rpoS* and *bolA* mRNA. Furthermore, the results are not linked to

parameters specific to the experiment and can be compared with independent results.

2.21.2 Optimization of the PCR

Optimizing the annealing temperature, the primer concentration and the magnesium chloride concentration increased the sensitivity of the PCR assay. The procedures to derive the optimum conditions for amplification are detailed in above sections.

The optimum concentration of magnesium chloride was found to be 1.5 mM and the optimum primer concentration was 0.3 µM. These concentrations were subsequently used in all real-time RT-PCR experiments to maintain reaction stringency, while still improving reaction efficiency. The optimum annealing temperature for the amplification of *rpoS* and *bolA* is 59 ºC. The actual temperature used in subsequent experiments was 60 ºC, because this reduced the formation of unwanted artefacts to undetectable levels.

2.21.3 The real-time PCR assay

The ABI 7500 instrument with the QuantiTect^{TM} SYBR® Green I PCR kit (Qiagen) was used for quantitative analysis of *rpoS* and *bolA* mRNA by real-time PCR. Initial assays were carried out according to the manufacturer's reaction conditions in conjunction with the optimum parameters determined using standard PCR. Immediately following the elongation step of every cycle the amount of product is measured as the fluorescence emitted from SYBR Green I bound to double-stranded DNA. As the amount of product increases, so does the amount of fluorescence because more SYBR Green I can bind to ds-DNA. As it is known, the amplification of DNA is logarithmic, until the

reaction becomes saturated, where the efficiency of the reaction is 100%, the number of copies of template/product doubles with each cycle. The baseline is the level of fluorescence from a sample where no amplification has occurred (noise level), measured between the first 3-15 cycles. Where no template has been added, no product should form and therefore the fluorescence remains at the baseline. A no template control is included in every experiment to ensure no contamination of the tubes takes place during preparation of the samples.

For the quantification of PCR products, a threshold level of fluorescence must be set (this is done manually using the ABI 7500 real-time software). This is adjusted to a level above the baseline of all the samples in a single experiment, where the first detectable increase in fluorescence takes place and below the plateau phase, where the reaction is saturated. Using this threshold the threshold cycle (C_T) is determined for each sample (where the amplification plot crosses the threshold), and is used in the absolute quantification of the starting amount of template. The earlier the amplification plot reaches the threshold cycle the greater the copy number of the starting template.

2.21.4 Melting curve analysis

The identity of PCR products was confirmed by melting curve analysis, which was performed after the amplification stage of every experiment. Melting curve analysis is used to determine the melting temperature of the product(s) of amplification. To carry out a melting curve analysis the temperature is cooled from 95 ºC (at the start of a new PCR cycle when DNA is denatured) to about 5 ºC above the primer annealing temperature. This is done in order to ensure complete formation of all the double stranded molecules. Following this, the

temperature is increased steadily (0.1 ºC/s) with continuous fluorescence detection to monitor the amount of SYBR Green I bound to DNA. As the temperature increases, the fluorescence will decrease slightly through the lower end of the temperature range, until the melting temperature of the products (both non-specific and specific) is reached where fluorescence will decrease more rapidly.

The fluorescence data collected is plotted as fluorescence against temperature, resulting in curves with peaks at the respective melting temperature (T_M) of the products formed.

2.22 Reference Genes

Reference genes represent by far the most common method for normalizing qRT-PCR data. This strategy targets RNAs that are, universally expressed, and whose expression does not differ between the experimental and control groups. Theoretically, reference genes are ideal as they are subject to all the variation that affects the gene of interest.

Reference genes (previously termed housekeeping genes) such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 16S rRNA are historical carry-overs from RNA measurement techniques that generate more qualitative results. These genes were found to be essential and, importantly, always switched on.

It is possible to detect reproducibly small changes in mRNA levels and provide numerical values that could be subject to statistical analysis. The increasing emphasis on quantification meant that the requirement for ubiquitous expression was no longer sufficient. Now its expression also had to be stable

and not be affected by experimental design. At best, a poorly chosen reference gene would reduce the resolution of the assay by introducing additional noise, and at worst, the reference gene would be directly affected by the experimental system; or present a completely false result. The choice of a suitable reference gene is dependent the on individual model and requires investigation of the appropriate literature. Throughout in this study, ribosomal gene 16S rRNA is used as a reference gene. It is a commonly used reference gene in many real-time RT-PCR experiments, and is used after proper literature study.

2.23 Assessment of Bacterial Respiratory Activity

The rate of O_2 consumption by stressed cells was measured by a polarographic oxygen sensor (model 53, Yellow Springs Instrument Co., Inc.) using a published procedure (Pereira et al. 2002;Thomas and Aune 1978). For each respirometry assay, 5 ml of bacterial cultures were placed in the temperature-controlled vessel of the BOM (T= 37 ºC ± 1 ºC). These parameters were varied for mimicking stress-induced conditions. The vessels contained dissolved oxygen (DO) probes connected to a DO meter. Once inside the vessels, the bacterial cultures were aerated for 30 min to ensure oxygen saturation. After reaching 100% saturation, the relative rates of O_2 consumption were determined from the linear portion of the continuous recording of O_2 consumption over 3 to 10 min and the decrease in oxygen concentration was monitored over time. To determine the oxygen uptake rate due to substrate oxidation, a small volume (12.5 µl) of a glucose solution (5 mg/l) was injected into each vessel. The parameter was expressed in mg of O_2 consumed *per* g of bacteria *per* time (mg O_2/g bacteria min^{-1}).

2.23.1 Stress response experiment for respirometry

2.23.1.1 Heat shock, cold shock, pH stress, and H_2O_2 stress

Aliquots of 0.1 ml of *E. coli* K-12 MG1655 culture (WT, Δ*bolA*, and Δ*rpoS*) were withdrawn at 2 min intervals and plated out directly on TSA plates to determine the viable cell numbers. Percentage survival was defined as the percentage change in the CFU counts per ml obtained after inoculation into TSB medium and incubation for 15 min following a sudden shift from optimal growth conditions, i.e., heat shock temperatures (42 and 46 °C), cold shock temperatures (5 and 20 °C) and different concentrations of H_2O_2 (3, 4, and 5 mM). Cells were washed three times in sterile distilled water. Cells were finally resuspended into 50 ml of distilled water and a volume of 5 ml of the cell suspension was pipetted the into respirometry cell. The respirometry cell was fitted in the respirometry chamber, and desired temperature was adjusted with the sample for 15 min to check the sudden change in respiratory activity of *E. coli*. For oxidative stress condition, the vessel of the biological oxygen monitor (BOM) was controlled at 37 ºC ± 1 ºC and H_2O_2 with final concentrations of 3, 4, and 5 mM was added for 15 min and respiring samples was monitored for 3 to 10 min.

2.23.2 Biological mass quantification (determining dry weight)

The dry mass of the biological samples was assessed by the determination of the homogenised bacterial suspensions. Aluminium foil cups were made and their initial weight was noted. 5 ml of the bacterial suspension was added in those cups and left them in a hot air oven at 105 ºC for 24 h to dry up all the liquid. After 24 h, cups were weighed for the final weight (i.e. including dry

cells in a cup). The difference in the weight was calculated and the results were expressed in gram of biomass per litre (g/biomass/L).

2.24 Scanning Electron Microscopy Observations

The scanning electron microscopy (SEM) observation was made of at least 15 fields of each biofilm-covered slide. Prior to the SEM observations, the biofilm samples were steadily dehydrated in an absolute ethanol (Merck) series at 15 min each, in 10, 25, 40, 50, 70, 80, 90 and 100 % v/v and then dried in a desiccator for 3 days. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10-15 kV. The slides were not fixed because fixation action involves the use of chemicals that be likely to react with some of the components in the biological matrix, the modifying the real biofilm structure, as has been documented by Azeredo *et al*. (1999). SEM observations were visualised through the acquisition of representative microphotographs.

Chapter 3

Gene expression under various stress-induced environments

This chapter describes the variation in expression of *rpoS* and *bolA* genes in *E. coli* after shifting the cells from optimal conditions to various stress-induced conditions in planktonic and biofilm phases.

3.1 Introduction

Stress is any damaging factor that unfavourably affects the growth or survival of microorganisms. Outcomes of stresses applied to microorganisms vary. Sub-lethal level of stress reduces or stops the growth of the microorganism and does not end result in viability loss (Vorob'eva 2004). In case of moderate stress environment, outcome leads to loss in cell viability and stops the growth of microorganism. Acute or extreme stress is lethal to cells, and causes the death of the mainstream of the population. The increase in resistance of an organism to one stress after application of a different and unrelated sub-lethal stress is known as cross-protection (Rowe and Kirk 1999).

Stress responses are extremely important to microorganisms as their habitats are subject to continuous change (Vorob'eva 2004). Cells respond in various ways when stress is applied to microorganisms. As a result, microorganisms might respond in following ways;

- Production of proteins that repair damage, maintain the cell, or eliminate the cell, or stress agent.
- Evasion of host organism defences.
- Cell transformation to a dormant state, i.e., spore formation.
- Transient increase in resistance or tolerance to deleterious factors.
- Adaptive genomic mutations.

The chemical contents of a bacterial cell (such as the DNA, RNA, and protein) vary with the environment, for example, growth of genetically identical bacteria in different nutrient media or at different temperatures can alter their chemical

composition (Marr and Ingraham 1962;Neidhardt 1963). Similarly, environmental conditions, such as starvation, temperature, osmolarity, and low pH, can trigger virulence mechanisms in pathogenic bacteria (Mekalanos 1992). Thus, bacterial behaviour and phenotype (including their ability to cause infection) is dictated by their surroundings. Hence, considerable attention should be given to the conditions under which these organisms grow (Harder and Dijkhuizen 1983).

In response to changes in their environment, bacteria have the ability to quickly regulate the expression of genes that control their growth and physiology (Hoch 2000). Because bacterial gene expression is tightly regulated at the transcriptional level (Rhodius et al. 2002) and prokaryotic RNAs have short half-lives (Conway et al. 2003), transcriptional profiling has been widely used in characterization of bacterial responses to various environmental conditions (Eriksson et al. 2003;Rhodius et al. 2002). Although there are various technologies to study gene expression, reverse transcription followed by quantitative real-time PCR (qRT-PCR) is a sensitive tool to quantitatively analyze RNA levels transcribed from a relatively large number of genetic regions. In addition, it can quantify low abundance RNAs and, with slight modification, can be applied to measure all categories of RNAs (Chen et al. 2005). Moreover, direct measurement of RNA levels from a set of responsive genes that either get induced or repressed under a specific environmental condition can reveal information about bacterial responses and be critical to understanding conditions in microenvironments around bacteria at the time of expression profiling.

The role of changes in microenvironments in determining bacterial responses varies with environmental conditions. In particular, microenvironments are logically impacted mostly under conditions where physical forces are altered; specifically, in this study the focus was on the cold stress condition. In addition, physico-chemical factors, such as pH, availability of oxygen (DO) and H_2O_2 concentrations, impact bacterial growth and gene expression. Simultaneous analysis of the above factors under different growth conditions provides information relevant to the specific gene in bacterial microenvironments.

3.2 Heat Shock Response

3.2.1 Overview

Cells subjected to heat shock, or a variety of other stresses, increase the synthesis of a set of proteins, known as heat shock proteins. This response is apparently universal, occurring in all forms of life from bacterial cells to mammalian cells (Sherman and Goldberg 1992). A large variety of stress conditions including physicochemical factors induce the synthesis of more than 20 heat shock proteins (HSPs). Heat shock response in *E. coli* transiently increases following an up shift from 30 to 42 °C inducing the rapid induction of HSPs, followed by an adjustment period where the rate of HSP synthesis decreases to reach a new steady-state level. Major HSPs are molecular chaperons which are in general important for cell survival, since they play a key role in preventing aggregation and refolding of proteins. Previous studies indicate that σ^{32}, which is a product of the *rpoH* gene, is directly responsible for regulation of the heat shock response (Straus et al. 1987). In *E. coli*, the expression of the *rpoS* regulon is known to be crucial for survival in liquid

cultures during the stationary phase (Saint-Ruf et al. 2004). But its role under heat shock in biofilm phase has not been investigated.

An adaptive or protective response may occur when microorganisms are stressed, which leads to increased tolerance of the microorganism to the same or to a different type of stress. This observable fact is also called an adaptive response, or induced tolerance, habituation, acclimatization, or stress hardening. The capability of a microorganism to adapt quickly to a shifting environment is very essential for growth and survival (Hengge-Aronis 1999). It can be short or longer term adaptation to an environment. A variety of stress related proteins may be induced that protect the cell during stress. *E. coli* has large numbers of signal transduction systems and regulatory mechanisms that allow it to respond to various environmental cues (Hengge-Aronis 1999).

Reduced growth rate, or induced entry into stationary phase, is indicative of general stress response (Cao and Sarkar 1997). Stress response is genetically regulated. Activation of the general stress response results in the expression of stress adaptive genes. Stress adaptive genes such as *bolA* which play a role in controlling cell morphology, *cfa* which is involved in cyclopropane fatty acid synthesis, and *uspB* which is important in ethanol resistance are examples of stress adaptive genes (Abee and Wouters 1999).

At the molecular level under different stress conditions, two sigma subunits of RNA polymerase, σ^S (*rpoS*) and σ^{70} (*rpoD*), coexist in *E. coli* cells. σ^S is subject to complex regulation in addition to long term stress adaptation different than σ^{70}. These two sigma factors control different genes, although they are very

similar both in their structure and function. The *rpoS* gene is a regulator for stationary phase gene expression which is responsible for the induction of a specific subset of genes. This gene is only expressed during stationary phase or under stress conditions and increases the resistance of the cell to a range of stresses (Rowe and Kirk 1999).

Sigma factor σ^{70} is encoded by *rpoD* gene and is considered as a housekeeping gene. The transcription of *rpoD* gene is growth dependent (Rijpens et al. 2002). *RpoD* confers promoter-specific transcription initiation on RNA-polymerase and it is vital for cell growth (Yamamoto et al. 2000).

The stress response system of a microorganism can be activated by several different stresses and may later protect against multiple stresses in the stationary phase. In the stationary phase, *E. coli* produces 30 different types of proteins that are regulated by RNA polymerase sigma factor (Arnold and Kaspar 1995). A varied range of stresses such as heat shock, cold shock, osmotic stress, ethanol, or starvation induces the same set of proteins, called general stress proteins. These proteins provide general stress protection (Vorob'eva 2004).

3.2.2 Gene expression and importance of sigma factor and its role in stress response of *E. coli*

The term gene expression refers to the entire process whereby the genetic information is decoded to produce a particular protein. Although it is a complex process involving many different steps, transcription initiation through

promoter clearance and release from the RNA polymerase is the most important control point in determining whether or not most genes are expressed.

Extracellular signals

(starvation, acetate, homoserine lactone etc.)

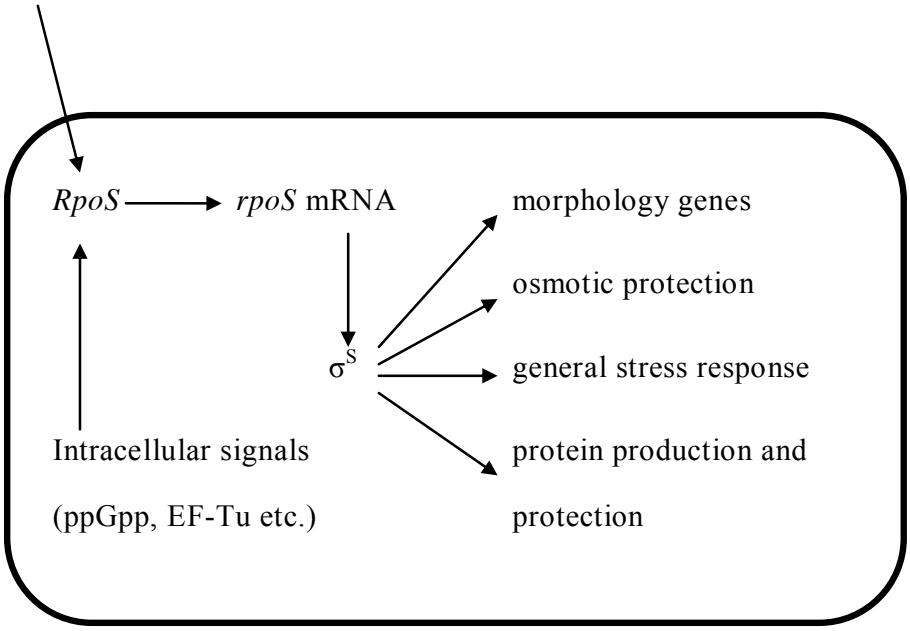


Figure 3.1: A preliminary representation of the *rpoS* regulon, adapted from (Huisman and Kolter 1994). The *rpoS* gene encodes a stationary phase specific sigma factor denoted by σ^S. Stressful environments and certain intracellular signals induce the expression of the gene and the production of the sigma factor. The sigma factor then initiates the transcription of the genes included in the *rpoS* regulon.

Different unfavourable environmental conditions trigger a bacterial expression of an appropriate set of genes in order to survive. External stimuli cause exchange of sigma subunits in the RNA polymerase (RNAP) complex and gene expression is adjusted to newly emerged needs. The RNA polymerase of *E. coli* is composed of the core enzyme (including *α2, β* and *β'* with the catalytic

activity of RNA polymerization), and one of seven different species of σ subunit (Table 3.1). Sigma species may clearly identify distinguishable gene promoters thereby activating distinct gene sets, known as regulons. The majority of the housekeeping genes expressed during exponential growth phase and are transcribed by the holoenzyme containing the *rpoD* gene product, while the holoenzyme σ^S is crucial for transcription of certain stationary-phase specific genes (Lange and Hengge-Aronis 1991b).

The σ^S or *rpoS* subunit is considered the general stress sigma factor. When a cell is in stationary or in exponential phase exposed to various stress conditions, it is strongly induced (Fig. 3.1). Various stress conditions include: ultra-violet radiation, hyperosmolarity, pH downshift, and non-optimal high or low temperature regimes (Hengge-Aronis 1999). The σ^S increase is often accompanied by a reduction or cessation of growth and provides cells with the ability to survive the actual stress as well as additional stresses not yet encountered, ("cross-protection"). The dual nature of the σ^S stress response is different from other specific stress responses, which are triggered by a single stress signal and result in the induction of proteins that allow cells to overcome only a specific stress.

Table 3.1: Various sigma subunits of *E. coli* with their number of genes under the control of each sigma factor (Lange and Hengge-Aronis 1991a;Lange and Hengge-Aronis 1991b).

.Sigma subunit	Protein size (aa)	Kd(nM) (RNAP-σ)	Intracellular concentration (molecules/cell)	Genes under the control of each sigma
RpoD	613	0.26	700	Growth related genes (~1000)
RpoN	477	1.55	110	Nitrogen regulated genes (~15)
RpoS	330	4.26	□1	Stationary phase/stress response (~140)
RpoH	284	1.24	<10	Heat shock/stress response (~40)
RpoF	239	0.74	370	Flagella-chemotaxis genes (~40)
RpoE	202	2.43	<10	Extreme heat shock/extracytoplasmic genes (~5)
Fecl	173	1.73	<1	Ferric citrate transport (~5)

3.3 Cold Shock Response

3.3.1 Overview

It was initially reported, when the *E. coli* growing at 37 °C was down shifted to 10 °C**,** that their growth was halted for 4 hours before renewed growth is established. During this lag period, a set of proteins, so-called cold shock proteins, is induced (Jones et al. 1987a). This response describes a specific pattern of gene expression in response to a downshift in temperature, which includes the induction of cold shock proteins, continued synthesis of transcriptional and translational proteins despite the lag period, and specific repression of heat shock proteins (Pamela and Masayori 1994). Since the initial discoveries in *E. coli*, cold shock responses and cold shock proteins have been

investigated in other prokaryotic as well as eukaryotic organisms (Berry and Foegeding 1997). Although many questions remain to be answered, including, information about the identification of many of the cold shock proteins, the induction of the response by other stimuli, the identification of possible regulators of some cold shock proteins, and the role of biofilm formation in cold shock conditions.

3.3.2 Cold shock response of *E. coli*

Response of *E. coli* to cold shock (10 °C) resulted in an induction of a specific set of cold shock proteins at rates 2 - 10 times greater than rates of synthesis at 37 °C (Jones et al. 1987b). The cold shock response, which occurs during the lag or acclimation period immediately after temperature downshift, is repressed when cells resume growth (Bae et al. 1997). The most studied response to cold temperature is the synthesis of cold shock proteins (CSPs). Increased amounts of small (7 kDa) proteins are synthesized by many bacteria when there is a shift to colder temperatures. A minimum of 15 various types of cold shock proteins were induced in *E. coli* (Jones et al. 1987a). These proteins were implicated in a variety of essential functions such as translation, transcription, mRNA degradation, recombination and protein synthesis in *E. coli* (Goldstein et al. 1990a;Graumann and Marahiel 1998;Jiang et al. 1997;Jones et al. 1987b). In pathogenic and non-pathogenic *E. coli*, CspA acts as a chief cold shock protein. It bound to RNA without apparent sequence specificity and with low binding affinity (Jiang et al. 1997).

Secondary structure in the RNA molecules is destabilized by CspA, which in turn rendered mRNA more prone to RNase degradation in *E. coli* (Jiang et al.

1997). Therefore, it works as an RNA chaperone and helps in facilitating the translation process at low temperatures. The expression of CspA at low temperature was regulated at various levels of transcription, mRNA stability, and translation (Phadtare et al. 1999). It has been reported that refolding of cold damaged proteins and proper folding of proteins was important after cold shock, as with heat shock–inducible molecular chaperones. Therefore, cold shock proteins functions as an RNA chaperones at low temperatures, while HSPs function as protein chaperones at high temperatures (Graumann and Marahiel 1998). However, a specific sigma factor has not been identified in the case of the cold shock response as is the case in heat shock response (Phadtare et al. 1999a).

In *E. coli*, CspA is the major cold shock protein, comprising 13% of the total protein synthesis (Goldstein et al. 1990a). It has been speculated that CspA functions as an RNA chaperone to prevent the formation of stable secondary structures in RNA molecules at low temperatures and thus assists translation of cellular mRNAs at low temperature (Pamela and Masayori 1994). In addition to CspA, *E. coli* contains a large family of CspA-like proteins from CspB to CspH, among which only CspB and CspG have been shown to be cold shock proteins (Bae et al. 1997). Other cold shock proteins found in *E. coli* include NusS (involved in both termination and antitermination of transcription), polynucleotide phosphorylase (involved in the degradation of mRNA), RecA (dual roles in recombination and the induction of the SOS response), H-NS and GyrA (both involved in DNA supercoiling) (Pamela and Masayori 1994), as

well as CsdA and RbfA (both important for ribosomal structure) (Abee and Wouters 1999).

3.3.3 Impact of cold shock on microorganisms

In order to survive low temperature stress, bacteria develop various mechanisms that permit low temperature growth (Russell et al. 1990). This involves maintenance of the structural integrity of macromolecular and macromolecule assemblies such as proteins and ribosomes. Another mechanism is a membrane modification which maintains membrane fluidity (Berry and Foegeding 1997b;Russell et al. 1990). Membrane lipid composition changes with decrease in growth temperature to an increased proportion of shorter and/or unsaturated fatty acids. These modulated activities of intrinsic proteins perform various functions such as ion pumping and nutrient uptake (Russell et al. 1990).

Temperature change serves as an effective signal to regulate gene expression in *E. coli* and other bacteria. *E. coli* are likely to encounter shifts to lower temperature, either for short term or long term gain during their life cycle (White-Ziegler et al. 2008). It is particularly important to understand how *E. coli* are able to adapt to low temperatures in all industrial settings where prevention of bacterial contamination is of the utmost important. Low temperature shift from 37 °C to 23 °C causes an increased expression of *rpoS* and *bolA* during the exponential phase (White-Ziegler et al. 2008). As *bolA* is considered to be an *rpoS* dependent gene (Lange and Hengge-Aronis 1991;M.Aldea et al. 1989), and it is expected to be induced at low temperature in exponential phase. Many bacteria synthesize increased amount of small

proteins known as cold shock proteins (CSPs) when there is a shift to cold temperature. This is an area which has been extensively studied (Jones et al. 1987b). Generally, there are 15 different types of cold shock proteins which have been induced in *E. coli* and they are all involved in essential functions like transcription, translation, protein synthesis, mRNA degradation and recombination in *E. coli* (Graumann and Marahiel 1998;Jiang et al. 1997). However, specific sigma factor has not been specifically identified in this case of cold shock response unlike heat shock response (Phadtare et al. 1999a).

It has been noticed that several genes with increased expression at 23 °C are involved in biofilm development (White-Ziegler et al. 2008). For example, *bolA* has been shown as one of the important genes in biofilm formation in *E. coli* and its transcription is induced in response to a variety of stresses. One study revealed that low temperature increases the expression of *bolA* 3.5 folds (White-Ziegler et al. 2008). These data suggest the importance of low temperature which increases the expression of genes that might have an important role in biofilm formation.

3.4 Acid Shock Response

3.4.1 Overview

Bacteria encounter a variety of potentially lethal acid stress conditions in a range of pathogenic and natural situations. To defy these stresses, pathogenic and commensal strains of *E. coli* possess remarkable and notable systems of acid resistance (AR) which is a programmed molecular response by which specific, stress-inducible proteins are synthesized (Mates et al. 2007). These

proteins presumably act to prevent or repair macromolecular damage caused by the stress. While some stress proteins are induced under many different conditions (e.g., universal stress proteins), others are induced only in response to a specific stress. Acid stress is defined as the combined biological effect of H^+ ion (pH) and weak acids including butyrate, propionate and acetate which can lead to fermentation (Bearson S et al. 1997;Gorden and Small 1993). This in turn results in an intracellular acidification that can damage the microbial biochemical processes (Bearson S et al. 1997).

Severe acidic stress e.g. (pH 3) results in faster proton leakage, and cells tend to lose their ability to maintain their homeostasis. Organic acids in their uncharged protonated form enter the cells resulting in acidification which causes cell death (Bearson S et al. 1997). How organisms survive during environmental stress is a fundamental question of biology. Understanding these systems will be crucial to the development of molecular biology where bacteria are asked to perform different tasks under stress.

The acid tolerance response (ATR) is defined as an induced protective response in microorganisms against acid stress (Gahan et al. 1996). The microbial response to acid stress is thought to include changes in membrane composition, increase in proton efflux, increase in amino acid catabolism, and induction of DNA repair enzymes (Beales 2004). Differences in ATR among different bacteria and between exponential and stationary phase cells have been reported (Hartke et al. 1996;Jordan et al. 1999). Intracellular or extracellular pH fluctuations can be a signal for induction of acid shock or stress adaptation proteins. External or periplasmic proteins may also be sensed by membrane

bound proteins (Foster 1999). Internal pH fluctuations may also affect gene expression or modulate a regulatory element that controls gene expression. *E. coli* for instance, will survive for hours in a pH 2 environment, whereas organisms such as *Vibrio cholera* and *Salmonella enterica* typically lose viability within minutes.

The inducible acid tolerance response (ATR) and acid shock response (ASR) which increases the resistance of stationary phase cells to acidic conditions were reported in *E. coli* (Garren et al. 1997;Garren et al. 1998). Acid tolerance response is a two-stage process involving an initial pre-shock exposure to a mild pH range between 5.0 and 6.0 followed by an acid challenge or shock exposure to a pH below 4.0 (Garren et al. 1998).

Acid shock response was performed by a rapid pH shift from a mild pH to a more strongly acidic pH, for example from 6.0 to 4.0 (Garren et al. 1998). Since stationary phase cells grown in a minimal glucose medium were used in these acid responses, it is possible that genes products resulting from the stationary regulation as *rpoS* could play a role in increased acid resistance (Garren et al. 1998). Additionally, it was found that a pH shift from 6.9 to 4.3 induced the synthesis of at least 16 polypeptides. Seven of these were specifically identified as acid shock proteins (Heyde and Portalier 1990). It has been suggested that the induction of acid shock proteins is associated with *rpoS* regulation and is required for ATR and ASR to provide acid stress protection to the cells (Garren et al. 1997;Garren et al. 1998).

Aaron *et al*., describe the cluster of 12 protein encoding genes located at 3652313 to 3665210 bp on the *E. coli* K-12 genome which has been termed an acid fitness island (AFI). These genes slpA through gadA are unique to *E. coli* and to its closely related genus *Shigella* which also exhibits some level of acid resistance (Mates et al. 2007).

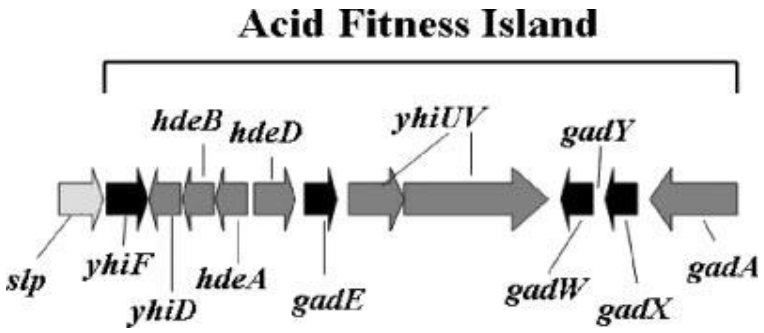


Figure 3.2: Diagrammatic representation of acid fitness island showing the cluster of 12 protein encoding genes in *E. coli* K-12 (Mates et al. 2007).

Enteroinvasive, enteropathogenic, and enterohaemorrhagic *E. coli* were significantly more acid tolerant than non pathogenic strains of *E. coli* K-12. Mechanistic and regulatory aspects of *E. coli* acid resistance have been intensively studied over the past decade and research has revealed two general forms of acid resistance. One form is amino acid dependent, while the other is amino acid independent (Mates et al. 2007). The mechanism of amino acid independent acid stress is also known as the glucose –repressed or oxidative acid resistance system. However, the amino acid dependent systems are known to require specific amino acid decarboxylases (GadA/B, AdiA, and CadA) and cognate antiporters (GadC, AdiC and CadC) that import amino acid substrates

(glutamic acid, arginine, or lysine) in exchange for exporting their respective decaroxylation products (γ-amino butyric acid, agmatine, and cadaverine) (Mates et al. 2007). Pathogenic and non pathogenic *E. coli* cells in the stationary phase were substantially more acid tolerant than the cells in the log phase.

3.4.2 Types of acid resistance systems

Depending on the growth phase, medium, and type of acid, different systems provide resistance to microorganisms. Three acid resistance systems have been identified in the stationary phase of all *E. coli* (Fig. 3.3). The induction of these systems is dependent on the type of medium and the growth conditions (Lin et al. 1996). The three complex medium dependent acid resistance systems included an oxidative system (AR1) and two fermentative acid resistance systems involving a glutamate decarboxylase (AR2) and an arginine decarboxylase (AR3) (Bearson S et al. 1997;Lin et al. 1995;Lin et al. 1996).

The oxidative system (AR1) is induced by growth in the stationary phase in Luria Bertani (LB) broth and is repressed by glucose. It is dependent upon σ^S whereas the arginine and glutamate acid resistance system is partially dependent on alternative sigma factor (Chung et al. 2006).

The glutamate (AR2) and arginine inducible amino acid decarboxylases (AR3) were associated with the accumulation of proteins in the growth medium. These amino acid decarboxylation systems play a key role in pH homeostasis and internal pH maintenance (Chung et al. 2006). The glutamate and arginine decarboxylase systems in *E. coli* are considered distinct systems. The Gad system encompassed three genes that are essential components of the glutamate

induced AR2. Two of the three genes GadA and GadB, encode highly homologous glutamate decarboxylase isoforms (protein that has the same function as another protein but which is encoded by a different gene) in *E. coli* and the third gene GadC encoded a putative glutamate: γ-amino butyric acid antiporter. Arginine decarboxylase, encoded by AdiA was responsible for AR3 based acid survival in *E. coli* (Chung et al. 2006;Lin et al. 1996).

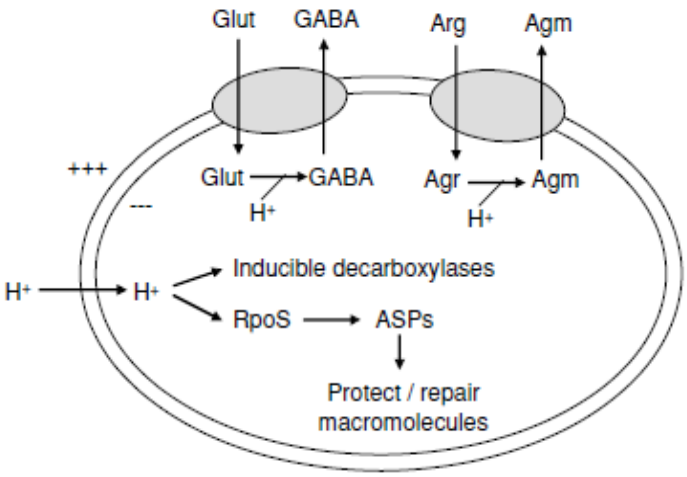


Figure 3.3: Diagrammatic representation of acid stress response in *E. coli*. The figure represents increase accumulation of *rpoS* that controls sets of acid shock proteins (ASPs). The ASPs function to prevent or repair acid-induced damage to macromolecules. Glut, (glutamate); Arg, (arginine) GABA, (gamma-amino-isobutyrate); Agm, (agmatine). Adapted from (Chung et al. 2006).

When *E. coli* cells enter the stationary phase, a depletion of nutrients or starvation conditions are encountered, and a number of morphological and physiological changes occur. Stationary phase cells became smaller and rounder, the cells accumulated storage compounds such as glycogen and

polyphosphate, and the DNA condensed (Nystrom 1995). There were a number of changes in the fatty acid composition of the inner membrane and in the protein composition of both inner and outer membranes in *E. coli* (Chung et al. 2006). *RpoS* is the major regulator of the stationary phase or general stress response in *E. coli* and other enteric bacteria. This alternative sigma factor has been recognized as a key factor in producing greater resistance of stationary phase and stressed cells.

E. coli in general has multiple genetic systems that respond to physical and chemical challenges that confer resistance to low and lethal pH. Data on acid tolerance response (ATR) are available for a number of food-borne bacteria or related to food microbiology but none are available for biofilms. In short biofilm production in relation to acid tolerance is a complex biological phenomenon, where different systems are involved which vary with organism, growth phase, medium, type of acid stress, and other environmental factors.

3.5 Oxidative Stress Response

3.5.1 Overview

The appearance of aerobic forms of life was an important step in the evolutionary process, since oxygen consumption leads to the production of ten-fold more energy from glucose than does anaerobic metabolism (Asad et al. 2004). However, this process imposes constraints on cell viability, because of the generation of reactive oxygen species (ROS) during respiration including superoxide anions, peroxide and hydroxyl radicals. These ROS are commonly involved in oxidative stress, which can damage proteins,

DNA and membranes. Oxidative stress can be functionally defined as excess of oxidants in cells (Fridovich 1978). The damage caused by H_2O_2 to *E. coli* cells and its involvement in biofilm production as an environmental stress response is unclear. Killing of *E. coli* by exogenous H_2O_2 revealed that *E. coli* was sensitive to both low concentrations of H_2O_2 (1-3 mM, mode one) and high concentrations of H_2O_2 (>20 mM, mode two), but less sensitive to intermediate concentrations (Imlay and Linn 1988). In mode one killing, actively growing cells were sensitive to low doses of H_2O_2, particularly in mutants lacking enzymes required for recombination or the base-excision DNA repair pathway (Storz et al. 1990).

In order to survive and proliferate, the bacteria have evolved effective mechanism to detoxify and repair damages caused by these ROS. The microbial oxidative stress response is an orchestrated set of reactions involving the synthesis of many proteins and small molecules (Demple 1991). The components of these responses can be divided into three categories. The first category is ROS detoxification: involving both antioxidant enzymes and molecules; for example, superoxide dismutase (SOD) breaks down superoxide anions to H_2O_2 that is further metabolized by monofunctional and bifunctional catalases to H_2O_2 and O_2. The second category of the response is reparation of damaged macromolecules involving various enzymes such as exonuclease III, which repairs oxidatively damaged proteins. The last component is process regulation, involving regulatory proteins such as *rpoS*, SoxRS and OxuR which regulate and coordinate the global responses to oxidative stress (Farr and Kogoma 1991;Rosner and Storz 1997;Storz and Imlayt 1999).

In many microbes, sub-lethal exposure to a stress can confer resistance to a lethal exposure to the same agent (adaptive response) or to unrelated agents (cross protection response). For example, *E. coli* pre-treated with a low dose of H_2O_2 acquire adaptive resistance to subsequent challenges to high doses of H_2O_2 and at the same time show cross resistance to heat stress (Christman et al. 1985;Farr and Kogoma 1991;Mongkolsuk et al. 1998). Similarly, *E. coli* cells pre-treated with low doses of superoxide generating compounds become resistant to the higher doses of the compound and heat stress (Greenberg and Demple 1989). These adaptive and cross protection responses result from coordinated induction of genes involved in both stress detoxification systems and damaged repaired processes. Thus, one stress signal may induce more than one regulon. Different microbes seem to evolve different mechanisms to protect themselves from oxidative stresses. These oxidative stress responses may be important to *E. coli* to overcome the stress and may lead to biofilm formation.

OxyR belongs to the LysR family of transcriptional regulators and is one of the first proteins shown to have oxidative stress sensing and transcriptional control activities (Kullik et al. 1995). LysR family members are all DNA-binding proteins which positively regulate the expression of their target genes, and many also repress their own expression (Schell 1993). *OxyR* has both DNA-binding and activation domains. The DNA-binding domain is responsible for *OxyR* binding to promoters of target genes, whereas, the activation domain recognises cell signalling for activation of gene expression. When bacterial cells are exposed to oxidative stress, inactive reduced *OxyR* is immediately converted into the oxidised form. Interestingly, in this case, only one protein

senses the biological stimulant (hydrogen peroxide treatment) and activates the cellular response (induction of gene expression). This is a common feature in bacteria; the cellular response to different stimulants involves one or few steps. It is convenient for a fast growing organism to have this kind of regulatory mechanism because it allows bacteria to rapidly adapt to new environmental conditions. Once the peroxide stress has been neutralized, oxidised *OxyR* must be converted back to the reduced form. Reduced *OxyR* can then act as a repressor of *OxyR* itself and some genes in the regulon (Mongkolsuk et al. 1998). The target genes that mediate oxidative stress – induction are mediated by a regulon named *OxyR* In *E. coli*, the *OxyR* regulon contains genes involved in hydrogen peroxide decomposition such as *katG* (catalase), *ahpCF* (alkyl hydroperoxide reductase), *gorA* (glutathione reductase) and *dps* (encoding a non specific DNA-binding protein that may protect against oxidative DNA damage and mutation (Farr and Kogoma 1991).

In this study, the focus will be on the expression of *rpoS* and *bolA* gene under different concentrations of hydrogen peroxide. To study the *E. coli rpoS* and *bolA* mutants is an essential step towards understanding its physiological role in the regulation of oxidative stress response which may result in biofilm formation.

3.5.2 Bacterial oxidative stress protective genes

The bacterial oxidative stress response is the result of well-programmed reactions involving the synthesis of many proteins and small molecules, which can be grouped into at least three categories:

1. those that regulate the process
2. those that detoxify ROS,
3. and those that repair damaged macromolecules.

The first category includes molecules that regulate genes involved in oxidative stress response (i.e., *OxyR*, SoxRS and *rpoS*) and regulatory proteins involved in signal transduction. The second group includes both enzymes and small molecules that are involved in detoxification of ROS such as superoxide dismutase (SOD) that breaks down superoxide anions to H_2O_2 and catalase that breaks down H_2O_2. Other proteins exist that do not have enzymatic activity but which can bind to macromolecules and protect them from ROS such as the non-specific DNA-binding protein. The last group includes enzymes involved in various repair processes of damaged cellular components such as exonuclease III (DNA repair) and methionine sulfoxide reductase (oxidatively damaged protein repair) (Farr and Kogoma 1991;Greenberg and Demple 1989;Mongkolsuk et al. 1998;Rosner and Storz 1997).

3.5.3 Transcriptional regulators of the oxidative stress response in Prokaryotes

Bacteria responded to a variety of stresses by the coordinate regulation of groups of genes (regulons). The common regulators are summarised in table 1. Multiple defences are known to protect aerobic organisms from toxic ROS produced by aerobic metabolisms. *E. coli* has independent multi-gene responses to two kinds of oxidative stress: excess H_2O_2 triggers the *OxyR* regulon, and excess superoxide anions or nitric oxide radicals trigger the SoxRS regulon which coordinates the transcriptional induction of at least 12 promoters

(Hidalgo and Demple 1996). In addition to *OxyR* and SoxRS, the *rpoS*-encoded σ^S subunit of RNA polymerase has also been recently shown to regulate the expression of antioxidant defence genes (Rosner and Storz 1997). The important transcription regulators in response to oxidative stress are summarised in table 3.2.

Table 3.2: List of antioxidant genes, activities and their regulators in *E. coli*.

Gene	***Activity***	***Regulators***
sodA	Manganese superoxide dismutase	$SoxRS^+$, AcrAB, FNR, IHF
fumC	Fumarase C	$SoxRS^+$, AcrAB, σ^S
acnA	Aconitase A	$SoxRS^+$, AcrAB, FNR, σ^S
zwf	Glucose-6-phosphate dehydrogenase	$SoxRS^+$
fur	Ferric uptake repressor	$SoxRS^+$, *OxyR*
micF	RNA regulator of ompF	$SoxRS^+$, OmpR, LRP
acrAB	Multidrug efflux pump	$SoxRS^+$
fpr	Ferredoxin reductase	$SoxRS^+$
nfo	Endonuclease IV	$SoxRS^+$
sodB	Iron superoxide dismutase	$SoxRS^+$
sodC	Copper-zinc superoxide dismutase	FNR, σ^S
katG	Hydroperoxidase I	*OxyR*, σ^S
ahpCF	Alkyl hydroperoxide reductase	*OxyR*
gorA	Glutathione reductase	*OxyR*, σ^S
grxA	Glutafedoxin 1	*OxyR*
dps	Non-specific DNA binding protein	*OxyR*, σ^S, IHF
oxyS	Regulatory RNA	*OxyR*
katE	Hydroperoxidase I	σ^S
xthA	Exonuclease III	σ^S

3.5.4 *RpoS* regulon

Cells in the stationary phase are constitutively resistant to a variety of stress conditions including exposure to high concentrations of H_2O_2, organic hydroperoxide or superoxide (Vattanaviboon et al. 1995). In *E. coli*, the expression of genes responsible for this increased resistance is controlled by a stationary phase-specific sigma factor σ^S or σ^{38}. This alternative sigma factor encoded by *rpoS*, formerly named *katF*, controls the expression of large number of genes involved in cellular responses to a diverse number of stresses, including starvation, osmotic stress, acid shock, cold shock, heat shock, oxidative DNA damage and transition to stationary phase. A list of over 50 genes whose expression is regulated by σ^S has been compiled. Among these genes are *katE* (HPII monofunctional catalase), *katG* (catalase peroxidase HPI), *gor* (glutathione reductase), *dps* (DNA binding protein), *rob* (DNA binding protein), *xth* (exonucleaseIII), all of which are important for resistance to oxidative stress. Although the transcription factor σ^S acts predominantly as a positive effector, it does have a negative effect on some genes (Loewen et al. 1998). The synthesis and accumulation of σ^S are controlled by mechanisms affecting transcription, translation, proteolysis and the formation of the holoenzyme complex (Loewen et al. 1998).

3.5.5 Catalase

Catalases or hydroperoxidases are an integral component of the bacterial cell's response to oxidative stress. Hydroperoxidases reduce the intracellular concentration of H_2O_2 by catalyzing the conversion of H_2O_2 to water and

oxygen (reaction 1) or by oxidising an intracellular reductant using H_2O_2 (reaction 2).

$$2\ H_2O_2 \xrightarrow{\text{Catalases}} 2\ H_2O + O_2 \quad \text{(Reaction 1)}$$

$$AH_2 + H_2O_2 \xrightarrow{\text{Peroxidase}} 2H_2O + A \quad \text{(Reaction 2)}$$

In many bacteria, there are two types of catalase enzymes, namely a monofunctional catalase HPII and a bifunctional catalase/peroxidise (HPI). Both catalase and peroxidase use H_2O_2 as a substrate, but catalase uses two electron transfer in dismutation of H_2O_2 to oxygen and water, whereas peroxidase are also capable of destroying H_2O_2, but requires NADH or NADPH as electron source.

In *E. coli*, HPI is encoded by *katG* and it is the most important cellular determinant for the resistance of H_2O_2. Although HPI possesses peroxidase activity, the intracellular substrate has not been identified. HPI is controlled at the transcriptional level by *OxyR*, which under normal conditions, is inactive in respect to *katG* transcription. However, under oxidative stress conditions (e.g. in presence of H_2O_2), two cysteine residues in *OxyR* are oxidised causing a conformational change in *OxyR*, who functions as a transcriptional activator of genes in the *OxyR* regulon including *katG*. In the case of *katG*, transcription is more than 10 folds higher when *OxyR* is in oxidised form. H_2O_2 mediated induction of *katG* transcription by *OxyR* is transient in nature since the redox

sensitive regulated protein is readily reduced to the inactive form in the cell, lasting only until H_2O_2 has been decomposed by catalase. In addition, an increase HPI catalase activity at stationary phase is due to *rpoS* – dependent transcription of *katG*. The second catalase HPII is encoded by *katE*. Its expression is not inducible by oxidative stress, but is regulated in a growth phase dependent manner. The synthesis of *katE* catalase requires the *rpoS* (katF) gene product as a positive regulatory factor. *RpoS* protein is recognised as an σ factor (σ^S or σ^{38}) whose synthesis is turned on during the stationary phase. It is believed that starvation triggers the synthesis of *rpoS*, which activates RNA polymerase to turn on transcription of the *katE* gene (Jenkins et al. 1988).

Less information was available on morphogene *bolA* in regards with its involvement in oxidative stress response of *E. coli*. Therefore, the role of *bolA* gene, during oxidative stress and in biofilm development is not yet fully understood. In this chapter, sudden change in expression of *rpoS* and *bolA* genes has been addressed under different hydrogen peroxide stress induced conditions with their involvement in biofilm formation in addition to heat, cold and acid stress.

3.6 Results and Discussion

3.6.1 Biofilm formation by *E. coli* K-12 MG1655

The growth curve of wild type and mutant strains in Luria-Bertani media in planktonic phase was developed as per section 2.5 in chapter 2. OD was measured at A_{600}. OD_{600}=1.0 was found to be exponential growth phase and OD_{600}=2.2 was considered to be stationary growth phase. It was found that *E. coli* can grow at the same growth rate even without *rpoS* and *bolA* gene in planktonic cells (Fig. 3.4).

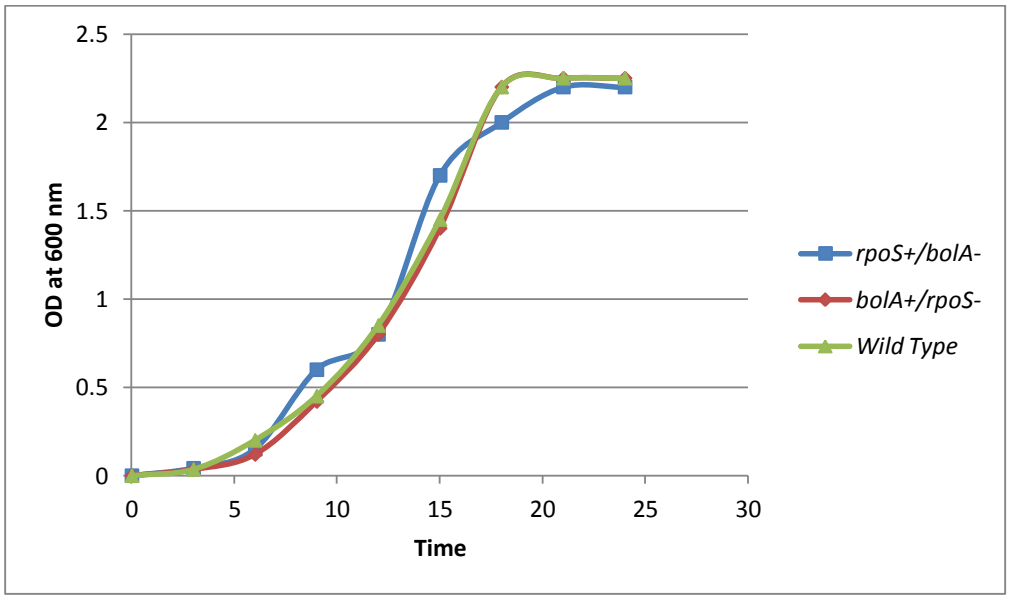


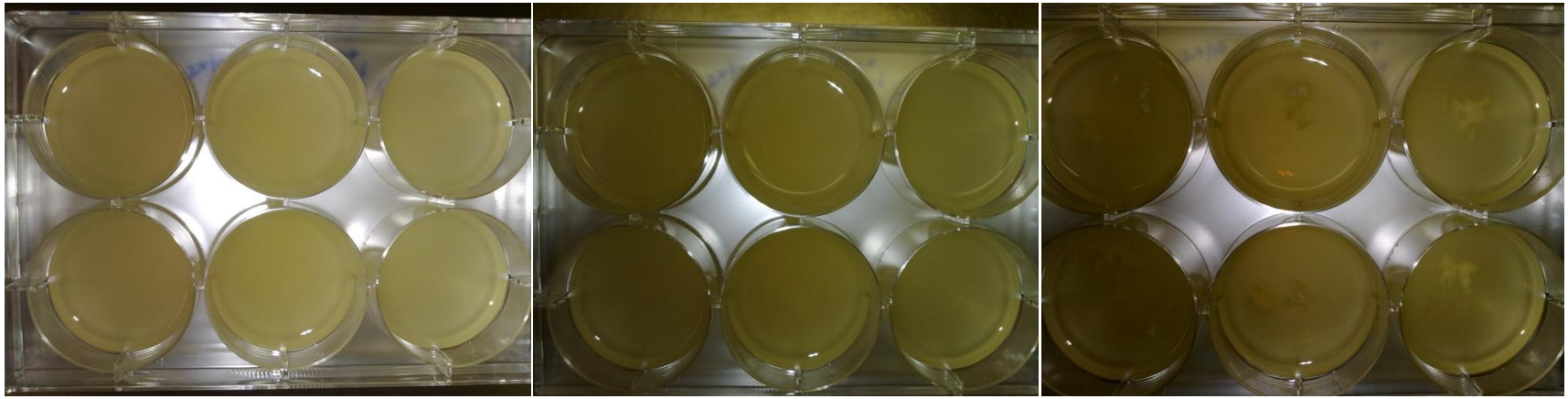
Figure 3.4: Planktonic growth curve of wild type (WT), *rpoS* mutant (Δ *rpoS*) and *bolA* mutant (Δ *bolA*) strains in LB media. Optical density was measured at A_{600}. OD_{600}=1.0 (exponential growth phase) and OD_{600}=2.2 (stationary growth phase). The data used is an average of three individual experiments.

Biofilm formation on the wall of the microtiter plate using wild type and mutant strains *(ΔrpoS and ΔbolA)* was carried out as per section 2.9 in chapter 2. Biofilm

mass produced by wild type and mutant strains was determined using crystal violet assay/Biofilm formation assay.

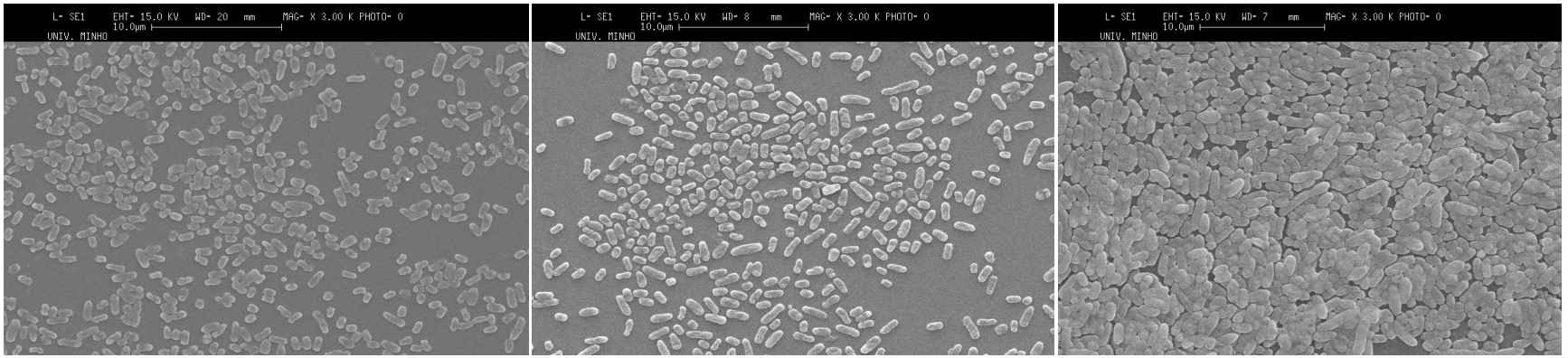
3.6.2 Adherence pattern

SEM confirms the adherence of the cells on the wall and increased amount of cells and initial biofilm formation was seen by WT, but not by Δ*rpoS* and Δ*bolA* strains (Fig. 3.6). The adherence pattern varies in *E. coli* K-12 MG1655 on different substrates (silicone, polypropylene and stainless steel), which is discussed in further chapter.



(A) **(B)** **(C)**

Figure 3.5: Cell aggregate/biofilm formation by *rpoS*$^{+}$/*bolA*$^{-}$ (A), *rpoS*$^{-}$/*bolA*$^{+}$ (B) and wild type i.e. *rpoS*$^{+}$/*bolA*$^{+}$ (C)



(D) **(E)** **(F)**

Figure 3.6: Scanning Electron Microscopy images of *rpoS*$^{+}$/*bolA*$^{-}$ (D), *rpoS*$^{-}$/*bolA*$^{+}$ (E) and wild type i.e. *rpoS*$^{+}$/*bolA*$^{+}$ (F), showing the increase in number of cells attached in the presence of *rpoS* and *bolA* genes.

From the scanning electron microscopic (SEM), experiments direct examination of the adherence patterns of the three strains were revealed. It was found that, the cells could not adhere to the bottom of the 6 well PVC tissue culture plate, but could attach to the wall of the wells. This was revealed after the scanning electron microscopic observation was done of the base and wall of the well. All the three strains including WT failed to produce biofilm on the base of the plate, but WT could grow as a cell aggregate in the media. This shows that biofilm formation and its development is substrate specific. PVC plates were not found to be a suitable substratum for *E. coli* K-12 MG1655 to attach and form biofilms. Therefore, adherence pattern was also studied and discussed in detail in chapter 5, using three different surfaces (silicone, polypropylene and stainless steel) inside the 6 well tissue culture plates and biofilm formation was seen under different environmental stress conditions on different substrate. These results suggest a new phenotype for *bolA* gene. In addition to its ability to produce a round morphology at stationary phase, *bolA* is implicated in biofilm development.

3.6.3 Preliminary conformational test

Before proceeding with Real-Time PCR expression analysis, preliminary experiment was done to ensure that the *E. coli* K-12 strain used in this study possessed a functional *rpoS* sigma factor apart from PCR and agarose gel electrophoresis. *RpoS*$^{+}$ Strains were screened for their ability to synthesize glycogen, as glycogen synthesis is under the direct control of *rpoS* to detect the *rpoS* mutant status. The glycogen synthesis in the *E. coli rpoS* mutant (white

colonies indicate the absence of glycogen), while the wild type strain was shown to be glycogen-positive (dark colonies) (Fig. 3.7).

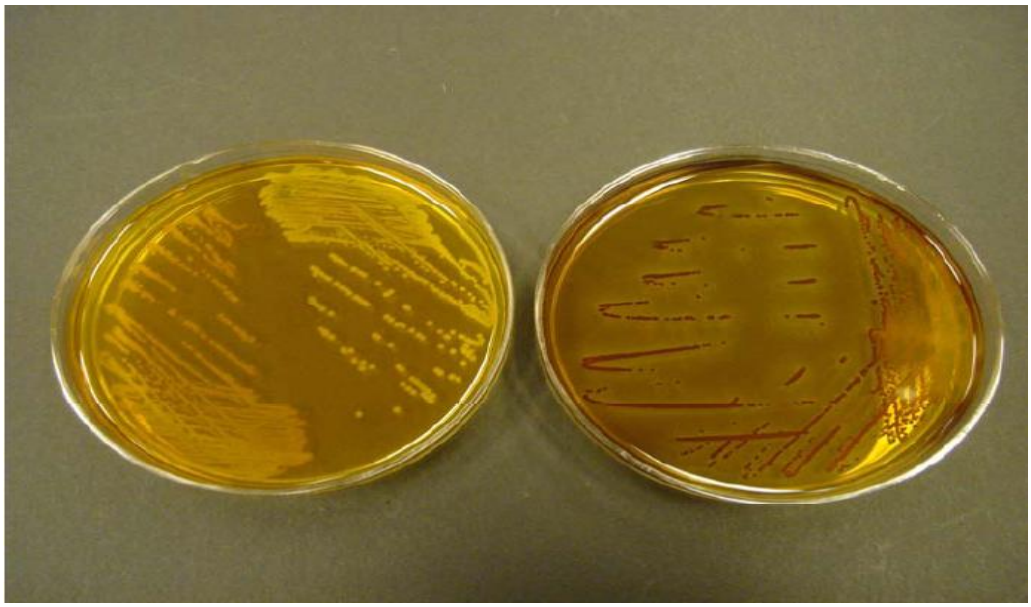


Figure 3.7: Glycogen phenotypes of wild-type *E. coli* and *rpoS* mutant strains reveal a functional and non functional status of *rpoS* sigma factor. Colonies were grown overnight at 37 °C and then stored at 4 °C for another 24 h. Intracellular glycogen in colonies was stained with iodine, resulting in two phenotypes: (i) dark brown colonies indicate glycogen-containing cells with a functional σ^S (wild type) and (ii) white colonies indicate glycogen-deficient cells with non-functional σ^S (mutant).

3.6.4 Primer designing

The PCR primers were optimised for the annealing temperature and were designed in house. The optimum annealing temperature for the amplification of *rpoS* and *bolA* was determined to be 60 °C (Fig. 3.8). The product sizes were 273, 216 & 201 bp for *rpoS*, *bolA* and for 16S rRNA respectively. Throughout

in this study, ribosomal gene 16S rRNA was used as a reference gene. The analysis of integrity of RNA was routinely checked using formaldehyde agarose gel electrophoresis (Fig. 3.9).

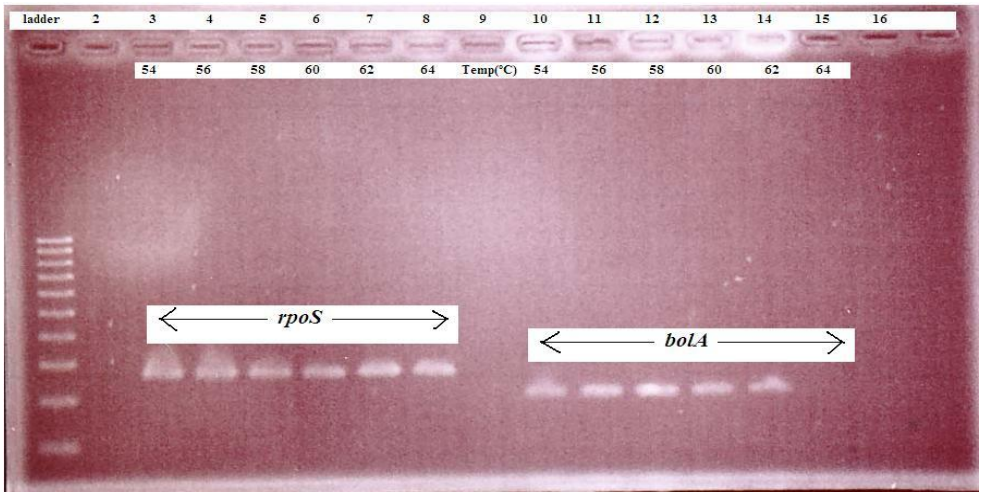


Figure 3.8: Agarose gel showing optimised primers for *rpoS* and *bolA* genes at different temperatures with a product size of 273 and 216 bp.

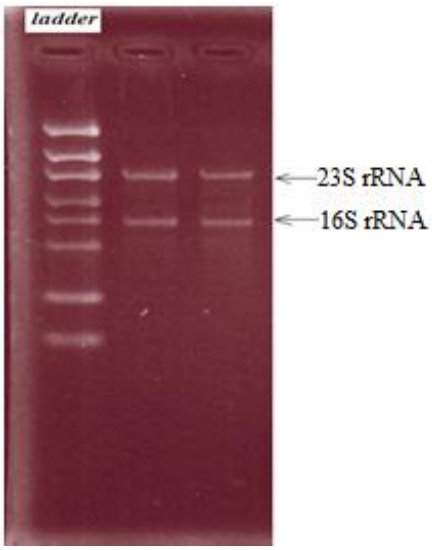


Figure 3.9: The analysis of the integrity of RNA, by formaldehyde agarose (1.5% w/v) gel electrophoresis, from the total RNA samples extracted from exponentially growing *E. coli* K-12 MG1655 cells. The size of 16S rRNA and 23S rRNA was 1.5 and 2.9 Kbp.

3.6.5 Real-Time PCR

Real time RT-PCR was used to examine the expression level of *rpoS, bolA* and reference gene (16S rRNA) in biofilms and planktonic cells. Total RNA was extracted, and was converted to cDNA with 30 min incubation at 42 ºC and 2 min inactivation at 95 ºC. The cDNA was processed to real time PCR with ABI PRISM 7500 (Applied Biosystems). Reactions were performed in a 12.5 µl reaction volume. Specific primers for 16S rRNA, *rpoS* and *bolA* were used and optimised using verity (Applied Biosystems) (Fig. 3.8).

A number of reference (housekeeping) genes have been described in the literature and are used at different frequencies. Reference genes represent the by most common method by far for normalizing qRT-PCR data. Reference genes (previously termed housekeeping genes) such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and 16S rRNA are historical carry-overs from RNA measurement techniques that generate more qualitative results. These genes were found to be essential and, importantly, always switched on. The choice of a suitable reference gene is dependent on the individual model and requires investigation of the appropriate literature.

3.6.6 Preparation of DNA standards and a standard curve for quantification using Real-Time PCR

Absolute quantification is the process that determines the absolute quantity of a single nucleic acid target sequence within an unknown sample. Absolute quantification was performed using Applied Biosystems® 7500™ Real-Time PCR. For the absolute quantification of the starting template present, a set of

standards is required. The concentration of seven standards was accurately determined and the number of copies was calculated. All seven standards were included in every real-time RT-PCR experiment conducted. The Ct value obtained for each standard was used to construct a calibration curve for the subsequent determination of the concentration of starting template in each of the samples (cDNA) analysed. This procedure was automatically performed using SDS software 1.3.1.

Relative quantification was employed for determination of the relative level of expression of the genes of interest and the housekeeping gene for all experimental samples after generating the Ct values using absolute quantification. The advantage of absolute quantification is the quality of results, which provide information on actual levels of a given mRNA, in this case *rpoS* and *bolA* mRNA. Furthermore, the results can be compared as independent results, and are not linked to parameters specific to the experiment.

The calibration curve was obtained during the runs performed with the DNA standards, and the original screenshot of a standard curve generated during the experiment was taken as an example (Fig. 3.10). The PCR amplification efficiency can be determined from the slope of the calibration curve. A slope equal to -3.3 indicates 100% efficiency. It should be noted that absolute quantities of each template are calculated based on individual calibration curves generated during individual PCR runs. The optimal baseline and threshold setting for each experiment was set to manual Ct (i.e., threshold 0.02). Ct values were generated for preparation of the standard curve for each standard using seven independent experiments.

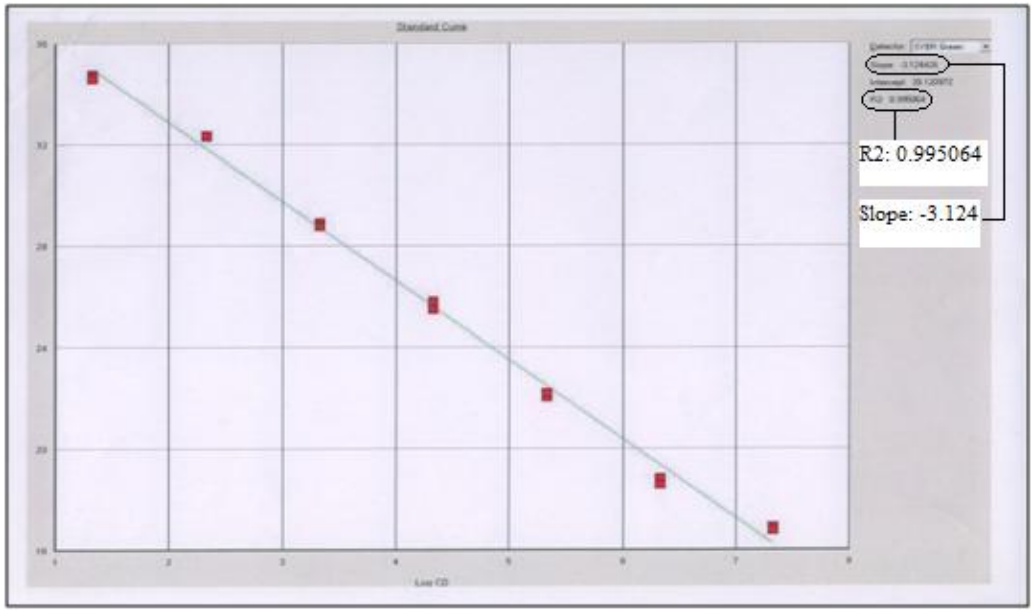


Figure 3.10: Diagrammatic illustration of the calibration curve generated from the average C_T values for each standard, obtained from all Real-Time RT-PCR determinations performed for analysis of *rpoS* and *bolA* mRNA transcription. This was done by SDS software 1.3.1 which calculates the amount of unknown samples by interpolating values from this standard curve.

3.6.7 Melting curve analysis

The melting temperature of the specific product amplified from the initial 16S rRNA, *rpoS*, and *bolA* mRNA template had a predicted melting temperature of 83, 84, and 80 °C respectively (Fig. 3.11). From the melting curve plot, it could be deduced that no primer dimers or secondary products were formed because only one peak was seen, which corresponds to the desired product. The products of all real-time PCR experiments presented in this report were confirmed using melting curve analysis and by agarose gel electrophoresis analysis.

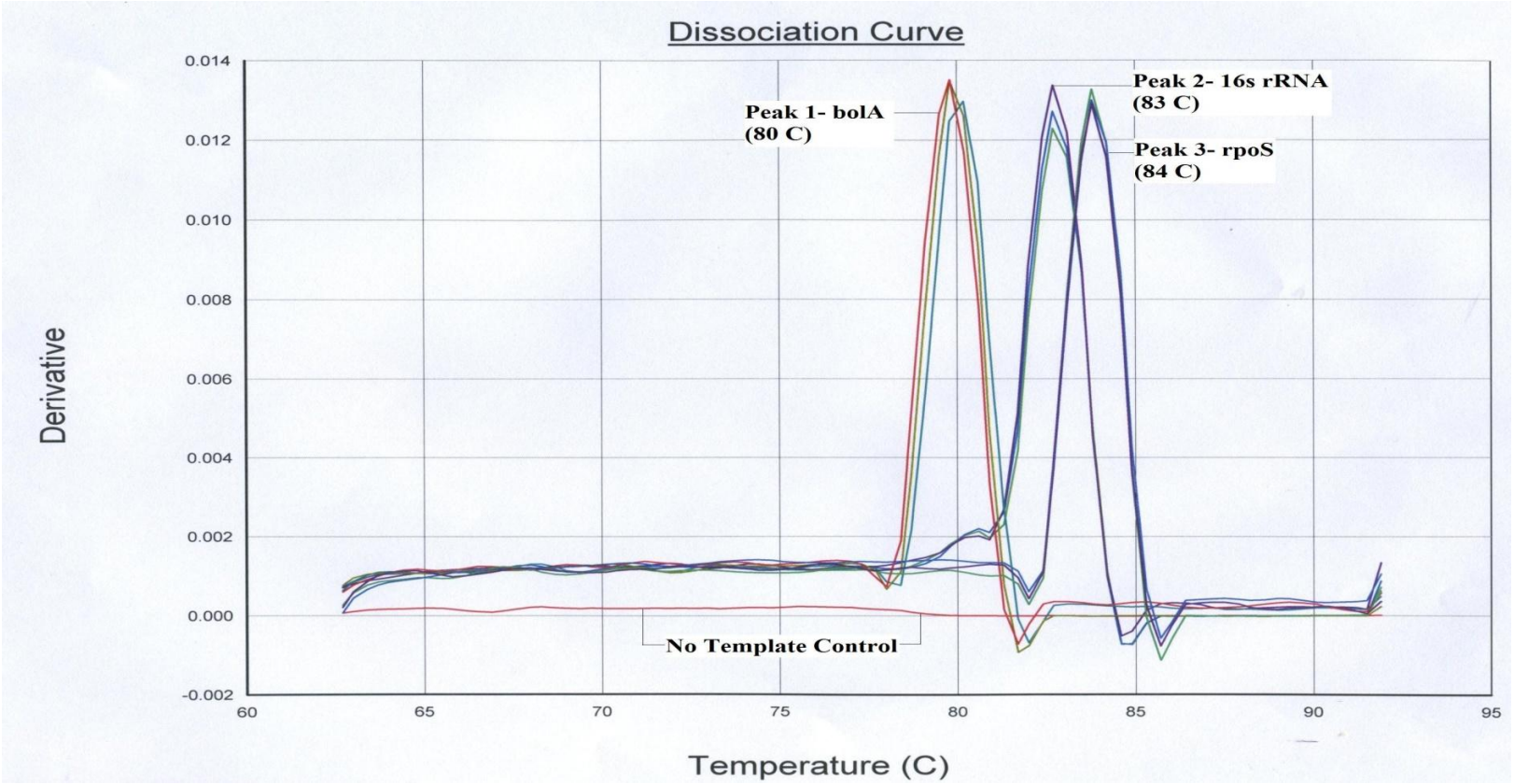


Figure 3.11: The above graph illustrates data from a typical real-time RT-PCR experiment with melting curve analysis. Two-step RT-PCR was carried out according to the optimised protocol described in section 2.21.3. It illustrates the calculated plot of fluorescence against temperature. Using this plot the melting temperature of the amplification product can be determined, which in this case is 83 ºC, 84 ºC and 80 ºC for 16S rRNA, *rpoS* and *bolA*. The data collected also includes no template control.

3.6.8 Expression of *rpoS* and *bolA* under non-stress conditions (37 ºC)

Expression of *rpoS* and *bolA* in WT and in mutated cells was measured at 37 ºC. It was found that expression of *rpoS* and *bolA* in WT strain was similar to the expression of *rpoS* and *bolA* in mutated strains (*rpoS*+/*bolA*- and *bolA*+/*rpoS*-).

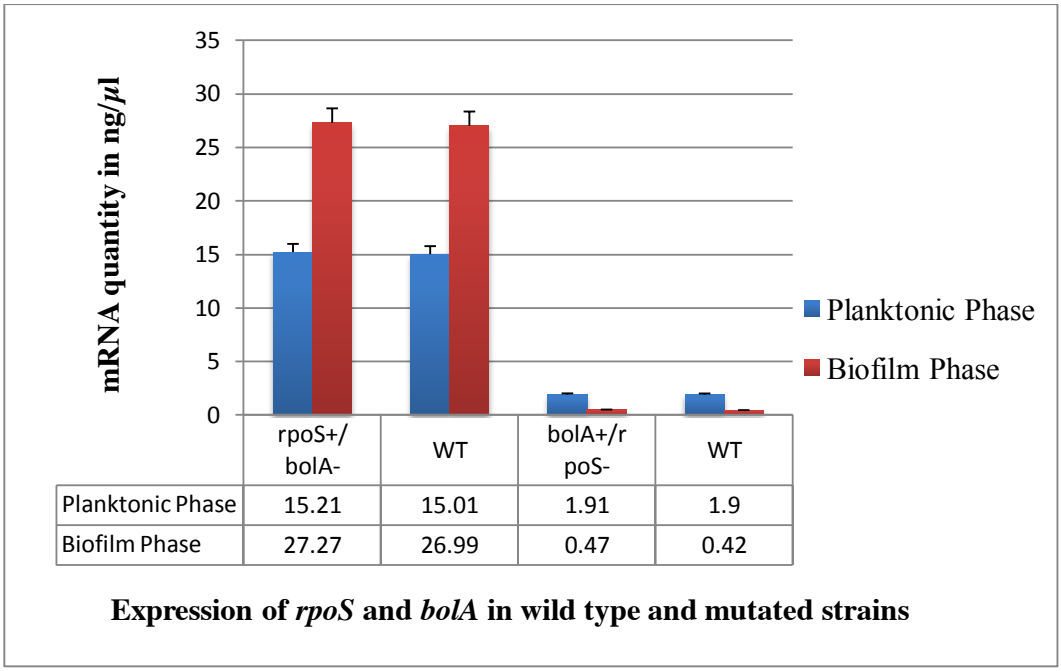


Figure 3.12: Bar graph representing the expression of *rpoS* and *bolA* in WT (*rpoS*+/*bolA*+) and mutated strains (*rpoS*+/*bolA*- and *bolA*+/*rpoS*-) at 37 ºC (control condition) in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA was measured in ng/µl.

3.7 Quantitation of mRNA Expression Level Using Standard Curve Method (Absolute Quantification) Under Heat Shock Condition

Earlier studies on *rpoS* and *bolA* genes have been done mostly with regard to long term stress conditions and biofilm formation under several forms of stress, including nutrient starvation in the stationary phase where the increased level of expression has been seen. This study was undertaken to determine whether *rpoS* and *bolA* genes can express under sudden stress condition (15 min of stress), i.e. a shift from optimal condition to heat stress (i.e. 37 °C to 42 °C and 46 °C) in both planktonic and biofilm phases of growth. Morphogene *bolA* is known to express in the stationary phase. Its expression in the biofilm phase at exponential level of growth and its possible role as a result of a sudden change in its environment were therefore interesting to investigate.

Real time RT-PCR was performed to quantify the expression level of the *rpoS* and *bolA* genes. As reported previously, 16S rRNA gene was used to analyze and compare the expression level with *rpoS* and *bolA* genes. 16S rRNA expression was found at nearly the same level under both stress and non-stress conditions in planktonic phase, as shown in the Figure 3.13. On the other hand, expression of 16S rRNA in biofilm phase was also found to be constant, but the quantity of mRNA was at very low level, approximately 20 folds less than planktonic cells (Fig. 3.13). The result shows that, there is a slight/no change in expression of 16S rRNA at 42 ºC and 46 ºC, when compared to 37 ºC.

Even though it was hypothesized that cells in biofilms were in stress conditions and expressed *rpoS* gene at high expression level, the *rpoS* gene expression

level was lower in biofilms than exponential planktonic cells. This suggests that cells in mature biofilms were in the stationary phase which show low activity and do not require the expression of the *rpoS* gene. The data indicates that gene expression within biofilm is different from that observed in standard planktonic growth cultures. After 15 min of heat stress i.e. a shift from 37 ºC to 42 ºC and 46 ºC, under planktonic form of growth, there was a sudden change in expression of *rpoS* and *bolA* genes (Fig. 3.14 and 3.15). 5 fold differences were observed in *rpoS* expression, when shift from 37 ºC to 46 ºC, while there was no obvious difference was seen in *bolA* expression under heat shock conditions. The level of expression of *rpoS* and *bolA* was found to be nearly 5 fold less in biofilms when compared with planktonic cells. There is no sudden change in expression of *rpoS* and *bolA* genes in the biofilm phase, which shows the transition of biofilm cells from exponential to stationary phase and the necessity of *bolA* gene for biofilm formation.

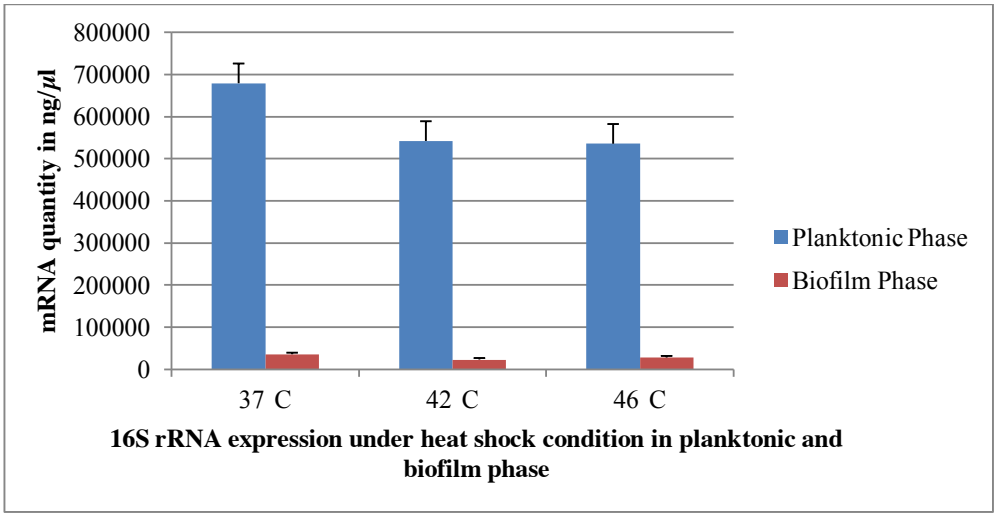


Figure 3.13: Bar graph representing the expression of 16S rRNA under heat shock conditions in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by heat shock and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA was measured in ng/µl.

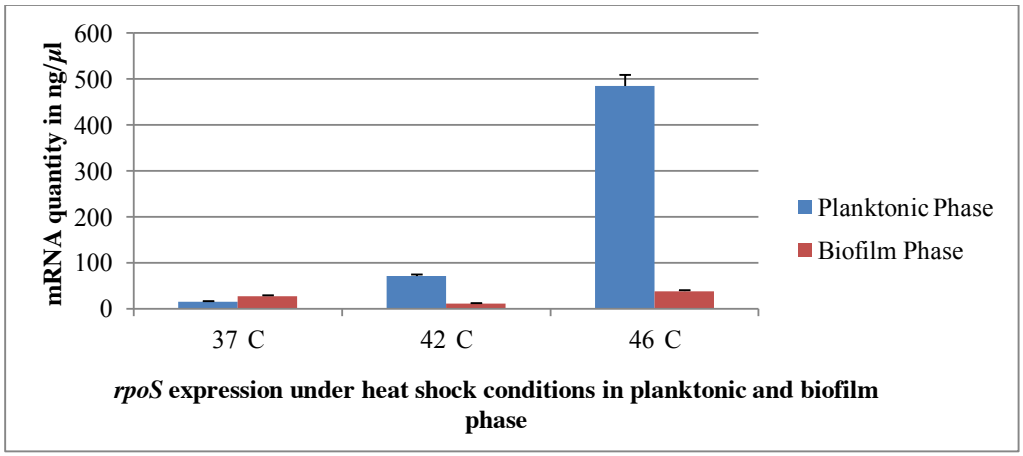


Figure 3.14: Bar graph represents the expression of *rpoS* gene (*rpoS*$^{+}$/*bolA*$^{-}$) under heat shock conditions in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37º C followed by heat shock and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

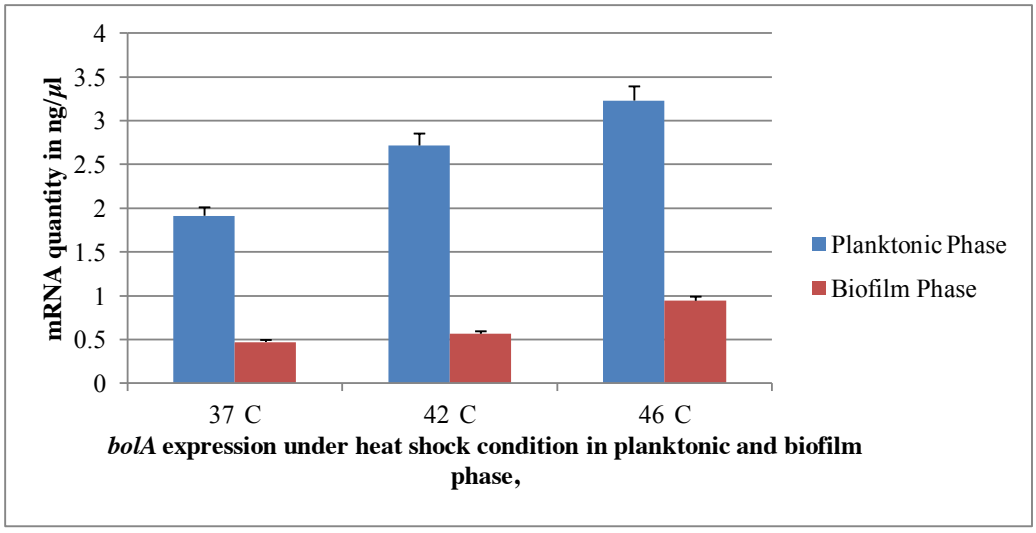


Figure 3.15: Bar graph represents the expression of *bolA* gene (*bolA*$^{+}$/*rpoS*$^{-}$) under heat shock conditions in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by heat shock and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

The comparison between wild type, *rpoS* and *bolA* mutant strains, showed that both genes contribute to the ability to respond and adapt to heat shock conditions. In this study, it is seen that, *rpoS* gene was expressed only outside the biofilms (i.e. planktonic cells), and cells inside the biofilms didn't show *rpoS* gene expression, which suggest that *rpoS* gene expression is down-regulated inside the biofilms even after 15 minutes of heat shock. The active cells outside the biofilms are probably responsible for the response to environmental stresses, which shows that *rpoS* can respond suddenly to a heat shock shift. In the case of *bolA*, its expression was increased with temperature even in biofilms, which suggests that *bolA* response to sudden heat shock

environment is constructive and leads in increased *bolA* mRNA level ending with biofilm formation and development.

3.8 Quantitation of mRNA Expression Level Using Standard Curve Method (Absolute Quantification) Under Cold Shock Condition

Bacteria have the ability to sense a multitude of environmental stimuli and use these cues to regulate gene expression to adapt cellular activities to the changing surroundings. Studies which have been previously done was in regards to expression of *rpoS* gene under long term cold shock conditions, while there is no data available on *rpoS* expression in regards with biofilms in cold shock conditions which may act as a protective mechanism for *E. coli*. On the other hand, same with the morphogene *bolA* where the increased level of expression of morphogene *bolA* has been seen and experimental data are available, but there is no data available on expression of *bolA* gene as a sudden response under cold shock environments which in turn may lead to biofilm.

This study aims, whether *rpoS* and *bolA* gene can express/response under sudden shift to stress environments (i.e. 15 min of stress), which states, shift from optimal condition to cold shock conditions (i.e. 37 °C to 20 °C and 5 °C) in both planktonic and biofilm phase. Morphogene *bolA* is known for expression under stationary phase. Question arises, can *bolA* gene expresses under biofilm phase at exponential level of growth in absence of *rpoS* gene, and may help in biofilm formation under cold shock condition? What can be the

possible role of *bolA* and *rpoS* gene under sudden change in environment outside stationary phase of growth?

3.8.1 Expression of 16S rRNA in cold shock conditions

Figure 3.15 shows the expression of 16S rRNA under cold shock environment, where 20 °C and 5 °C were used for cold shock conditions. It was found that expression of 16S rRNA was nearly constant in the planktonic phase at 37 °C and at 20 °C and 5 °C as well. No difference was found in the expression of 16S rRNA in the planktonic phase, but there was a major difference in expression in the biofilm phase (Fig. 3.16). Low quantities of mRNA were found in biofilm mode of growth, and it can be seen that that 16S rRNA expression was affected under biofilm phase in cold shock environment, and there was no sudden response in biofilm phase by 16S rRNA as in heat shock conditions. Overall result shows that, there is no change in expression of 16S rRNA at 20 ºC and even at 5 ºC, when compared to 37 ºC. Decrease in the expression of 16S rRNA in biofilm phase is a matter for speculation.

3.8.2 Expression of *rpoS* in cold shock conditions

The cells in biofilms were in stress conditions and expressed *rpoS* gene at high level. The *rpoS* gene expression level was lower in biofilms than exponential planktonic cells. There was a sudden variation in expression of *rpoS* at 20 ºC and 5 ºC in planktonic cells (Fig. 3.17). Nearly a 5 fold increase was seen suddenly in the expression level of *rpoS* after shift form 37 ºC to 5 ºC, as in the heat shock condition, where there was a 5 fold increase was seen suddenly after a shift form 37 ºC to 46 ºC. There was a rapid increase in the expression of *rpoS* at 37 ºC in biofilm mode but no increase at 20 ºC and 5 ºC when compared to

planktonic mode of growth. A 2-3 fold increase was seen in the mRNA quantity of *rpoS* in biofilm phase after shift from 37 ºC to 20 ºC and 5 ºC, which shows that *rpoS* can respond suddenly to cold shock environment even in biofilm phase. Slow rate of expression concludes that the cells are in biofilm phase, and this mode of growth serves as a protective barrier to stress environment for bacteria, which further shows the transition of biofilm cells from exponential to stationary phase/slow growth in presence of *rpoS*.

3.8.3 Expression of *bolA* in cold shock conditions

Interesting results were seen in case of *bolA* expression under cold shock environment. The necessity of *bolA* gene for biofilm formation and sudden response to cold shock condition was seen when *bolA* expression was measured in WT strain i.e. in presence of *rpoS*. It was found that *bolA* is dependent on *rpoS* gene in cold shock environment and cannot respond to cold shock conditions in absence of *rpoS*. 1 to 1.5 fold decreases in expression level was seen in *bolA* in planktonic phase after shift of bacterial cells from 37 ºC to 20 ºC and 5 ºC. When the planktonic phase expression results was compared with the biofilm phase, it was found to be same i.e. 1 to 1.5 fold decrease in *bolA* expression. On the other hand, when the expression of *bolA* was seen in wild type strain (i.e. in presence of *rpoS*), it was found that there was a sudden change in the expression of *bolA*, nearly 3 to 3.5 fold increase in the expression was seen after shifting of cells from 37 ºC to 20 ºC and 5 ºC in biofilm mode of growth. As compared to planktonic phase cells, *bolA* expression was approximately 20 fold increased at 5 ºC, 2 fold increase at 20 ºC and 3 fold increase at 37 ºC. This shows the importance of *rpoS* regulon in particularly cold shock environments which regulated the expression of *bolA* gene and may

lead to biofilm which is a result of stress surroundings.

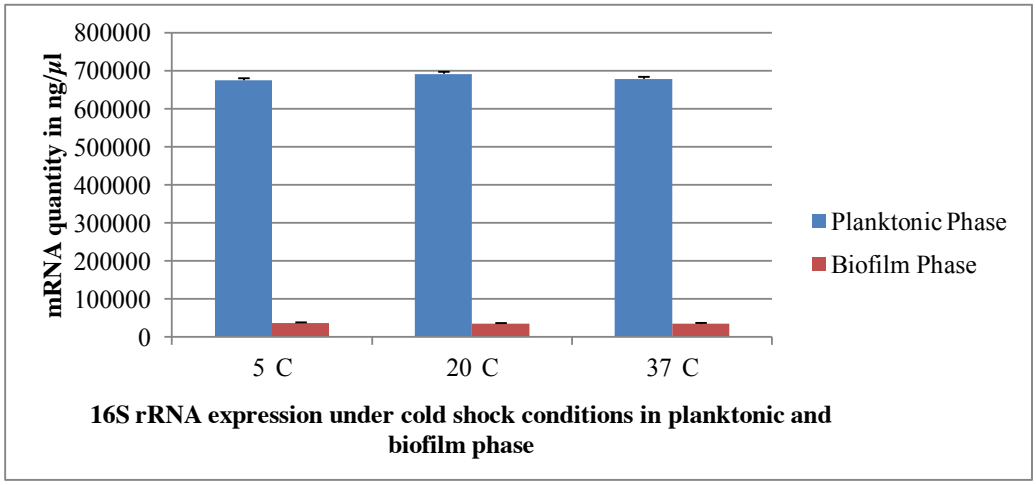


Figure 3.16: Bar graph represents the expression of 16S rRNA under cold shock conditions in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by cold shock and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

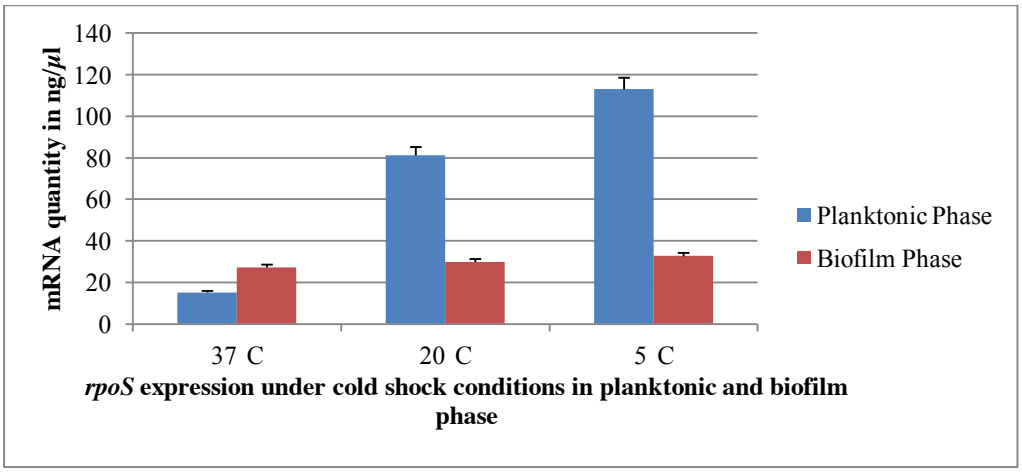


Figure 3.17: Bar graph represents the expression of *rpoS* gene (*rpoS*$^{+}$/*bolA*$^{-}$) under cold shock conditions in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by cold shock and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

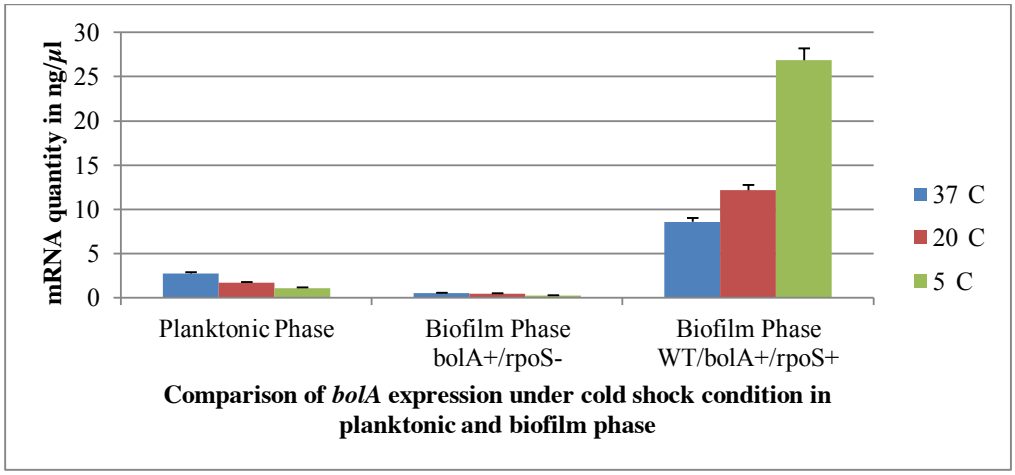


Figure 3.18: Bar graph represents the expression of *bolA* gene (*bolA*$^{+}$/*rpoS*$^{-}$) with and without *rpoS* under cold shock conditions in planktonic and biofilm phase. The cultures were grown Overnight in LB media at 37 ºC followed by cold shock and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

The results here suggest the importance of *bolA* and *rpoS* genes in *E. coli* to cope with the stress environment and adaptation to the cold shock environment. The results show that expression of *bolA* is under the transcriptional control of σ^S (encoded by *rpoS*). The presence or absence of σ^S has an impact on biofilms (Corona-Izquierdo and Membrillo-Hernandez 2002). Interestingly, the data suggests that *bolA* can be a major factor in biofilm formation under cold stress conditions, as it has responded in sudden stress. Its expression gradually increased within 15 minutes in the external stress environment. Because the levels of *bolA* depend on σ^S, it can easily be still hypothesized that *bolA* may facilitate the biofilm development.

Bacteria have the ability to respond to temperature as a primary cue to regulate gene expression. In mesophiles, the cold-shock response is characterized by the transient, increased production of cold-shock proteins after a temperature decrease (generally >10 ºC) that subsequently facilitates adaptation to prolonged growth at low temperature. Cold shock response in *E. coli* was widely studied at 23 ºC to 10 ºC. This study aims to study the response *E. coli* in at 5 ºC, specifically in relation to *rpoS* and *bolA* genes. The comparison between wild type, *rpoS* and *bolA* mutant strains, showed that both gene contributes to the ability to respond and adaptat to cold shock conditions.

Overall, the study here demonstrates that temperature has a dramatic effect on gene expression, signifying that adaptation to low temperature requires a co-ordinated, multifunctional response. This study implicates *rpoS* gene and its coordinated expression with *bolA* gene at 20 ºC and 5 ºC which may lead to biofilm development as environmental stress response.

3.9 Quantitation of mRNA Expression Level Using Standard Curve Method (Absolute Quantification) Under Different pH

Overall, growth profiles of the three strains with mutations appeared to be similar. Microbial numbers recovered on LB agar plate at different intervals after acidic shock for 15 minutes also appeared similar. Unlike heat and cold shock, in all cases here, including 16S rRNA results appeared more robust. To determine whether any of the genes had a specific role at different acidic pH, expression analysis of *rpoS* and *bolA* gene was also measured at different alkaline pH for data comparison.

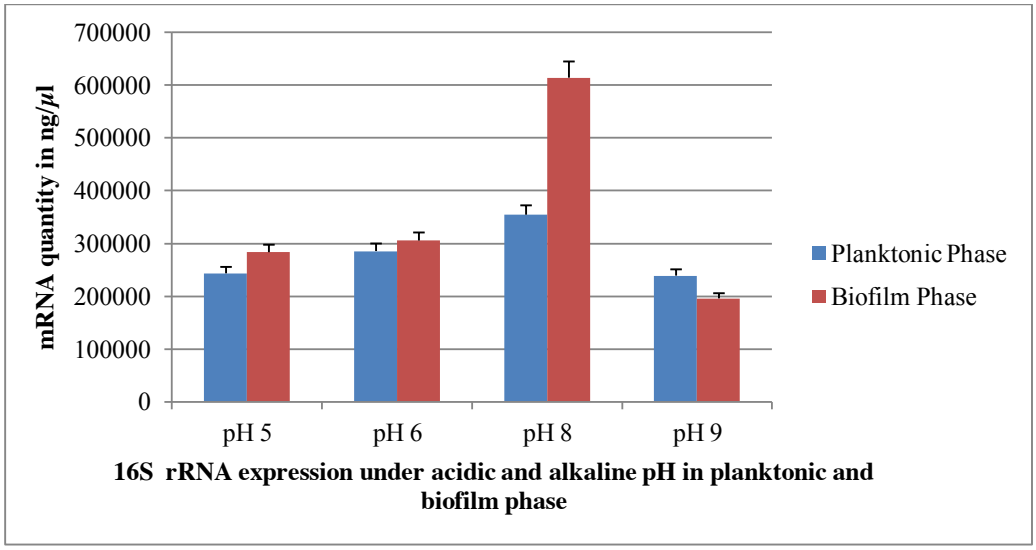


Figure 3.19: Bar graph represents the expression of 16S rRNA gene under different pH in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by pH stress and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

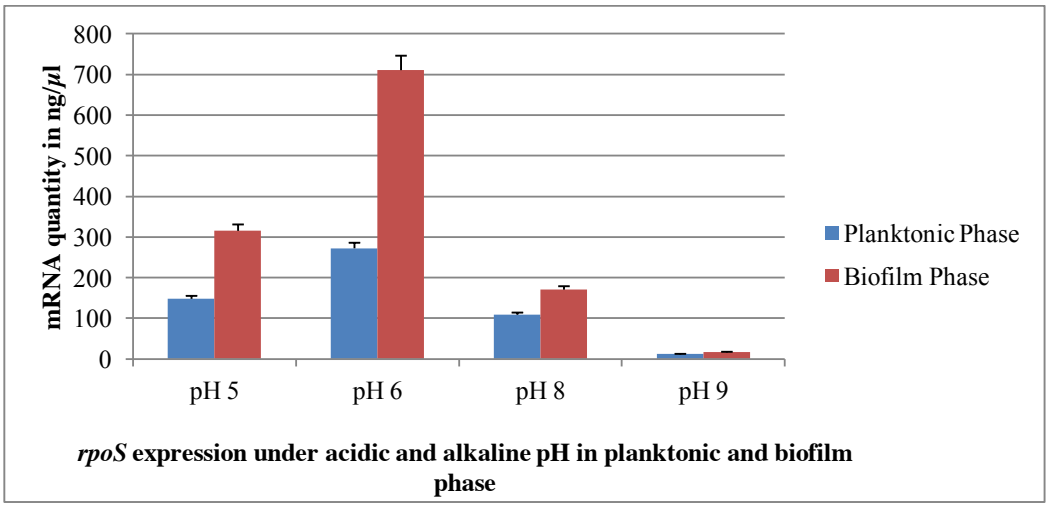


Figure 3.20: Bar graph represents the expression of *rpoS* gene (*rpoS*$^{+}$/*bolA*$^{-}$) under different pH in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by pH stress and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

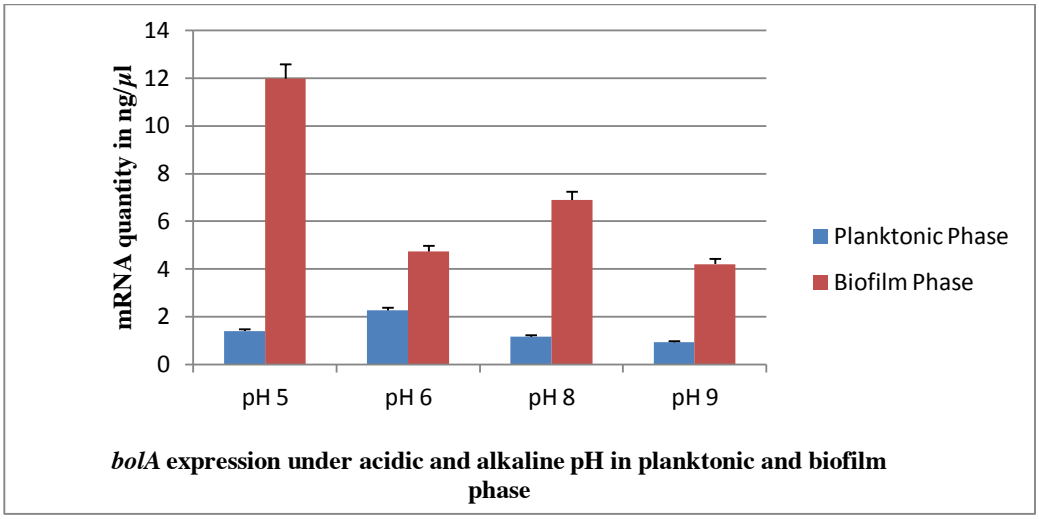


Figure 3.21: Bar graph represents the expression of *bolA* gene (*bolA*$^{+}$/*rpoS*$^{-}$) under different pH in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by pH stress and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

Based on the expression analysis, we found that response of 16S rRNA, *rpoS* and *bolA* within 15 min of stress at various pH was sudden under biofilm conditions when compared to the planktonic form of growth. Increased level of expression of morphogene *bolA*, *rpoS* and 16S rRNA under biofilm mode of growth has been seen at pH 5, 6, 8 and 9, which shows the involvement of these two genes in resistance to acidic and alkaline stress conditions with a sudden response under different pH shock environments.

Morphogene *bolA* is known to express in the stationary phase, but its expression in the biofilm phase at the exponential level of growth in absence of *rpoS* gene has not been studied. Here the data shows that it may help in biofilm formation under pH stress conditions because of its sudden response to different pH levels, with a 10-15 fold increase in the expression of *bolA* gene under biofilm

phase (Fig. 3.21). This shows its possible role and expression outside the stationary phase conditions.

Results from figure 3.20 and 3.21 shows that expression of *rpoS* and *bolA* genes was dramatically increased in the biofilm phase at pH 6 and pH 8 when compared to optimum pH 7. However, at the same time it is seen that the expression of 16S rRNA and *rpoS* was 2 to 4 fold less when pH was increased to more acidic and alkaline pH i.e. pH 5 and pH 9, but *bolA* expression under pH 5 was higher than pH 6, which shows the ability of *bolA* to respond under severe acidic conditions. Under more alkaline and acidic pH (pH 9 and pH 5), expression of 16S rRNA, *bolA* and *rpoS* gene was less when compared to less alkaline and acidic environment (pH 8 and pH 6) in both biofilm and planktonic form of growth. This shows that the level of expression decrease to 1 to 2 fold when pH increases. In this study the potential for various pH shock treatment was examined to assess the ability and involvement of *rpoS* and *bolA* genes under acid induced environments using non-pathogenic strain of *E. coli* K-12 MG1655. From this study the following results can be concluded:

Acidic and alkaline shock environments appeared to enhance the expression of *rpoS* and *bolA* genes in acidified LB broth in biofilm and planktonic phases, regardless of pH variation. Different pH treatment did result in enhanced expression of *rpoS* and *bolA*, which may result in acid habituation and survival of *E. coli*, and formation of biofilms, which is still a matter of speculation. In poor growth conditions, *bolA* was shown to be essential for normal cell morphology during exponential growth and stationary phase and in response to sudden carbon starvation. There was no data available to show that *bolA* can

respond to sudden pH shift environments. It has been shown that *bolA* modulates cell morphology by increasing the transcription of genes encoding enzymes involved in cell wall biosynthesis and cell division.

Here, the data shows that *bolA* can also respond to sudden acidic and alkaline stress conditions. Acid resistance is an important property of *E. coli*, because it enables the organism to survive gastric acidity. Acid resistance is also important for colonization of the lower intestine where the presence of short chain fatty acids can cause cytoplasmic acidification even at neutral external pH. Based on the expression analysis, it appears that the expression of these genes under the various pH conditions was completely different when compared with heat and cold shock environments.

3.10 Quantitation of mRNA Expression Level Using Standard Curve Method (Absolute Quantification) Under Different Hydrogen Peroxide Concentrations

E. coli K-12 MG1655 was exposed to hydrogen peroxide (3, 4 and 5 mM final concentration) in LB broth. When a concentration of 3, 4 and 5 mM hydrogen peroxide was used to provide stress to bacterial cells, same time the viable count was measured after 15 min of stress. The total count did not decrease suggesting that cells do not get lysed after 15 min of hydrogen peroxide exposure. After the cells had been exposed to different concentration of hydrogen peroxide, levels of mRNA were quantified using Real-Time RT PCR.

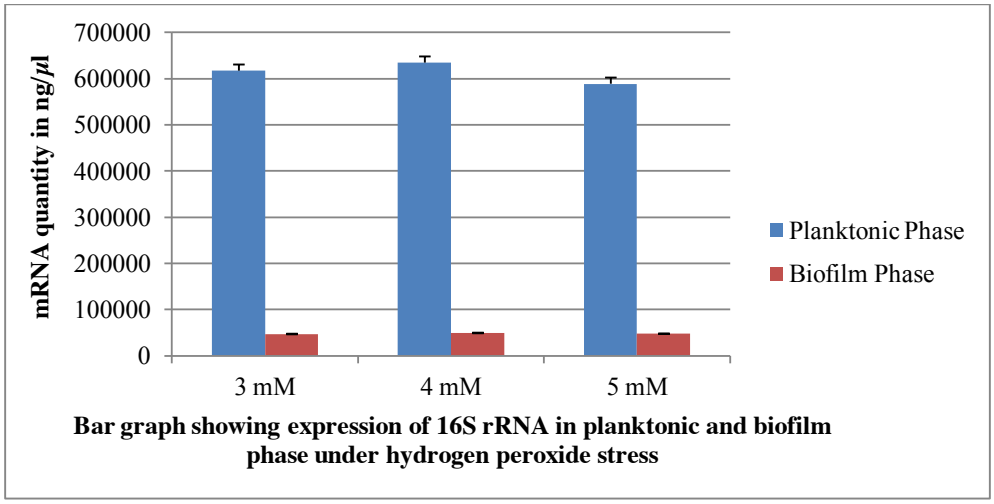


Figure 3.22: Bar graph represents the expression of 16S rRNA gene under different hydrogen peroxide concentration in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by oxidative stress and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

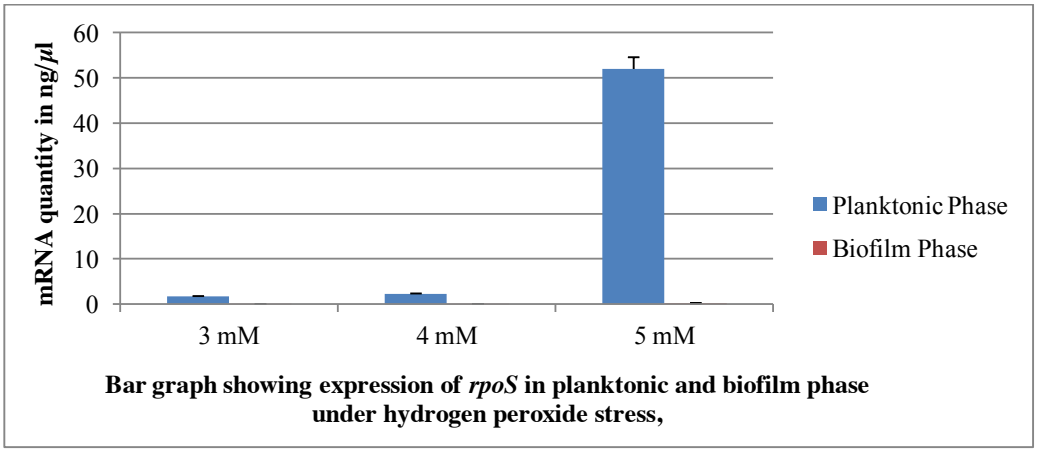


Figure 3.23: Bar graph represents the expression of *rpoS* gene (*rpoS*$^{+}$/*bolA*$^{-}$) under different hydrogen peroxide concentration in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by oxidative stress and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

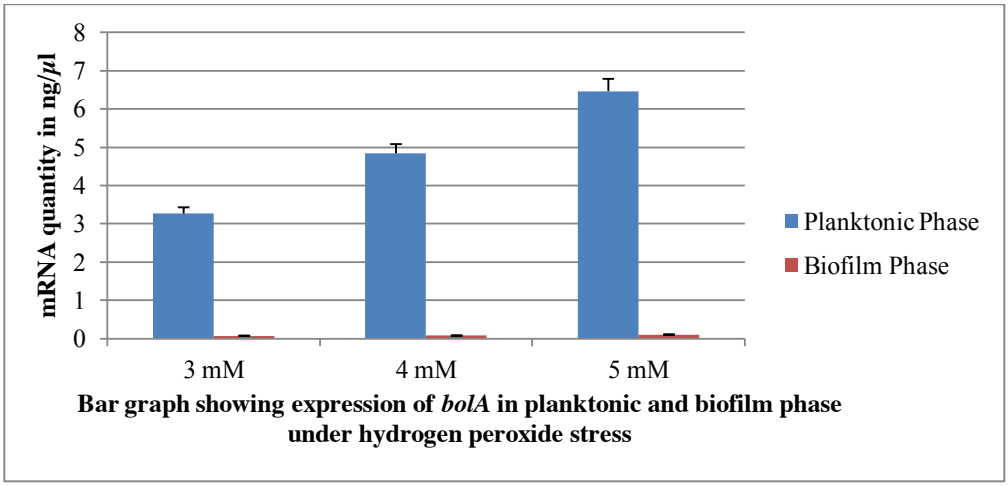


Figure 3.24: Bar graph represents the expression of *bolA* gene (*bolA*$^{+}$/*rpoS*$^{-}$) under different hydrogen peroxide concentration in planktonic and biofilm phase. The cultures were grown overnight in LB media at 37 ºC followed by oxidative stress and percent survival was calculated as described in chapter 2. The values shown are the means of three independent experiments and the error bars indicate the range. The level of mRNA quantity was measured in ng/µl.

Expression of 16S rRNA, *rpoS* and *bolA* was measured at 3, 4 and 5 mM hydrogen peroxide concentrations. Constant expression by 16S rRNA after 15 min of stress at various concentration of hydrogen peroxide was seen, both in the planktonic and biofilm phase (Fig. 3.22). Expression of 16S rRNA in the biofilm phase was much less than when compared to planktonic form of growth. This is because of the stationary/no growth state of cells in biofilm, which shows that there is no variation in 16S rRNA expression at various concentrations of hydrogen peroxide, except its expression goes slower in biofilm phase.

Results from figure 3.23 and 3.24 shows that expression of *rpoS* and *bolA* genes was dramatically increased from 3 mM to 5 mM concentration of hydrogen peroxide in the planktonic phase. A near 5 fold increase in the expression of *rpoS* was measured when cells were exposed to 5 mM hydrogen peroxide concentration from 3 mM under planktonic conditions. Not much difference was seen between 3 and 4 mM of hydrogen peroxide concentration. There was a vast difference between the expression of *rpoS* under planktonic and biofilm condition was seen. Under biofilm phase nearly no expression was measured after 15 min of exposure of hydrogen peroxide, which concludes that, there is no sudden response by *rpoS* under oxidative stress in biofilm phase.

Figure 3.24 represents the expression of *bolA* under oxidative stress. It was seen that expression of *bolA* in planktonic phase was similar to that for rpoS, with nearly 2 fold increases at 3, 4 and 5 mM concentration of hydrogen peroxide under planktonic mode of growth, but there was no sudden response to oxidative stress of *bolA* was seen under biofilm phase. This shows that *bolA* has

an ability to respond suddenly to oxidative stress in the planktonic phase, but not in the biofilm mode of growth. It might respond after a long duration in order to make biofilm stable under oxidative stress in biofilm mode. Cultures were also tested qualitatively for catalase activity by applying 6% (wt/vol) H_2O_2 directly onto colonies on LB agar plates. Vigorous bubbling indicated wild-type *rpoS* activity and positive reaction to hydrogen peroxide.

Oxidative stress can cause a wide range of damage to all living cells through their detrimental effects on many key macromolecules. Bacteria have evolved multiple systems to protect themselves from oxidative stress, some of which are regulated by *OxyR*, a global regulator for the peroxide stress response. In *E. coli* and other bacteria, *OxyR* regulates many genes involved in detoxification (*katG*, *ahpC*, *ahpF*) and protection (*dps, gor, grxA, trxC*). In many other stress conditions, *rpoS* is considered as a master regulator of stress response and controls the transcription of many other genes.

In this investigation, response of *rpoS*, and *bolA* to oxidative stress was measured and was characterized after the mutants of these genes were constructed. The results in this study will provide the knowledge for clarifying the genes involved in biofilm formation under oxidative stress and the molecular mechanism underlying in it. This finding is consistent with the notion that *rpoS* and *bolA* are somehow involved in responding to oxidative stress at sudden and may result in biofilm formation. These findings suggest that *rpoS* and *bolA* expression was actually reduced in biofilm phase because of slow/no growth of the cells, but they respond well against oxidative stress. This favours

the idea that exposure to hydrogen peroxide simply induces the expression of stress-induced genes and may lead to biofilm formation.

3.11 Gene Expression Using the $2^{-\Delta\Delta CT}$ Method (Relative Quantification)

The polymerase chain reaction is an exponential process whereby the specifically amplified product ideally doubles each cycle. As such, the measured Ct value is a logarithmic value that needs to be converted into a linear relative quantity. The average Ct was calculated for both the target genes and 16S rRNA and the ΔCt was determined as (the mean of the triplicate Ct values for the target gene) minus (the mean of the triplicate Ct values for 16S rRNA). The ΔΔCT represented the difference between samples. However, in this quantification method, only one reference gene can be inserted in the equation for analysis of the gene of interest. In this case reference gene is 16S rRNA and the genes of interest are *rpoS* and *bolA*.

To determine the relative level of expression of the genes of interest and the housekeeping gene for all experimental samples, mathematical calculations was done using the Ct values generated using standard curve method. Normalize the expression level of the gene of interest by dividing by the relative expression level for the housekeeping gene for the same sample. Finally to calculate the fold-change in gene expression, divide the normalized number for the experimental sample by the normalized number for the control sample. This was simply perform to replicate reactions for each gene of interest and for the housekeeping gene for each sample under conditions known to yield Ct values

that lie on the standard or calibration curve. To compare gene expression between each sample, calculate the ΔΔCt. A ΔCt value is calculated for each sample as the difference between the Ct values for the gene of interest and the housekeeping gene in each sample. The ΔΔCt value is the difference between the ΔCt values of an experimental sample and the control sample. The fold-change in gene expression is equal to $2^{-\Delta\Delta Ct}$.

3.11.1 Analysis of *rpoS* and *bolA* gene expression using the relative quantification method ($2^{-\Delta\Delta Ct}$) under heat, cold, pH, and oxidative stress

A noticeable difference in gene expression of *rpoS* and *bolA* gene under various stress-induced environments in both the planktonic and biofilm phases was seen. In this study, the data are presented as the fold change in target gene expression in various stress-induced environments normalized to the internal control gene (16S rRNA) and relative to the normal control. The *N*-fold differential expression in the target gene of a stress-induced samples compared with the normal sample counterpart was expressed as $2^{-\Delta\Delta Ct}$ in this study. The *rpoS* and *bolA* gene expression level was seen higher in biofilms than the exponential planktonic cells. Expression analysis of mRNA of *rpoS* and *bolA* genes under various stress environments was performed using relative quantification method. Results showed the *N*-fold change in the expression of both *rpoS* (Fig. 3.25) and *bolA* (Fig. 3.26) genes under heat shock temperatures (42 and 46 °C), cold shock temperatures (5 and 20 °C), pH stress levels (pH 5, 6, 8, and 9), and different concentrations of H_2O_2 (3, 4, and 5 mM).

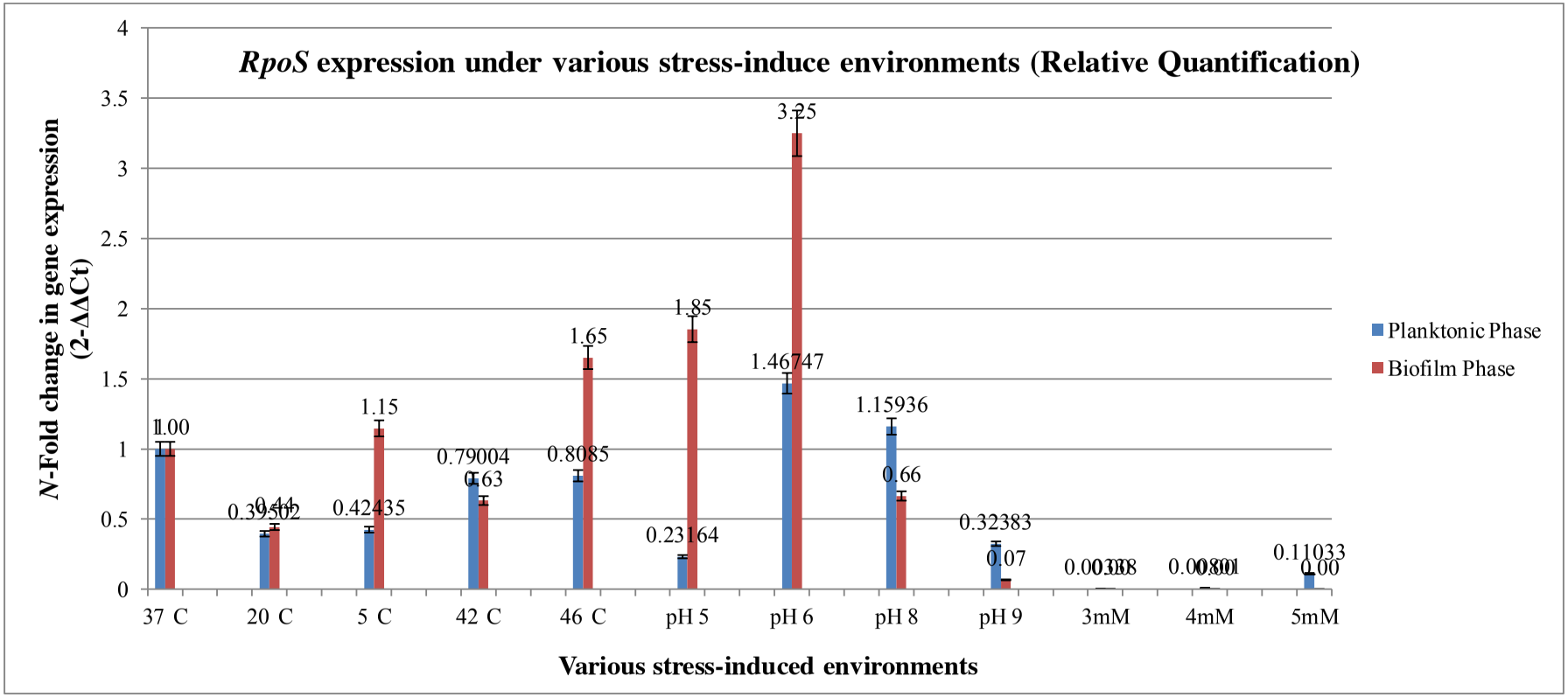


Figure 3.25: Bar graph represents the expression of *rpoS* gene (*rpoS*$^{+}$/*bolA*$^{-}$) under various stress conditions in planktonic and biofilm phase. The cultures were grown overnight in LB at 37 °C followed by various stresses and percent survival was calculated. The values shown are the means of three independent experiments and the error bars indicate the range. Increased mRNA expression was defined as *N*-fold >1.0, ‘‘normal’’ expression (control) was an *N*-fold = 1, and decreased mRNA expression was *N*-fold <1.0. Conditions used in this study are heat shock conditions (42 °C and 46 °C), cold shock conditions (20 °C and 5 °C), various pH (5, 6, 8, 9) and hydrogen peroxide concentrations (3mM, 4mM and 5mM).

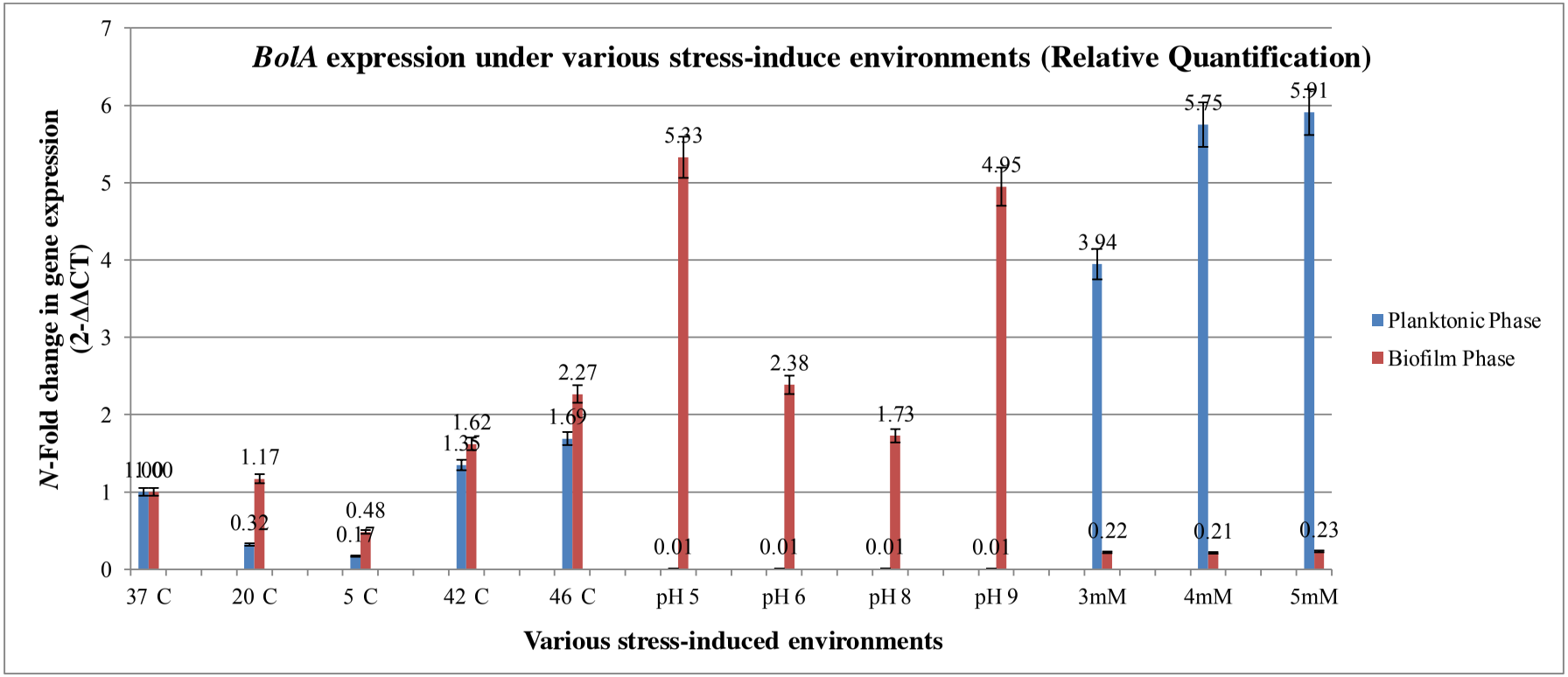


Figure 3.26: Bar graph represents the expression of *bolA* gene (*bolA*$^{+}$/*rpoS*$^{-}$) under various stress conditions in planktonic and biofilm phase. The cultures were grown overnight in LB at 37 °C followed by various stresses and percent survival was calculated. The values shown are the means of three independent experiments and the error bars indicate the range. Increased mRNA expression was defined as *N*-fold >1.0, ‘‘normal’’ expression (control) was an *N*-fold = 1, and decreased mRNA expression was *N*-fold <1.0. Conditions used in this study are heat shock conditions (42 °C and 46 °C), cold shock conditions (20 °C and 5 °C), various pH (5, 6, 8, 9) and hydrogen peroxide concentrations (3mM, 4mM and 5mM).

Earlier studies on *rpoS* and *bolA* genes have investigated long-term stress conditions and biofilm formation under several forms of stress, including nutrient starvation at stationary phase, where the increased level of expression has been seen. This study assessed whether *rpoS* and *bolA* gene could express under suddenly changing stress conditions, i.e., 15 min intervals from optimal condition to the various stress-induced conditions (i.e., heat, cold, pH fluctuation, and oxidative stress) in both planktonic and biofilm phases. Morphogene *bolA* is known to express in the stationary phase. Its expression in the biofilm phase at exponential level of growth and the possible role of *bolA* gene under sudden change in environment was therefore investigated.

E. coli frequently encounters various types of stresses in natural and man-made environments. In this study, real-time RT-PCR was performed to investigate the expression profiles of *rpoS* and *bolA* genes in response to similar stresses. The stress-induced conditions used in this study were chosen to represent some scenarios that this bacterium may encounter during natural shifts. These results indicate that the *bolA* and *rpoS* respond to different conditions quite distinctly, and have distinct expression patterns under various stress conditions.

RpoS is a conserved stress regulator that plays a significant role in survival under stress conditions in *E. coli*. The *rpoS* mutation had a pronounced effect on gene expression in stationary phase, and more than 1,000 genes were differentially expressed. Even in exponential phase when *rpoS* is expressed at low levels, mutation in *rpoS* affects the expression of a large set of genes. On the other hand, *bolA* expression is also confined to stationary phase. Its involvement in biofilm formation and expression in the stationary phase is two

different events, which are related to stress. So the purpose here was to analyse the expression of *rpoS* and its dependent gene *bolA* under biofilm mode of growth, as a sudden response to stress.

3.11.2 Expression of *rpoS* and *bolA* in various stress conditions

No activity of *rpoS* was found under oxidative stress, which suggests that cells in mature biofilms do not require expression of the *rpoS* gene under oxidative stress in either the planktonic or in biofilm phases (Fig. 3.25). *RpoS* might be able to respond in later stages/higher concentration (H_2O_2) to oxidative stress but not suddenly (in this study). An interesting result was seen in the case of *bolA*, which showed a 5–6-fold increase in expression under oxidative stress in the planktonic phase when compared with *rpoS* expression. Decreased expression of *bolA* in the biofilm phase is seen under oxidative stress when compared with the planktonic phase, which shows that cells can respond well in the planktonic phase in presence of *bolA* but not in biofilms, whereas *rpoS* cannot respond in either phase. The data indicate that gene expression within biofilm is different from that observed in standard planktonic growth cultures. Nearly, 1.6-fold increase in the expression of *rpoS* and 2.2-fold increase in the expression of *bolA* was seen after 15 min of heat stress, i.e., shift from 37 to 46 °C, under the biofilm mode of growth. In the planktonic phase, a minor change was seen after the shift to 42 and 46 °C from 37 °C (Fig. 3.25). Sudden decrease in the expression of *rpoS* and *bolA* both under cold shock condition suggests that low temperature does not induce the expression of both genes, or it can be said that *rpoS* and *bolA* cannot respond suddenly to the cold shock condition, whereas on the other hand, variation in the pH change induces the expression of

rpoS and *bolA* up to 3.5- and 5.5-fold increase under biofilm mode of growth, which in turn shows the necessity for both genes when the pH is changed. It also hypothesizes that cells in biofilms were in stress conditions and requires the expression of *rpoS* and *bolA* as a sudden response to environmental change.

Overall, results from this study suggest a new phenotype for the *bolA* and *rpoS* gene. In addition to its ability to produce round cell morphology, *bolA* is implicated in biofilm development. The fact that *bolA* is expressed under unfavourable conditions (i.e., stress and stationary phase) suggests that biofilm formation is a mode of action by which the bacteria protect themselves against the environment. The expression of *bolA* is under the transcriptional control of σ^S (encoded by *rpoS*). The presence or absence of σ^S has an impact on biofilms (Corona-Izquierdo and Membrillo-Hernandez 2002). In *rpoS* mutant strains, the biofilm cell density is reduced by 50%, and there are differences in biofilm structure (Adams and McLean 1999). Interestingly, deletion of *bolA* also reduces biofilm formation by *E. coli* K-12 MG1655. Considering the fact that the levels of *bolA* depend on σ^S, we can still hypothesize that *bolA* may facilitate the biofilm development. As the expression level of *bolA* was higher than that of *rpoS* alone shows that the sudden change in environment could increase the expression of *bolA*. This might indicate that σ^S may act through *bolA* to facilitate biofilm development.

The study showed that both *rpoS* and *bolA* genes can respond and express under sudden change in environment. Change in pH suggests the importance of *rpoS* and *bolA* and their response to the pH fluctuation is constructive, which may lead to increased *bolA* and *rpoS* mRNA levels resulting in biofilm formation

and development. In general, the study demonstrated that temperature, pH, and hydrogen peroxide have a dramatic effect on gene expression, signifying that adaptation to various environmental change conditions requires a coordinated multifunctional response. This study concludes that *rpoS* gene and its coordinated expression with *bolA* gene possibly play a major role in biofilm development.

3.12 Key Findings

1. Importance of *rpoS* and *bolA* genes under cold shock conditions. Sudden response was seen by WT cells under cold shock, when compared to mutant cells.

2. It was found that *bolA* can express without *rpoS* under various pH in biofilm phase.

3. Mutation in *bolA* leads to no expression of *rpoS* under hydrogen peroxide stress.

4. *BolA* can respond well without *rpoS* under various stress conditions both in planktonic and biofilm phase, which shows that σ^S may act through *bolA* to facilitate biofilm development.

Chapter 4

Respiratory activity of *E. coli* K-12 MG1655 in planktonic phase under various stress-induced environments

This chapter describes the bacterial respiratory (metabolic) activity measured by oxygen uptake rate due to glucose oxidation under various stress-induced environments and validates the respirometric method as an indicator of the cellular metabolic state in relation with *bolA* and *rpoS* genes.

4.1 Introduction

Flexibility of gene expression in bacteria permits their survival in varied environments. The genetic adaptation of bacteria through systematized gene expression is not only important, but also clinically relevant in their ability to respond under various stress environments (Latasa et al. 2006). Stress-induced responses enable their survival under more severe conditions, thus enhancing resistance and/or virulence (Costerton et al. 1987).

When subjected to stress, bacteria respond by metabolizing intracellular substrates. This endogenous respiration presumably supplies energy to maintain cell viability and substrates to repair damage caused by auto degrading enzymes (Boylen and Ensign 1970). Temperature change serves as a good signal to regulate gene expression in *E. coli* and other bacteria. *E. coli* is likely to encounter shifts to either low or high temperatures either for short term or long term duration during their life cycle (White-Ziegler et al. 2008). Stress response genes are induced whenever a cell needs to adapt and survive under adverse growth conditions (Vieira et al. 2004). Morphogene *bolA* in *E. coli* is one such example. It was first thought to play a role in adaptation to stationary growth phase (Lange and Hengge-Aronis 1991;M.Aldea et al. 1989). However its function is still not fully understood and is not confined to the stationary phase. In fact its expression might be induced by different forms of stress, such as heat shock, pH stress or cold shock which result a high level of expression of *bolA* mRNA (Lange and Hengge-Aronis 1991;M.Aldea et al. 1989). It also has a major effect on the bacterial envelope and, therefore, is probably involved in cellular protection under adverse growth conditions (Santos et al. 1999).

In the study of the cell envelope i.e. cytoplasmic membrane, outer membrane and periplasm, protein expression in response to environmental stresses, has been described as the extra cytoplasmic stress response (ESR) (Rowley et al. 2006). The ESR due to environmental changes and stresses has been studied in many bacterial pathogens, but there has been no investigation into respiratory activity of bacteria under various environmental stress conditions in relation to *bolA* and *rpoS* genes. Perturbations in either the external or internal environments such as heat and cold shock, pH fluctuations or nutrient starvation, must be communicated to the cytoplasm so that gene expression and post-translational responses can be modified to ensure the survival of the organism (Hengge-Aronis 1996).

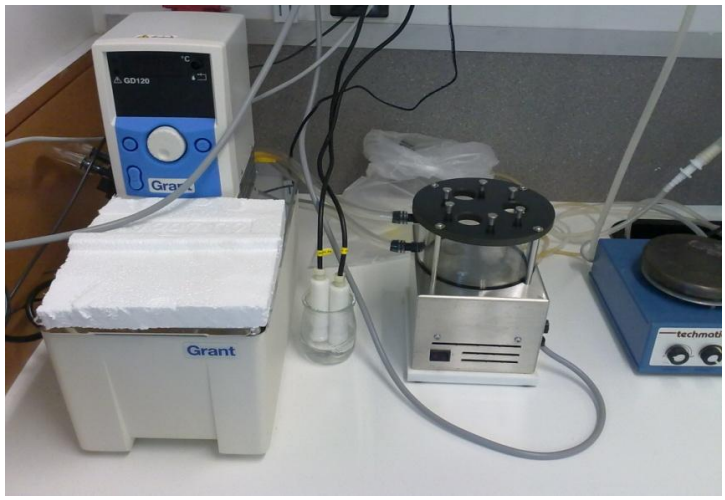
Respiratory activity, measured by oxygen uptake rate due to glucose oxidation, has already been used to assess the potential of antimicrobial agents (Simoes et al. 2005). However, no reports are available concerning the use of this technique in studying gene expression and its involvement in respiration or metabolic activity. Respirometric measurement, based on the rate of oxygen uptake needed to oxidize glucose is a reliable and fast method to assess various genes and their contribution in respiratory activity of bacteria (Simoes et al. 2003;Simoes et al. 2005). This study was designed to measure the differences in respiratory activity of *E. coli* under various stress-induced environments. This study used $rpoS^{+}$/$rpoS^{-}$ (defective in the stress regulator sigma S) and $bolA^{+}$/$bolA^{-}$ strains to study the respiratory activity of *E. coli* with different induced stress environments.

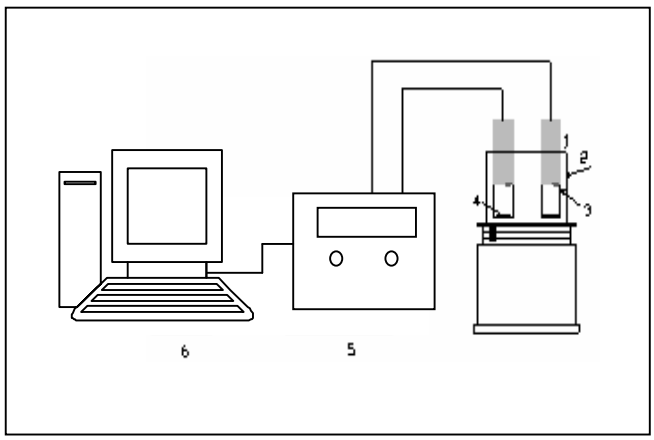
Respirometry is a rapid and non-destructive method, which provides a better understanding of the stress tolerance response of *E. coli* involving *bolA* and *rpoS* genes in different environmental conditions. Determining the respiratory activity can

be a possible criterion in understanding the mechanism behind the stress tolerance response. The subjects of endogenous respiration, the utilization of endo-cellular reserves, and starvation survival, have been dealt with before (Boylen et al. 1970). In this study, the effects of a variety of stresses including oxidative stress have been investigated as they might alter the cell envelope, increasing the susceptibility of *E. coli* to peroxidise bactericidal action on respiration of *E. coli* in presence and absence of *rpoS* and *bolA* genes. Future work should explore the *rpoS* and *bolA* regulation in detail, in order to fully understand the physiological role of these two genes.

4.2 Metabolic activity - respiratory activity

Respirometry is defined as, determination of respiratory/metabolic activity of a bacterial population by measuring the consumption of oxygen occurred in a specific period of time (Stewart et al. 1994). Respiratory activity of samples are evaluated by measuring oxygen uptake rates in a biological oxygen monitor (BOM) in short-term assays (Pereira et al. 2002). The assays are performed in a Yellow Springs Instruments (Model 5300A), represented as (Fig. 4.1).

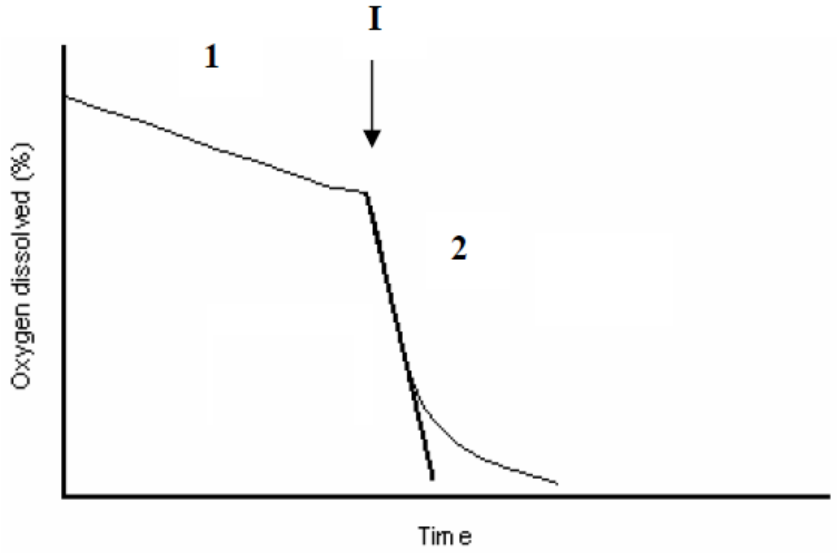




1 – Oxygen electrode
2 – Respirometry chamber
3 – Respirometry cell
4 – Magnetic stirrer
5 – YSI 5300A (display screen)
6 – Data acquisition (computer)

Figure 4.1: Schematic representation of a biological oxygen monitor.

A typical plot characterizing the oxygen uptake measured in respirometry assays is shown in figure below (Fig. 4.2). The difference between the two respiration rates gives the specific oxygen uptake rate due to the glucose oxidation, herewith referred as "respiratory activity" (Simoes et al. 2005), and expressed in mg of O_2 consumed per gram of biomass per minute (mg $O_2/g_{biomass}$ min^{-1}).



1-Oxygen consumption due to endogenous metabolism

I-Nutrient addition

2-Oxygen consumption due total metabolism (endogenous and exogenous)

Figure 4.2: Typical profile of oxygen uptake in an assay of respiratory activity* according to Stewart *et al*. (1994).

*The initial linear decrease observed (1) corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, a small volume (12.5 µl) of a glucose solution (5 g/l) was injected within each vessel (point I), (2) corresponds to the total respiration rate.

4.3 Results

The bacterial respiratory activity was evaluated by the measurement of the oxygen uptake rate due to glucose oxidation. Since cells exhibit metabolic activity, the value of the respiratory activity (exogenous and endogenous) per gram of cells varies under different stress conditions. The aim of this investigation was to compare the rates of endogenous and exogenous respiration of *E. coli* K-12 MG1655 suspended cultures (Table 4.2) subjected to stress-induced conditions. Our data show that, overall metabolic activity, i.e. oxidation of glucose in presence and absence or *rpoS* and *bolA* genes varies from one stress condition to another.

Figure 4.3 shows the effect of various shock conditions i.e. heat shock temperatures (42 and 46 °C), cold shock temperatures (5 and 20 °C) and different concentrations of H_2O_2 (3, 4, and 5 mM), on the respiratory activity (RA) of *E. coli* wild type (WT), *bolA* ($bolA^+/rpoS^-$) and *rpoS* ($rpoS^+/bolA^-$). The results show that, at 37 °C and in presence of both $bolA^+$ and $rpoS^+$ gene RA is higher when compared to that obtained in the absence of both genes. This shows the importance of both genes in the respiratory activity of *E. coli*. Under heat shock conditions, *bolA* responds well when compared to *rpoS*, while cold shock temperatures are found to inhibit respiration in *E coli*. From the results, it appears that there is a higher rate of respiration in *E. coli* under heat shock in the presence of the *bolA* gene. Interestingly, on the other hand, only the wild type can respond to oxidative stress, which shows that *rpoS* and its coordinated expression with *bolA* are required to respond under hydrogen peroxide stress, and that this varies at different concentration of H_2O_2 (Fig 4.3). The use of respirometry with *rpoS* and *bolA* genes showed that when bacteria were exposed to various stresses, metabolic activity varied.

4.3.1 Determining dry weight of the homogenised planktonic cells.

Table 4.1: Determination of the dry weight of the homogenised bacterial suspensions to measure the respiratory activity to get the average total biomass. The results were expressed in grams of biomass per litre (g/biomass/L).

Sample	Sample volume (ml)	Initial weight (g)	Final weight (g)	Total solid weight (g) (Final-Initial)	Total dry biomass g/biomass/L
WT at 37 °C	4	1.212	1.219	0.007	1.825
bolA^{+}/rpoS^{-} at 37 °C	4	1.304	1.375	0.071	17.725
rpoS^{+}/bolA^{-} at 37 °C	4	1.456	1.525	0.069	17.175
WT at 42 °C	4	1.383	1.449	0.067	16.625
bolA^{+}/rpoS^{-} at 42 °C	4	1.655	1.659	0.004	0.95
rpoS^{+}/bolA^{-} at 42 °C	4	1.678	1.758	0.081	20.125
WT at 46 °C	4	1.452	1.521	0.069	17.3
bolA^{+}/rpoS^{-} at 46 °C	4	1.525	1.528	0.004	0.875
rpoS^{+}/bolA^{-} at 46 °C	4	1.438	1.504	0.066	16.425
WT at 20 °C	4	1.455	1.530	0.075	18.775
bolA^{+}/rpoS^{-} at 20 °C	4	1.801	1.877	0.076	19.075
rpoS^{+}/bolA^{-} at 20 °C	4	1.355	1.397	0.042	10.45
WT at 5 °C	4	1.318	1.365	0.047	11.75
bolA^{+}/rpoS^{-} at 5 °C	4	1.343	1.411	0.068	16.975
rpoS^{+}/bolA^{-} at 5 °C	4	1.633	1.689	0.057	14.125
WT at 3 mM	4	2.629	2.633	0.004	1
bolA^{+}/rpoS^{-} at 3 mM	4	2.955	3.106	0.151	37.75
rpoS^{+}/bolA^{-} at 3 mM	4	2.852	2.955	0.103	25.75

WT at 4 mM	4	3.028	3.032	0.004	1
bolA^{+}/rpoS^{-} at 4 mM	4	2.735	2.843	0.108	27
rpoS^{+}/bolA^{-} at 4 mM	4	3.019	3.138	0.119	29.75
WT at 5 mM	4	2.737	2.889	0.152	38
bolA^{+}/rpoS^{-} at 5 mM	4	2.828	3.010	0.182	45.5
rpoS^{+}/bolA^{-} at 5 mM	4	3.005	3.153	0.148	37

4.3.2 Determining respiratory activity of the planktonic cells.

Table 4.2: Determination of the respiratory activity of the *E. coli* wild type and mutant strains under various stress-induced environments using dry weight of the homogenised bacterial suspensions from table 4.1. Respiratory activity (RA) was expressed in mg $O_2/g_{biomass}$ min^{-1}.

Sample	Endogenous RA (%O_2/sec)	Total RA (%O_2/sec)	Exogenous RA (%O_2/sec) Total-Endo	Exogenous RA (mg O_2/L/sec) Total-Endo	Total dry biomass (g/biomass/L)	RA (mg O_2/g biomass/min)
WT at 37 °C	0.0072	0.3612	0.3540	0.0326	1.825	1.0706
bolA$^+$/rpoS$^-$ at 37 °C	0.0032	0.3341	0.3309	0.0304	17.725	0.1030
rpoS$^+$/bolA$^-$ at 37 °C	0.0051	0.3374	0.3323	0.0306	17.175	0.1068
WT at 42 °C	0.0235	0.5077	0.4841	0.0445	16.625	0.1607
bolA$^+$/rpoS$^-$ at 42 °C	0.0036	0.3644	0.3607	0.0332	0.95	2.0960
rpoS$^+$/bolA$^-$ at 42 °C	0.0135	0.4094	0.3959	0.0364	20.125	0.1086
WT at 46 °C	0.0528	0.5487	0.4959	0.0456	17.3	0.1582
bolA$^+$/rpoS$^-$ at 46 °C	0.0091	0.4077	0.3986	0.0367	0.875	2.5144
rpoS$^+$/bolA$^-$ at 46 °C	0.0387	0.4509	0.4121	0.0379	16.425	0.1385
WT at 20 °C	0.0152	0.1060	0.0908	0.0084	18.775	0.0267
bolA$^+$/rpoS$^-$ at 20 °C	0.0012	0.1086	0.1067	0.0098	19.075	0.0309
rpoS$^+$/bolA$^-$ at 20 °C	0.0046	0.0984	0.0938	0.0086	10.45	0.0495
WT at 5 °C	0.0406	0.0101	0.0304	0.0028	11.75	0.0143

bolA^{+}/rpoS^{-} at 5 °C	0.0096	0.0023	0.0073	0.0007	16.975	0.0024
rpoS^{+}/bolA^{-} at 5 °C	0.0217	0.0102	0.0114	0.0011	14.125	0.0045
WT at 3 mM	0.0053	0.3814	0.3760	0.0346	1	2.0757
bolA^{+}/rpoS^{-} at 3 mM	0.0040	0.1244	0.1204	0.0111	37.75	0.0176
rpoS^{+}/bolA^{-} at 3 mM	0.0165	0.3345	0.3181	0.0293	25.75	0.0682
WT at 4 mM	0.0125	0.4032	0.3907	0.0359	1	2.1564
bolA^{+}/rpoS^{-} at 4 mM	0.0019	0.0804	0.0785	0.0072	27	0.0160
rpoS^{+}/bolA^{-} at 4 mM	0.0051	0.3338	0.3286	0.0302	29.75	0.0610
WT at 5 mM	0.0045	0.3858	0.3812	0.0351	38	0.0554
bolA^{+}/rpoS^{-} at 5 mM	0.0157	0.0757	0.0601	0.0055	45.5	0.0073
rpoS^{+}/bolA^{-} at 5 mM	0.0016	0.2544	0.2529	0.0233	37	0.0377

4.3.3 Respiratory activity (RA) in planktonic phase under heat shock, cold shock and hydrogen peroxide stress.

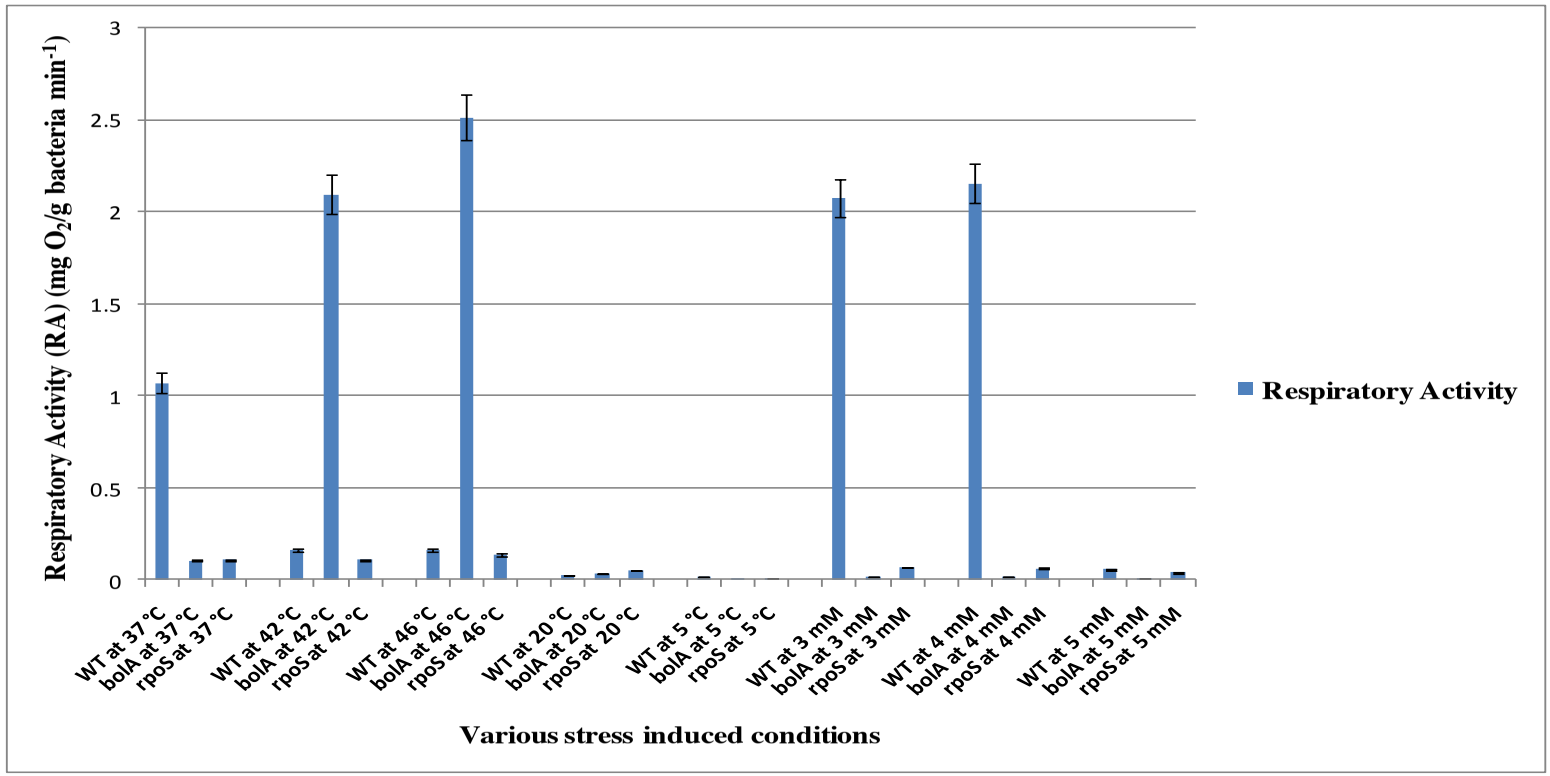


Figure 4.3: Bar chart showing respiratory activity (RA) of *E. coli* in presence of *rpoS* and *bolA* gene under various stress-induced conditions. **WT:** Wild Type, ***bolA:*** $bolA^{+}/rpoS^{-}$, ***rpoS*****:** $rpoS^{+}/bolA^{-}$. Conditions used in this study are heat shock conditions (42 °C and 46 °C), cold shock conditions (20 °C and 5 °C) and hydrogen peroxide concentrations (3 mM, 4 mM and 5 mM).

4.4 Discussion

Respirometry was undertaken to investigate the metabolic/respiratory activity of *E. coli* under various stress-induced environments. This was done using a well studied and genetically tractable organism, *E. coli* to rapidly identify the significance of *rpoS* and *bolA* genes in respiration under stress. Differences in the respiration rate was observed using wild type and mutant strains. In addition the work with *E. coli* may serve as a model for the study of bacteria less responsive to genetic and molecular approaches.

In a previous work short-term respirometry proved to be a rapid, reliable, economic and easy methodology that can be used to evaluate respiratory activity in various stress conditions and its efficacy against aerobic, heterotrophic, carbon-consuming bacteria (Simoes et al. 2003). The specific physiology of bacterial cells helps to explain both their extraordinary phenotypic and genotypic properties when compared with other bacteria or in varied environments. Planktonic cells presented more differential features including elongated shape, more activity, and a higher content of proteins and polysaccharides per cell (Simoes et al. 2003).

This novel study, employing respirometry has demonstrated that *bolA* can respond to external environments in absence of *rpoS*. Also it appears that *bolA* is expressed under unfavourable conditions (i.e., stress and stationary phase). The expression of *bolA* is under the transcriptional control of σ^S (encoded by *rpoS*). The presence or absence of σ^S has an impact on *bolA* and mutation in *bolA* results in less uptake of oxygen in various stress induced environments. This indicates that σ^S might act through *bolA*. The overall study concludes the importance of *bolA* gene than *rpoS* in

respiratory activity of *E. coli*, under heat shock condition, where consumption of oxygen and oxidisation of glucose is at faster rate.

4.5 Key Findings

1. Mutation in *rpoS* leads to an increase rate of oxygen uptake and glucose oxidation under heat shock conditions.

2. *RpoS* and *bolA* genes were found to be very important under hydrogen peroxide stress. Cells can respire at a faster rate in presence of these two genes under 3 mM and 4 mM hydrogen peroxide stress.

Chapter 5

Imaging of biofilm formation by *E. coli* on various surfaces under different stress-induced environments

This chapter reviews the adherence pattern/biofilm formation of *E. coli* K-12 MG1655 on various surfaces under different stress conditions with special emphasis on *rpoS* and *bolA* genes using scanning electron microscopy (SEM).

5.1 Introduction

As late as 1987, the biofilms were regarded as surface-attached microbes embedded in their extracellular polymeric substance (EPS) and producing unorganized slime layers on the surfaces (Stoodley et al. 2002). In other words, EPS is produced when bacteria colonize the surface and helps in attaching the cells to the surface and eventually forms the biofilm matrix. EPS are composed of polysaccharides, but may also contain proteins, nucleic acids and polymeric lipophilic compounds. EPS represents the major structural constituent of biofilms in terms of weight and volume being responsible for the interaction of microbes with each other as well as with interfaces (Flemming 2002; Neu et al. 2001). The current thinking on biofilm formation is much more complicated and is considered to be a well-controlled phenomenon. Increasing evidence suggests that bacteria possess different modes of growth and that cells in biofilms differ profoundly from planktonically growing cells of the same species (Kuchma and O'Toole 2000;Stoodley et al. 2002).

Biofilm formation occurs by at least three mechanisms: the redistribution of surface-attached but motile cells, the multiplication of attached cells, and by recruiting cells from the bulk fluid (Stoodley et al. 2002). These mechanisms depend on the organisms involved, the substratum, and the environmental conditions. The maturation of a biofilm, resulting in the complex architecture with water channels, is influenced by a number of biological factors and by hydrodynamic features (Stoodley et al. 2002). The biological factors include cell-to-cell signalling between the biofilm bacteria, growth rates of the bacteria, extent of EPS production, motility of the biofilm bacteria as well as possible competition or cooperation between the bacteria.

5.2 Bacterial Behaviour on Surfaces

Both microbial adhesion and biofilms are of great importance from the industrial point of view, especially in the food industry where it occurs on a high variety of surfaces in contact with food. Microbial adhesion occurs when microorganisms deposit and attach onto surfaces, initiating a growth process (Zottola et al. 1994). Bacteria are capable of sensing surfaces (O'Toole and Stewart 2005). Contact with the surface initiates a complex differentiation programme resulting in the synthesis of alginate. Genes necessary for production of alginate in *Pseudomonas aeruginosa* were shown to be up-regulated 15 min after attachment (Dunne, Jr. 2002). Various changes in gene regulation cause the biofilm cells to become phenotypically and metabolically different from their planktonic counterparts (Kuchma and O'Toole 2000;Stoodley et al. 2002;Watnick and Kolter 1999;Whiteley et al. 2001). This difference has been persuasively shown in *Escherichia coli*, *Pseudomonas*. *aeruginosa, Pseudomonas putida,* and *Bacillus cereus*. *B*. *cereus* is a well-known food-poisoning organism (Granum and Lund 1997) that produces biofilms on stainless steel in protein-rich media such as milk (Lindsay et al. 2000;Peng et al. 2001).

5.2.1 Initial Adhesion between Bacteria and Non-Living (Biologically Inert) Surfaces

Initial adhesion between bacteria and living surfaces is usually mediated through specific molecular docking mechanisms, whereas, adhesion between bacteria and non-living surfaces is usually accomplished by non-specific (e.g. hydrophobic) interactions (Dunne, Jr. 2002). Later on during the biofilm growth, on non-living surfaces, cell-to-cell adhesion can be mediated by specific adhesins such as the

polysaccharide intercellular adhesin (PIA) of *Staphylococcus epidermidis* (Dunne, Jr. 2002;Rupp et al. 2001). Another important phenomenon in the initial adhesion of bacteria to non-living surfaces, e.g. to stainless steel, is surface conditioning (Carpentier and Cerf 1993;Korber et al. 1995). It means that when clean surface is immersed, interactions between the surface and the liquid phase begin immediately.

Physiochemical variables are responsible for the interaction between the cell and the conditioned surface. These are explained by two different theories (Korber et al. 1995). Firstly there is the "surface free energy/hydrophobicity" or "wetting" theory, which is based on surface thermodynamics. If the total free energy of the system is reduced by cell contact with a surface, then adsorption will occur. This theory relies on determining critical surface tension of the bacteria and substratum, and does not take electrostatic interactions into account (Carpentier and Cerf 1993). Secondly there is the extended DLVO theory which equates the repulsive and attractive forces acting on an adhering particle (Busscher et al. 1998;Poortinga et al. 2001). Microbial adhesion is described as a balance between attractive Van der Waals forces, electrostatic forces (often repulsive as most bacteria and conditioned surfaces are negatively charged), short range Lewis acid-base interactions and Brownian motion forces.

5.3 Parameters Affecting Biofilm Development

A multitude of studies exist where bacterial attachment, to different surfaces, has been studied with different bacterial species in different ionic strength, flow and nutrient conditions. For example the adhesion of *Bacillus* spp. has been studied extensively due to their deleterious impact in the dairy industry. *Bacillus* spores adhered as monolayers on many kinds of surfaces, hydrophobic spores of *B. cereus*

being the most adhesive (Husmark and Ronner 1992). Various mechanisms exist by which different species of microorganisms are able to come into closer contact with a surface, attach firmly, promote cell to cell interactions and grow as a biofilm (Bryers 1991). In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Donlan and Costerton 2002). Properties of the cell surface, particularly in the presence of extracellular appendages together with, the interactions involved in cell to cell communication and the production, by the microorganisms, of extracellular polymeric substances, are the chief factors that may possibly provide a competitive benefit for one microorganism where a mixed community is involved (Donlal 2002). Table 5.1 summarizes important variables which are involved in cell attachment and biofilm formation.

Table 5.1: Variables important in cell attachment, biofilm formation/development

<table>
<tr><th>Bulk fluid properties</th><th>Cell properties</th><th>Surface properties</th></tr>
<tr><td>Flow velocity</td><td>Cell surface hydrophobicity</td><td>Texture or roughness</td></tr>
<tr><td>pH</td><td>Signalling molecules</td><td>Hydrophobicity</td></tr>
<tr><td>Temperature</td><td>Extracellular polymeric Substances</td><td rowspan="4">Conditioning film</td></tr>
<tr><td>Presence of antimicrobial agents</td><td rowspan="2">Extracellular appendages</td></tr>
<tr><td>Ions</td></tr>
</table>

5.4 Biofilms are Harmful and Hard to Destroy

Sessile cells in biofilms are less mobile and more adhesive than their planktonic counterparts. They stick together to form a complex community and carry out different roles. Often, biofilms are harmful to industry, the environment and human health (Costerton et al. 1999). For example, anaerobic bacteria in biofilms reduce sulfur to hydrogen sulfide to corrode pipes, whilst aerobic bacteria use oxidation to corrode metal. On computer chips, biofilms serve as conductors which interfere with electronic signals. More than half of the infectious diseases caused by *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, and other bacteria involve biofilms (Potera 1999). Biofilms are resistant to current modes of removal such as corrosive chemicals, bacteriophages, antibiotics and immune cells (Kolter et al. 1993;Watnick and Kolter 1999). Therefore, biofilms are robust, diverse, and hard to destroy. Various techniques have been performed to manage and eliminate biofilms, such as chemical treatments, heat, and cleaning regimens.

5.5 Methods Directly to Examine Adherent Microorganisms

5.5.1 Cultivation of biofilms

The potential of bacteria to produce biofilms can be measured in the laboratory using microtiter plates (Danese et al. 2000b;Kolari et al. 2001). The growth medium and the bacterial inoculum are dispensed in the wells of the plate, and incubated at a chosen shaking rate and temperature for a specific period of time. The wells are then emptied, washed and the biofilm that has accumulated on the walls of the wells is stained using crystal violet. The intensity of the colour of the attached cells can be measured by a plate reader. This method is simple and allows a large number of analyses to be carried out simultaneously. However, there are limitations to this

technique in that commercially available substrata (microtiter plates) are limited to a number of different types of polystyrene. Therefore, the only method used in this study is described in detail below.

5.5.2 Microscopy

The most common method for the enumeration and morphological observation of microorganism on various surfaces is microscopy. This includes direct counting methods such as light microscopy, epifluorescence microscopy, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Scanning electron microscopy is considered the most appropriate technique for evaluating the interaction of microorganisms in the biofilm matrix. Samples are fixed with the help of a chemical agent, such as glutaraldehyde, pararformaldehyde and osmium, or cryo-fixed through quick freezing, to avoid cell damage by ice crystals (Kumar et al. 1998; Zoltai et al. 1981). In this study SEM was used for the investigation of biofilms on various substrates under various stress conditions.

5.5.3 Scanning electron microscopy

Scanning electron microscopy (SEM) is a technique, which is used to produce magnified images of small selected areas of solid samples (Patrick et al. 3rd edition). Imaging is produced by scanning the sample with an electron beam while displaying the signal from the electron detector on a TV screen or computer monitor (Charles et al. 4th edition). The imaging system relies principally on the specimen being adequately electrically conductive, to ensure that the bulk of the incoming electrons are the grounded (Reimer et al. 2nd edition). In order to emit the high energy electrons beams that are needed for imaging, SEM generally operates under a high vacuum and dry environment. One of the most critical factors in the success of SEM

is that images of three-dimensional samples are usually amenable to interpretation by the observer (Reimer et al. 2nd edition). SEM image formation relies on collecting the different signals that are scattered as a result of the high-energy beam interacting with the sample (Lloyd et al. 1981). The two major image signals that are used for image formation are backscattered electrons and secondary electrons. These are generated within the primary beam and with sample interaction (Patrick et al. 3rd edition). Backscattered electrons are used to show compositional variations, where secondary electrons are used to reveal topographic features of the specimen (Reimer et al. 2nd edition).

5.5.3.1 Focusing, magnification and resolution

Correct focus setting can be attained by adjusting the control to acquire the sharpest realistic image with detail of the specimen (Reimer et al. 2nd edition). Setting a high magnification value usually allows this. Magnification values can be expressed as the ratio of the size of the image as viewed to that of the raster scanned by the beam on the specimen (Patrick et al. 3rd edition). Resolution is expressed as the smallest feature clearly visible depending upon the size of the image (Patrick et al. 3rd edition). Resolution is limited to both the diameter of the electron beam and the interaction between the electron and the specimen. Shorter working distances produce a higher resolution image.

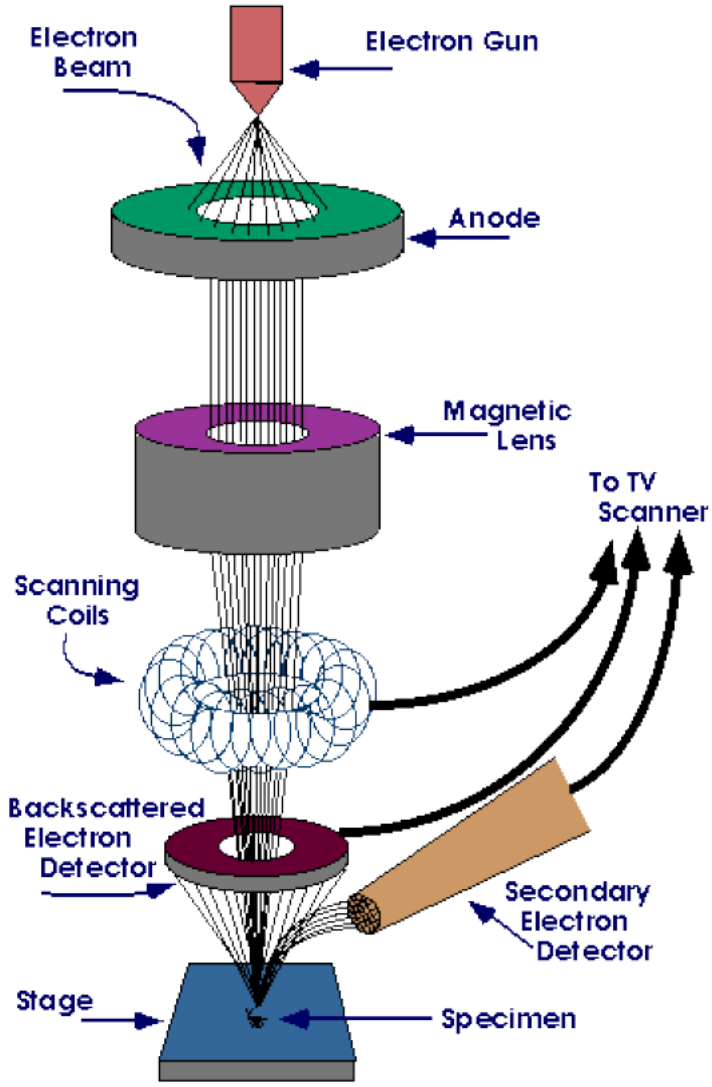


Figure 5.1: Schematic diagram of scanning electron microscope

5.5.3.2 Instrumentation

5.5.3.2.1 Electron gun

An electron gun, also known as an electron emitter, is an electrical component which is capable of producing an electron beam. Inside the electron gun, there is a major component called the filament which is the source of the electrons (Patrick et al. 3rd edition). An electron gun is usually composed of a tungsten filament that emits electrons (Patrick et al. 3rd edition). The standard filament or cathode is composed of

V-shaped wire with an approximate diameter of 100 μm (Charles et al. 4th edition). By applying a high voltage approximately between 20 keV to 40 keV, electrons can be pushed out of the outer most shell of the tungsten filament. The electron gun performance is highly dependent on the operating temperature. At the optimal temperature, the electron gun can easily produce electrons. This prolongs the life of the filament. The mean life of a tungsten filament is roughly 25 hours for older models and 200 hours for newer models which contain a good vacuum system in the microscope (Patrick et al. 3rd edition). The major causes of filament failure include poor vacuum, high voltage and air contamination due to age (Patrick et al. 3rd edition). The electron gun also contains negative potential (Wehnelt cap). The Wehnelt cap is responsible for forcing electrons into the proper direction (Patrick et al. 3rd edition). The Wehnelt cap is a negative potential through which electrons get repelled to the anode. The anode is attached to the ground into which highly negative electrons get pulled towards it. The negative shield and the anode act as an electrostatic lens to produce an image of the electron source near the electron (Patrick et al. 3rd edition).

5.5.3.2.2 Electromagnetic lenses

Electromagnetic lenses are important to capture electrons that travel off the axis. Electromagnetic lenses have two poles that are intended to focus electrons in the correct direction. Electromagnetic lenses demagnify the beam of electrons into a small area known as a spot. The spot size can be adjusted by a control, called the spot size controller which is an essential tool of SEM (Charles et al. 4th edition; Patrick et al. 3rd edition).

5.5.3.2.3 Detector

Unique detectors must be installed in order to collect backscattered electrons. Installation of detectors affects the performance for collecting electrons. The efficiency of collecting electrons can be enhanced by increasing the surface area of the detectors and positioning them above the specimen in which backscattered electrons most probably will be encountered (Patrick et al. 3rd edition). There are different kinds of detectors for backscattered electrons. From among these, the one most commonly used is the solid state detector. Solid state detectors are less expensive, sensitive to differences in atomic number and easy to operate (Charles et al. 4th edition). In addition, a secondary electron detector, in particular a cathodoluminesence is used mainly for conventional imaging utilizing topographic contrast. A cathodoluminesence detector has a better sensitivity. Other detectors that are used in SEM include a scintillator-photomultiplier and specimen current detector.

The ultimate goal of this attachment study was to invent colonization resistant surfaces, to determine the adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel surfaces under various stress induced environments in presence and absence of *rpoS* and *bolA* genes. While surfaces or surface coatings that retard bacterial adhesion have been described, none have been developed that totally prevent it (Dunne, Jr. 2002).

5.6 Results and Discussion

The results here show the adherence pattern of *E. coli* K-12 MG1655 on stainless steel, silicon and polypropylene under various stress-induced conditions i.e. heat shock: 46 °C, cold shock: 5 °C and oxidative stress (H_2O_2 at 3 mM) in presence of *rpoS* and *bolA* genes. *E. coli* was used as a model organism to the study the biofilm formation on various surfaces. This model represents well the biofilms in nature, as reflected by in the strong adhesion to the solid surface and the growth at an air-liquid interface.

The availability of nutrients and oxygen is essential for cell survival. In this study cells were grown up to 72 hours, the growth medium being changed every 24 hours. This renewed supply of nutrients helps minimize the decrease in *E. coli* cell density after its preliminary growth and supports a plateau in the growth of bacteria over the course of 3 days. After the time and stress conditions employed, the biofilms formed were always thickest near the air-water interface suggesting that on the continuously submerged surface regions the availability of oxygen may have limited biofilm formation of this aerobic bacterium. This implies an abundance of organic molecules and oxygen in the air-liquid interface, which allowed planktonic cells to form clusters and release EPS to form rigid biofilms. It has been suggested that the role of surfaces is important for attachment and growth of heterotrophic bacteria (Williams et al. 1995). Because of the nature of this study, which means, attachment of *E. coli* on various surfaces in different stress-induced conditions, the results here vary. In some cases the effect of mutation in *rpoS* and *bolA* gene and change in substrate under stress condition has an impact on biofilm growth.

5.6.1 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (24 h @ 37 °C).

The conditions used in this study are listed in Table 5.2. For the images presented, the following designations are used. For example, P1WTSI 1/2 should read as,

P1 - Plate 1 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

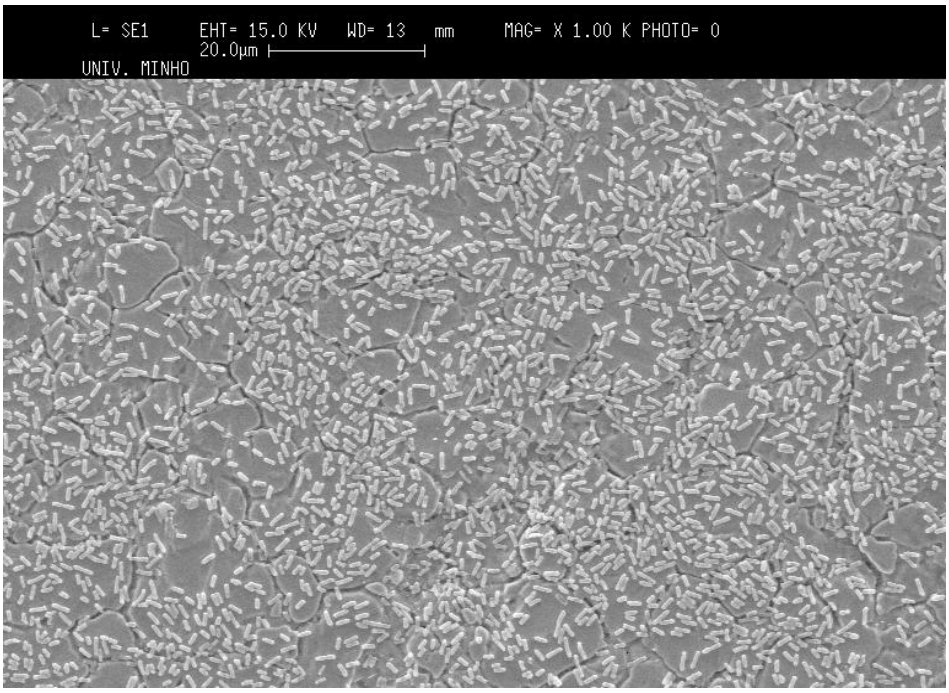
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.2: Condition used in this study with the designated names and remarks

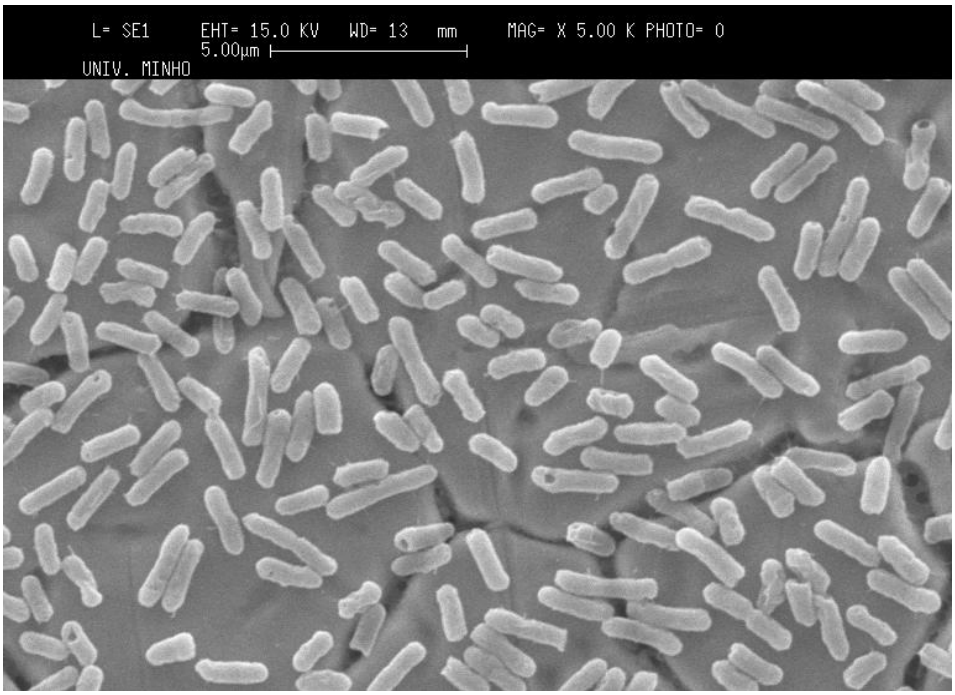
Plate	Strain	Material	Name	Figure	Remarks
Plate 1 **24 h @ 37 °C**	*WT*	Stainless Steel	-	NA	Nothing Visible
		Silicone	P1WTSI 1/2	5.8	Thick biofilm
		Polypropylene	P1WTPP 1/2	5.7	Less attachment
	bolA+/rpoS-	Stainless Steel	P1BACO 1/2	5.2	Very well attached
		Silicone	P1BSI 1/2	5.4	Very well attached
		Polypropylene	P1BPP 1/2	5.3	Less attachment
	rpoS+/bolA-	Stainless Steel	P1RACO 1/2	5.5	Very well attached
		Silicone	P1RSI 1/2	5.6	Very well attached
		Polypropylene	-	NA	Nothing visible

After 24 hours of growth at 37 °C, it was found that mutation in *bolA* did not support the cells to attach to stainless steel and polypropylene surfaces (results not shown). In presence of *bolA*, *E. coli* cells were able to attach to the stainless steel very well and could form biofilms (Fig. 5.2). All three stains were able to attach and grow as biofilms on silicon (Fig. 5.4, 5.6 and 5.8). After 48 hours of growth, cells were able

to grow on the surface of stainless steel and polypropylene. This indicates that *bolA* responded in the stationary phase of growth and can play a major role in presence and absence of *rpoS*. After 24 hours at 37 °C polypropylene was not found to be the main surface for *E. coli* cells to attach to and to form as biofilms (Fig. 5.3 and 5.7).

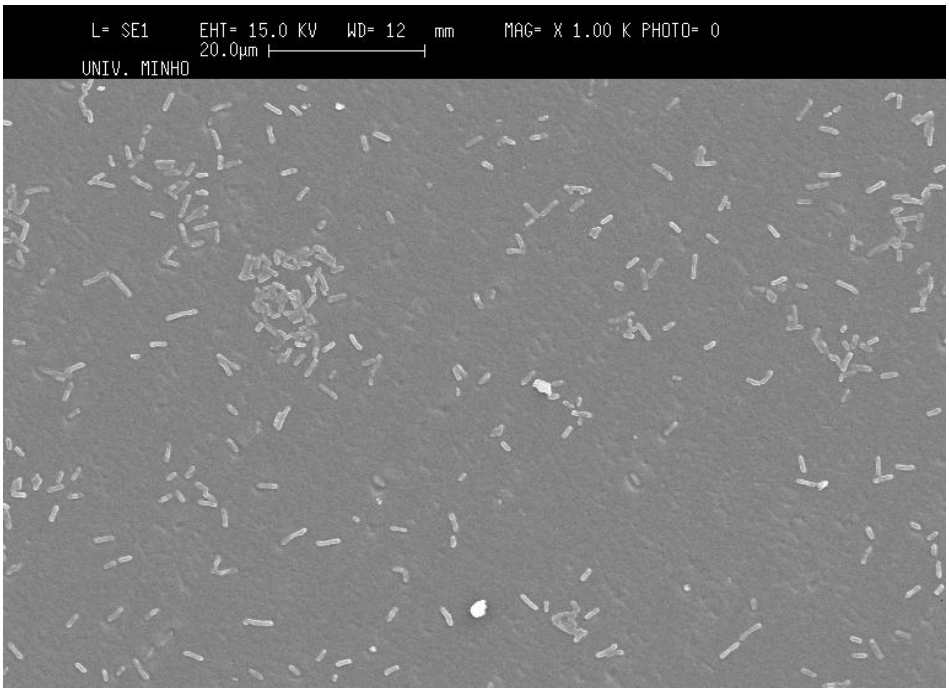


Name: P1BACO 1 (1000 X)

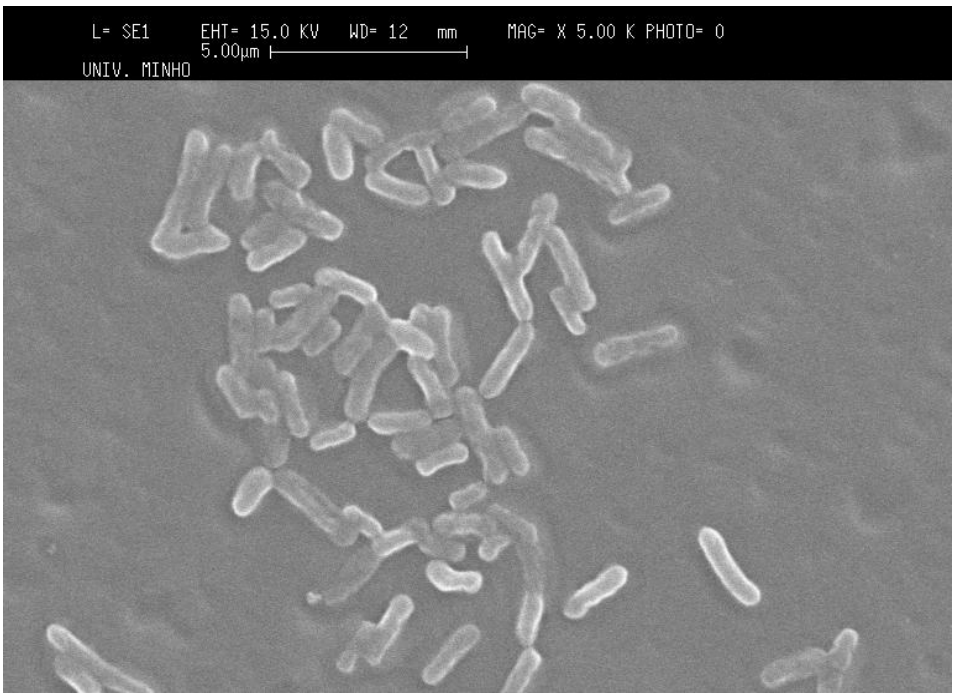


Name: P1BACO 2 (5000 X)

Figure 5.2: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 24 hours at 37 °C on stainless steel. This shows that cells were able to attach well on stainless steel surface.

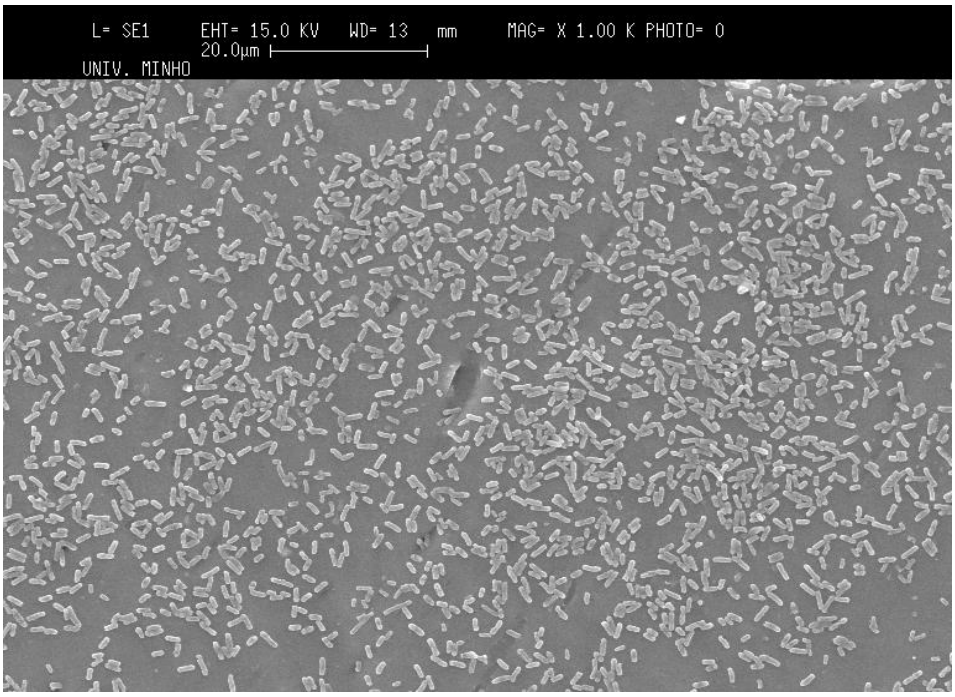


Name: P1BPP 1 (1000 X)

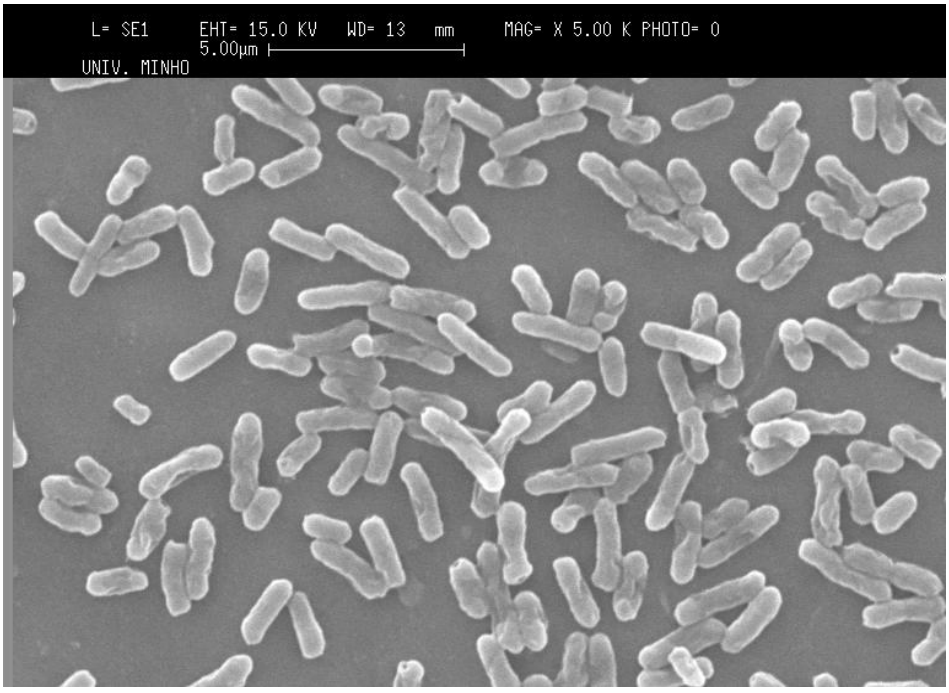


Name: P1BPP 2 (5000 X)

Figure 5.3: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 24 hours at 37 °C on polypropylene. This shows that cells can attach to the polypropylene surface, but less abundantly.

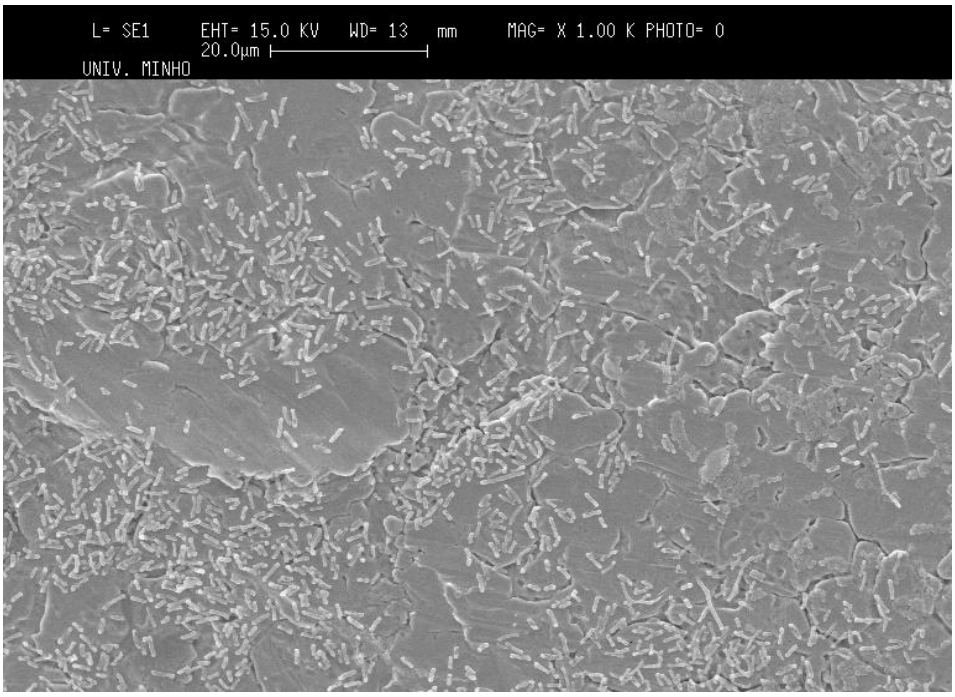


Name: P1BSI 1 (1000 X)

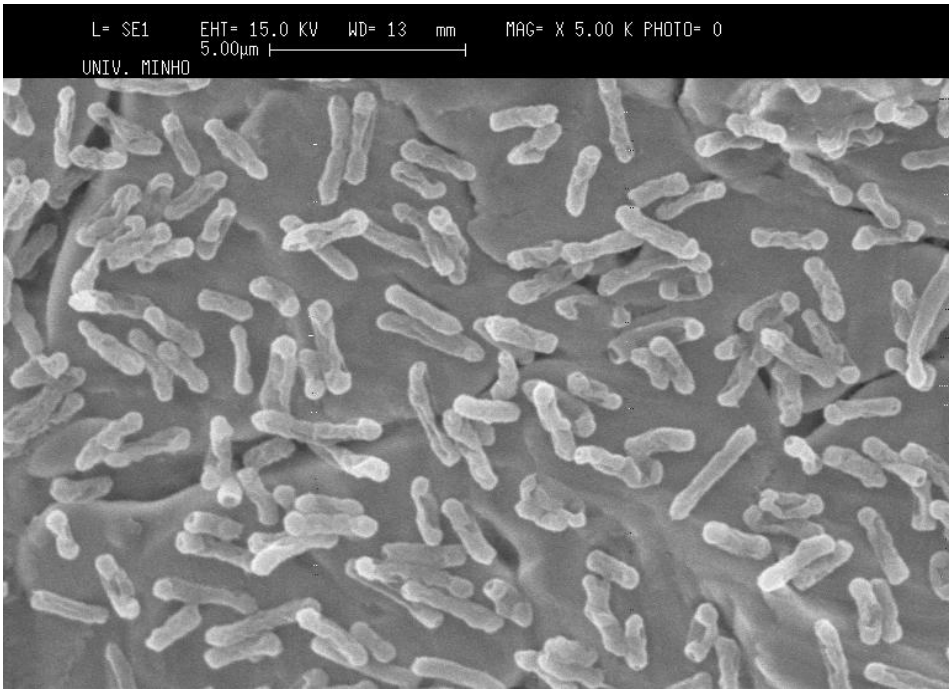


Name: P1BSI 2 (5000 X)

Figure 5.4: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 24 hours at 37 °C on silicon. This shows that cells can attach and can grow as biofilms on silicon surface.

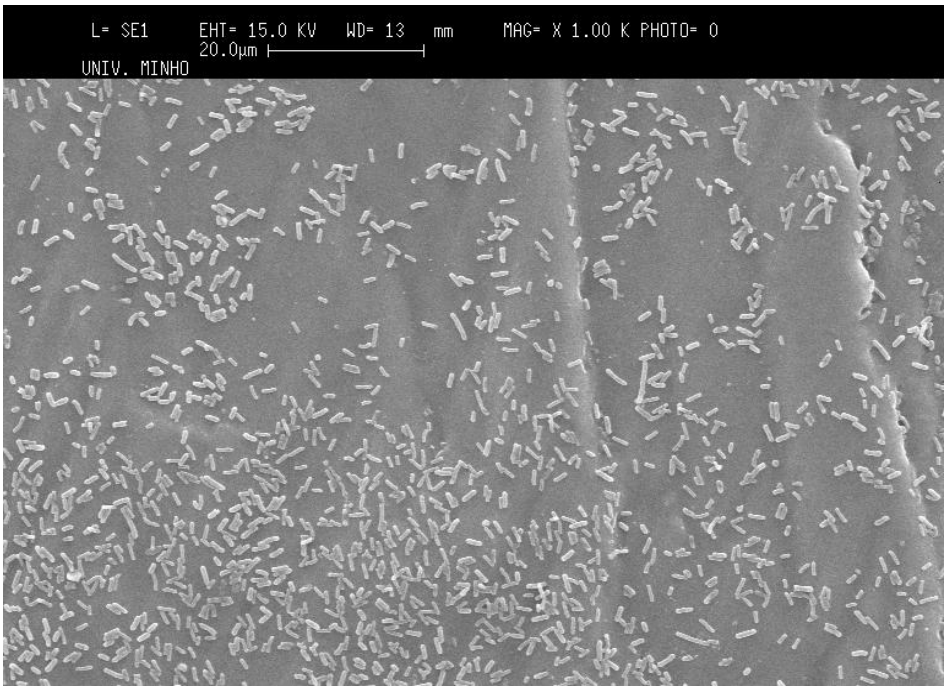


Name: P1RACO 1 (1000 X)

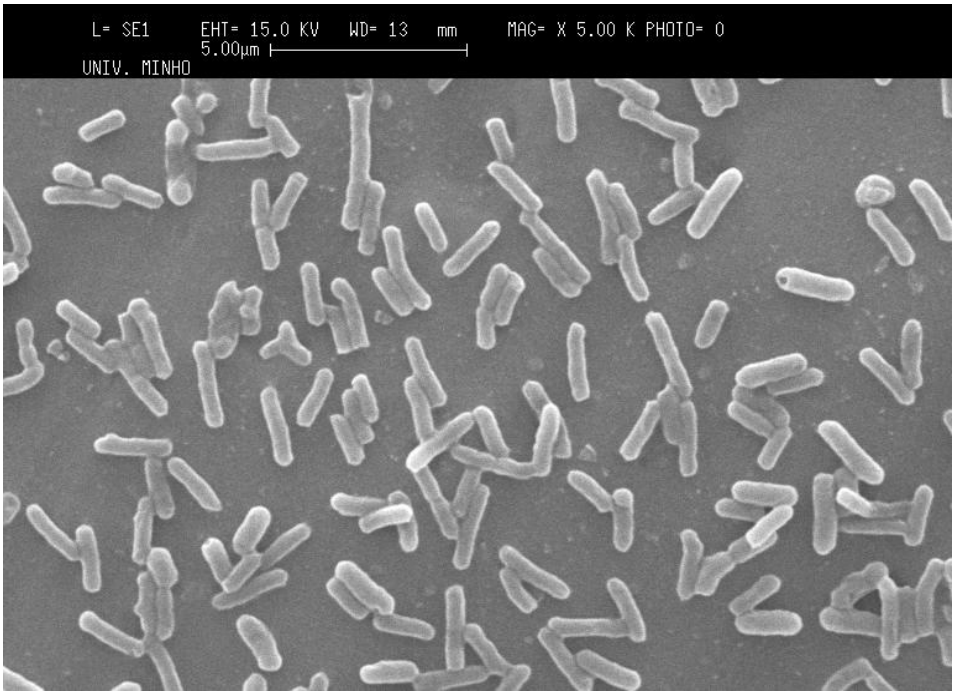


Name: P1RACO 2 (5000 X)

Figure 5.5: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 24 hours at 37 °C on stainless steel. This shows the importance of stainless steel for the cells to attach and grow as biofilms in later stages.

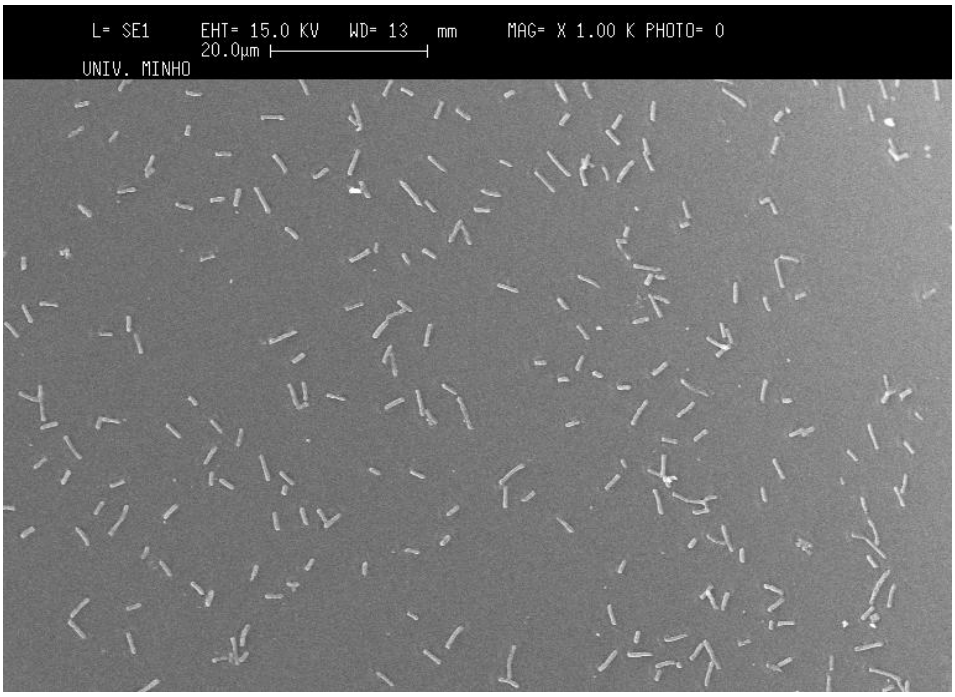


Name: P1RSI 1 (1000 X)

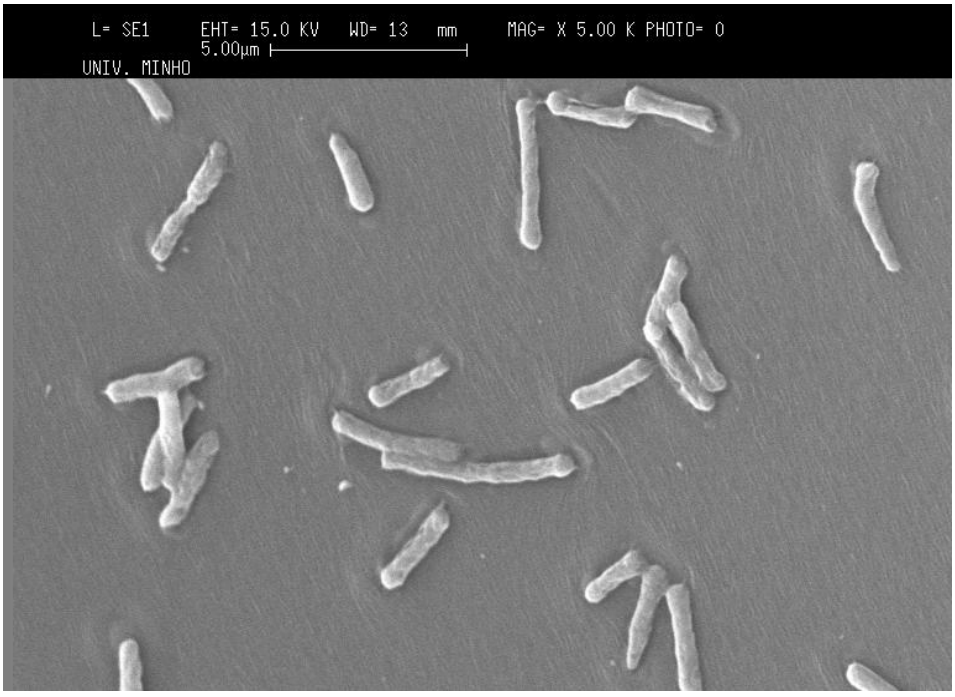


Name: P1RSI 2 (5000 X)

Figure 5.6: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 24 hours at 37 °C on silicon. Silicon also found to be suitable surface for the cells to adhere.

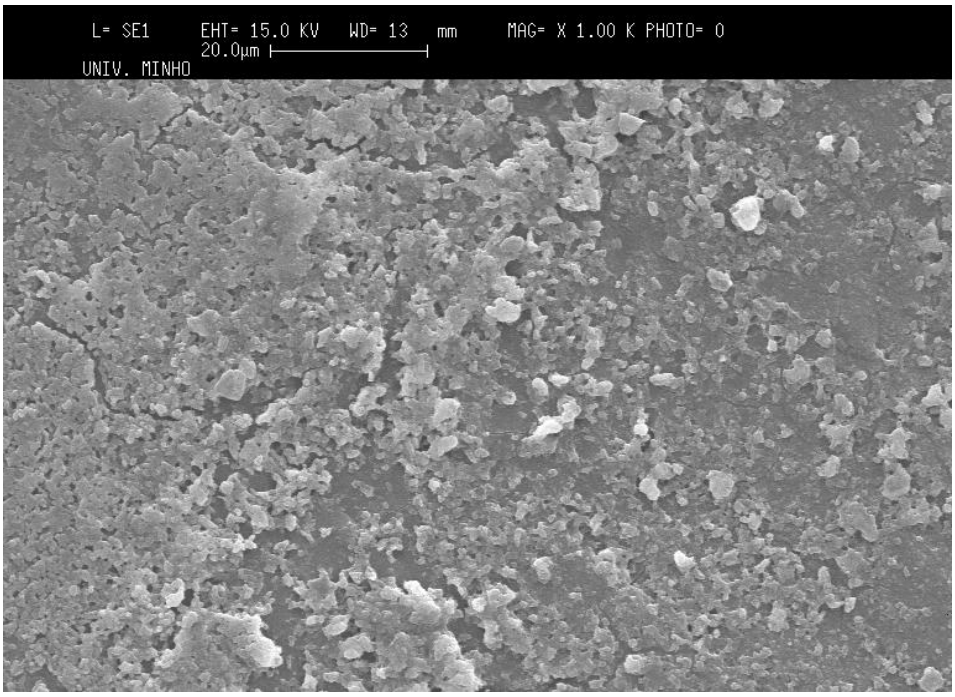


Name: P1WTPP 1 (1000 X)

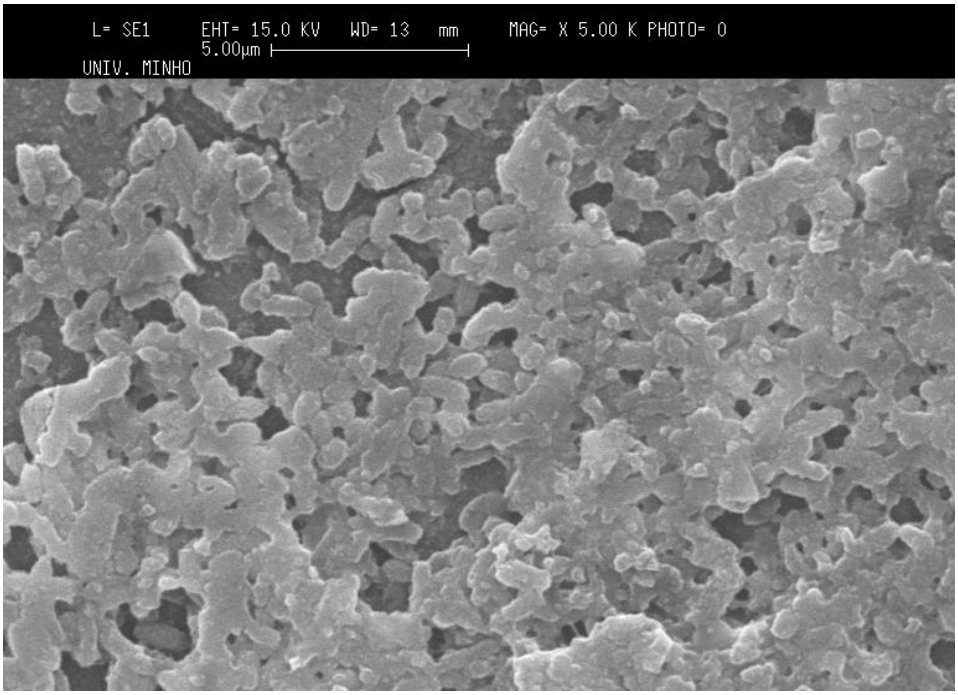


Name: P1WTPP 2 (5000 X)

Figure 5.7: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 24 hours at 37 °C on polypropylene. Polypropylene surface was found to be not suitable for the cells to adhere.



Name: P1WTSI 1 (1000 X)



Name: P1WTSI 2 (5000 X)

Figure 5.8: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 24 hours at 37 °C on silicon. Thick biomass was seen in presence of both *rpoS* and *bolA* genes, which shows the importance of these two genes.

5.6.2 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (48 h @ 37 °C).

The conditions used in this study are listed in Table 5.3. For the images presented, the following designations are used. For example, P2WTSI 1/2 should read as,

P2 - Plate 2 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

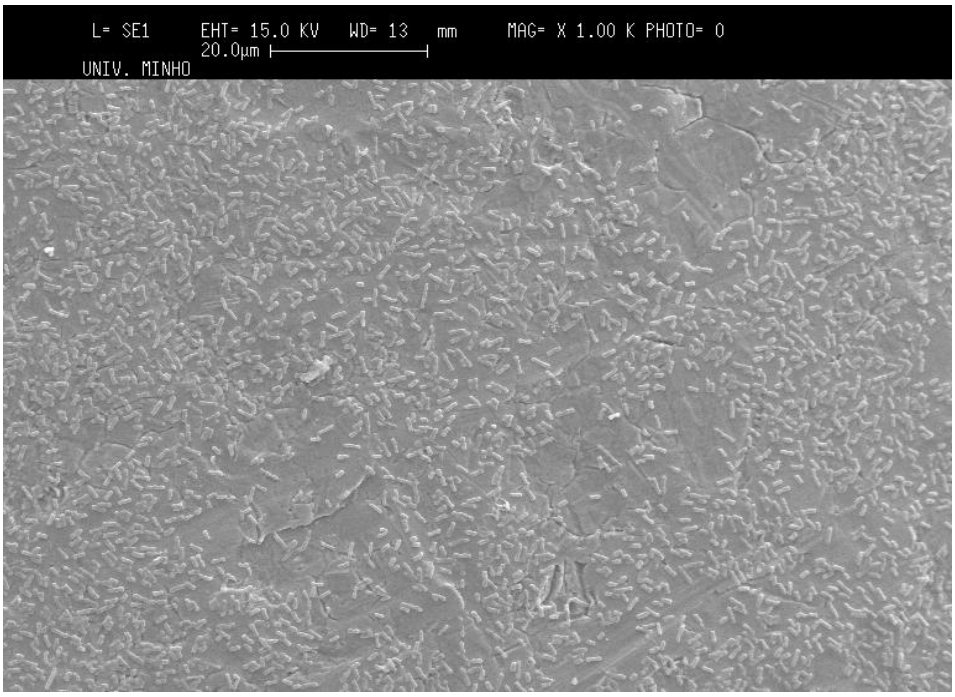
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.3: Condition used in this study with the designated names and remarks

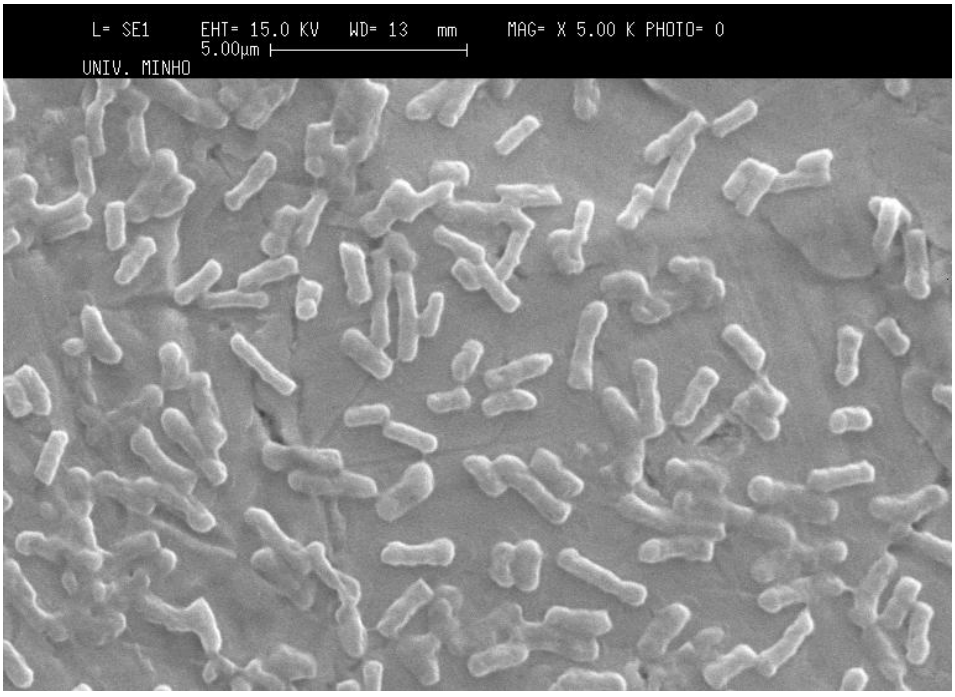
Plate	Strain	Material	Name	Figure	Remarks
Plate 2 **48 h @ 37 °C**	*WT*	Stainless Steel	P2WTACO 1/2	5.15	Very well attachment
		Silicone	P2WTSI 1/2	5.17	Thick biofilm
		Polypropylene	P2WTPP 1/2	5.16	No attachment/artifact
	bolA+/rpoS-	Stainless Steel	P2BACO 1/2	5.9	Well attached
		Silicone	P2BSI 1/2	5.11	Well attached/cell aggregation
		Polypropylene	P2BPP 1/2	5.10	Less attachment
	rpoS+/bolA-	Stainless Steel	P2RACO 1/2	5.12	Less attachment
		Silicone	P2RSI 1/2	5.14	Well attached
		Polypropylene	P2RPP 1/2	5.13	Very less attachment

Even after 48 hours of growth at 37 °C, the *rpoS* and *bolA* mutated cells and the WT were not able to attach to the surface of polypropylene, which shows again that it is not a suitable substratum for *E .coli* to attach and to form a biofilm (Fig. 5.10, 5.13 and 5.16). Cells which are seen in SEM images may be artefacts. After 24 hours of exposure to cells, stainless steel proved to be the best surface for cells to attach. However, as was discussed earlier in this chapter, this could have been because of

the conditioning film formed on stainless steel after the initial adhesion of bacteria (Carpentier & Cerf 1993; Korber *et al*. 1995). When a clean surface is colonised, physiochemical variables immediately facilitate the interactions between the surface and the liquid phase. A clear difference was seen at 48 hour compared to 24 hours of growth (Fig. 5.15). It can be seen that there was greater production of EPS at 48 hour especially on stainless steel and silicon surfaces (Fig. 5.9, 5.11, 5.15 and 5.17). Thick biofilm was seen to be produced by WT (Fig. 5.17) and in presence of *bolA* on silicon (Fig. 5.11), while the cell density and thickness of biofilm decreased in absence of *bolA* (Fig. 5.14). This clearly shows that in absence of *rpoS*, *bolA* is responding well and acting through a different network, which allows the cells to attach and multiply.

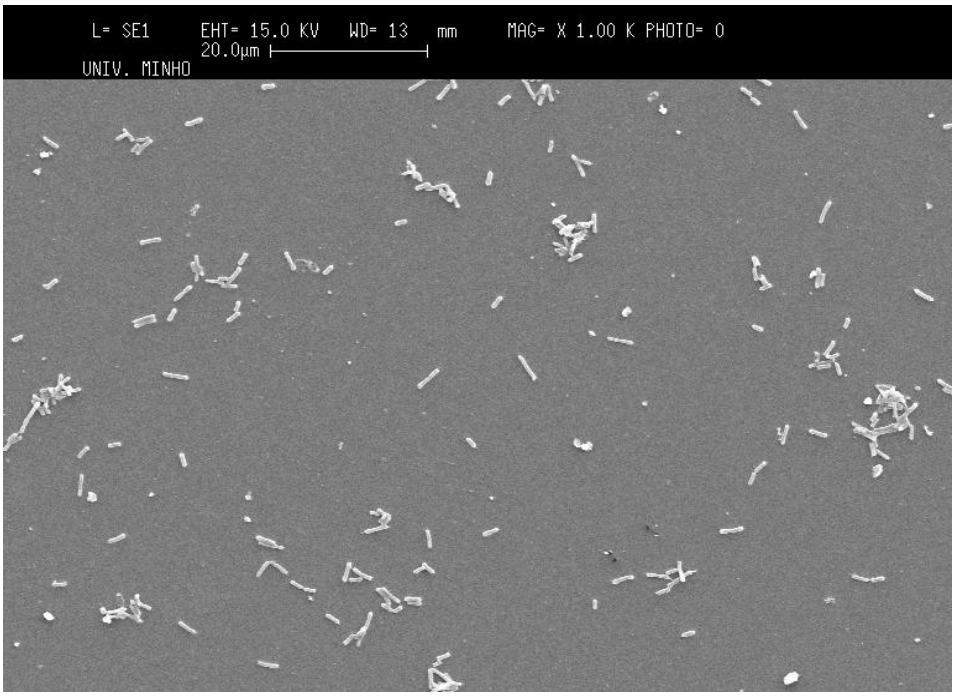


Name: P2BACO1 (1000 X)

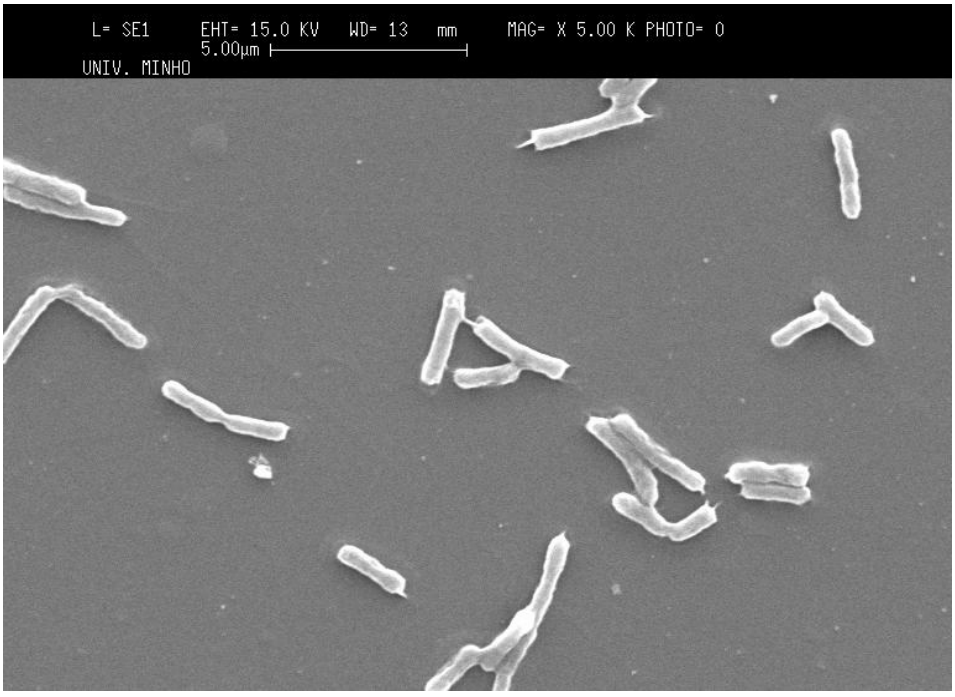


Name: P2BACO2 (5000 X)

Figure 5.9: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 37 °C on stainless steel. It can be seen here that cells can adhere certainly on stainless steel surface.

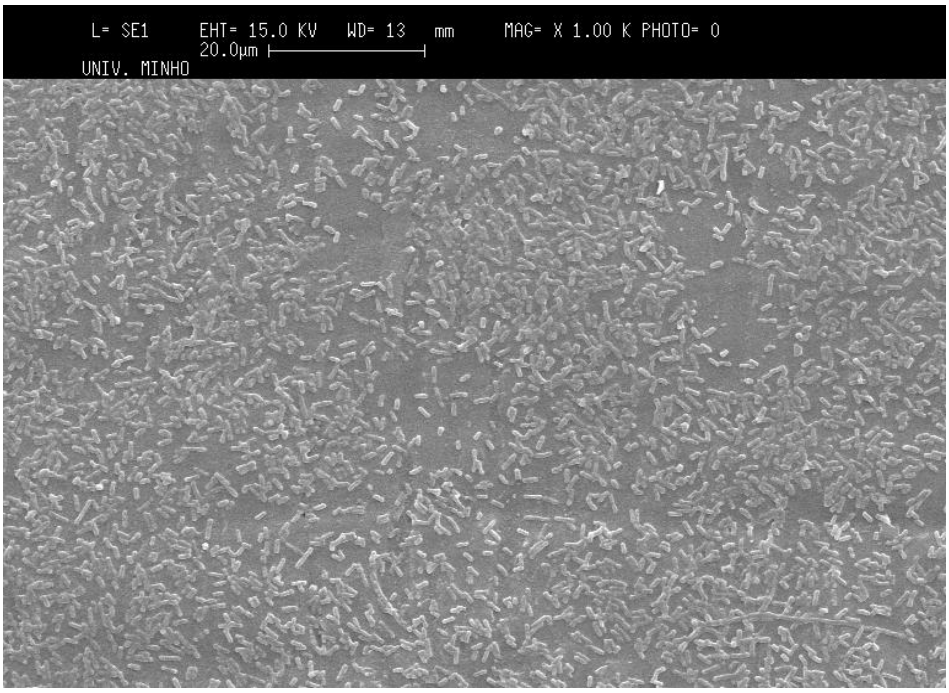


Name: P2BPP1 (1000 X)

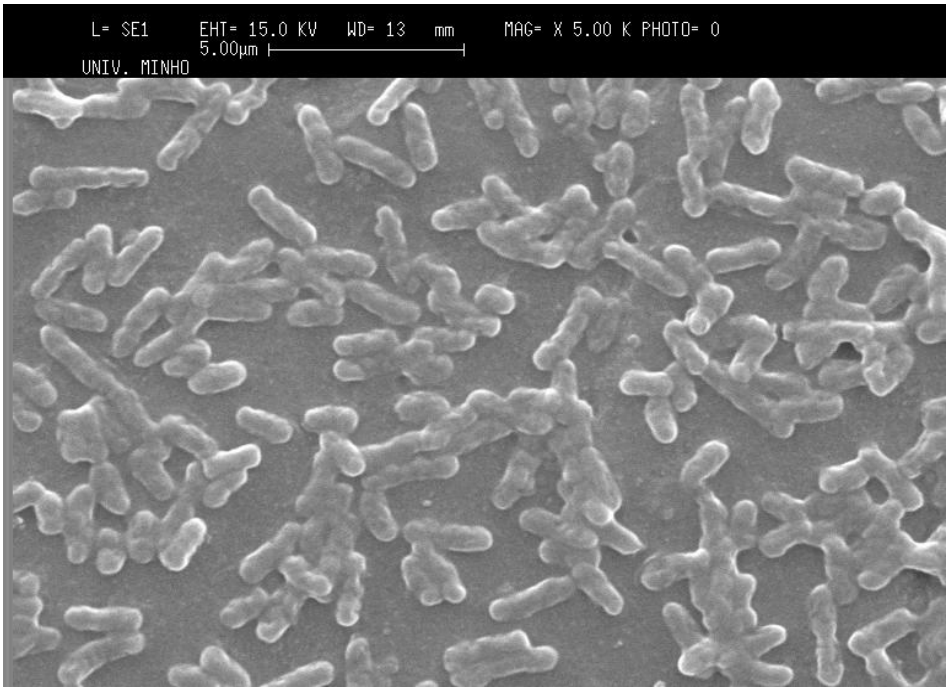


Name: P2BPP2 (5000 X)

Figure 5.10: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 37 °C on polypropylene. As in earlier cases, polypropylene surface was found to be not suitable for the cells to attach.

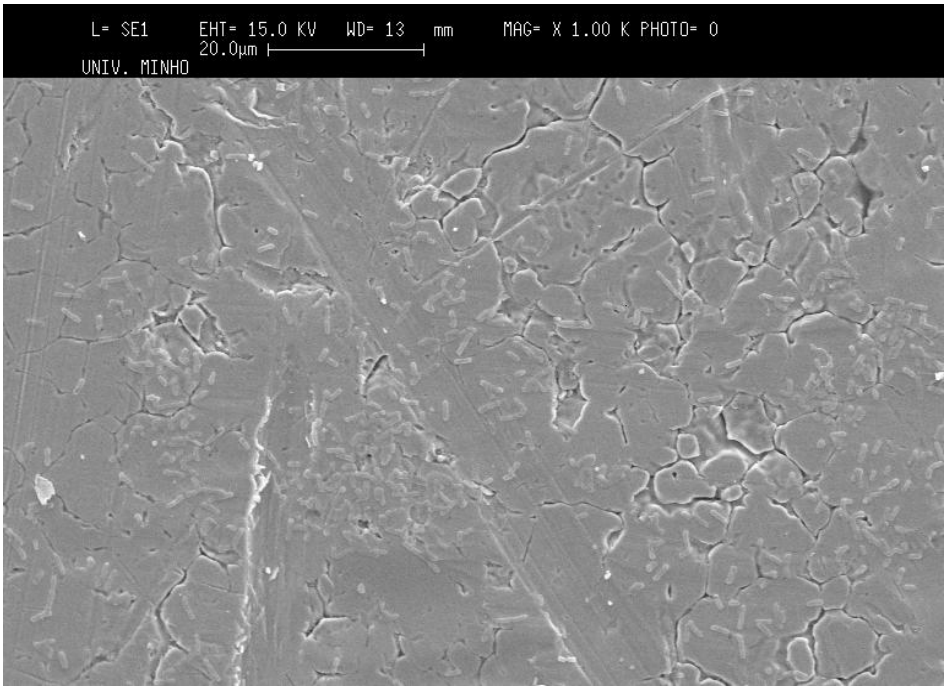


Name: P2BSI1 (1000 X)

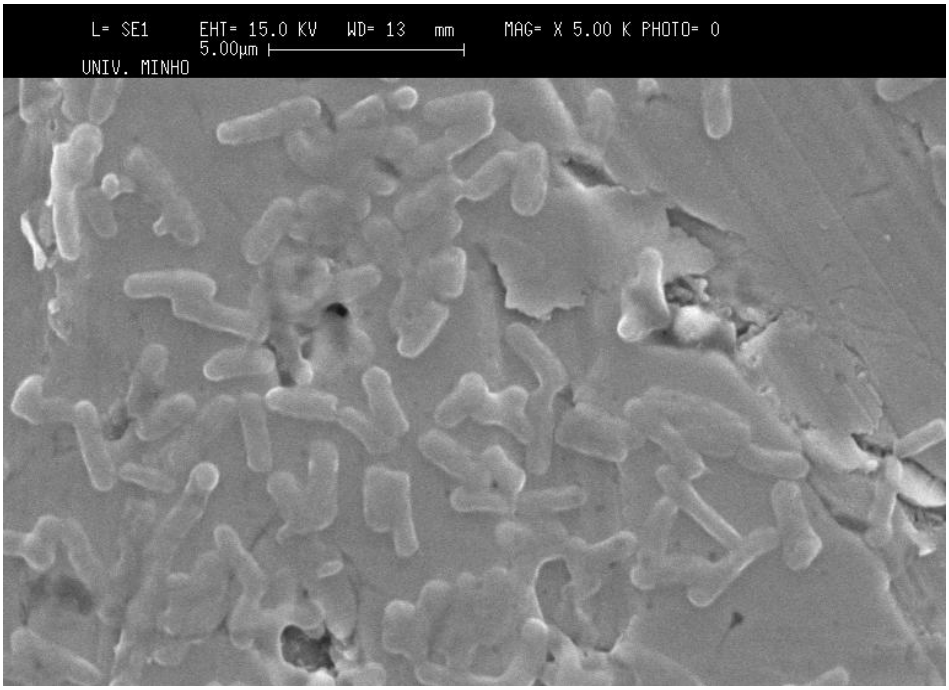


Name: P2BSI2 (5000 X)

Figure 5.11: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 37 °C on silicon. Cells can attach well on silicon surface and might grow as biofilms under stress conditions.

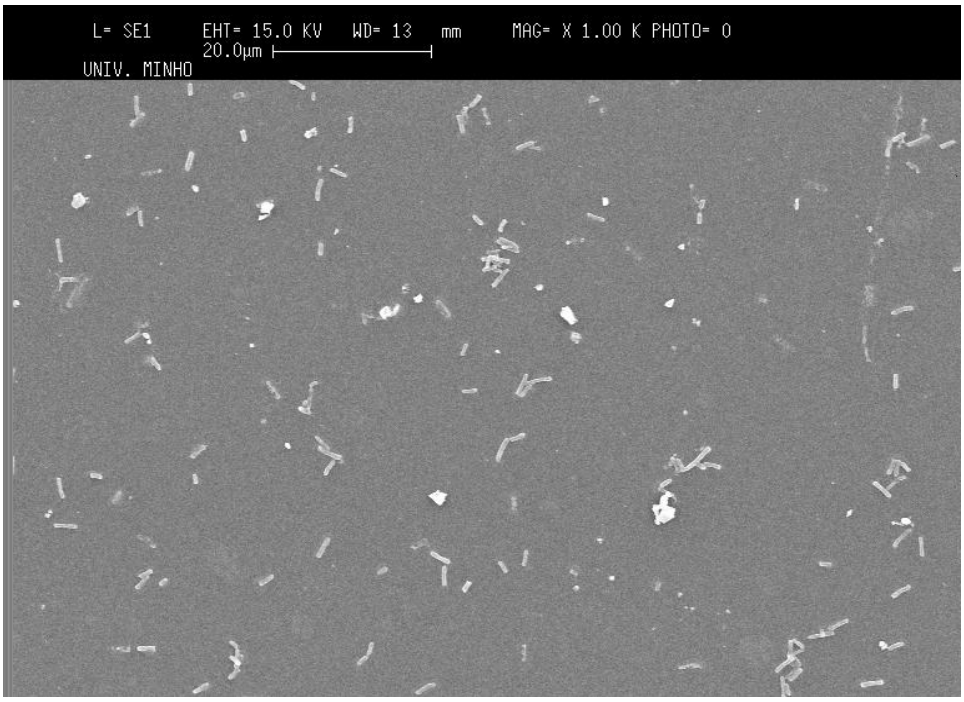


Name: P2RACO1 (1000 X)

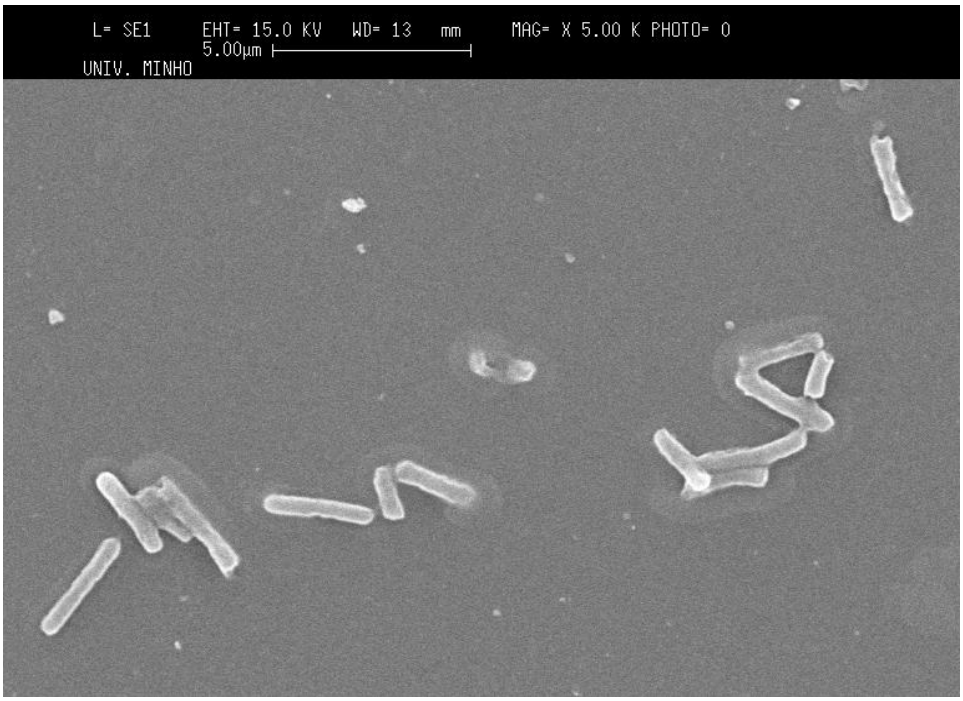


Name: P2RACO2 (5000 X)

Figure 5.12: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 37 °C on stainless steel. It was found that presence of *bolA* is important for attachment to stainless steel surface.

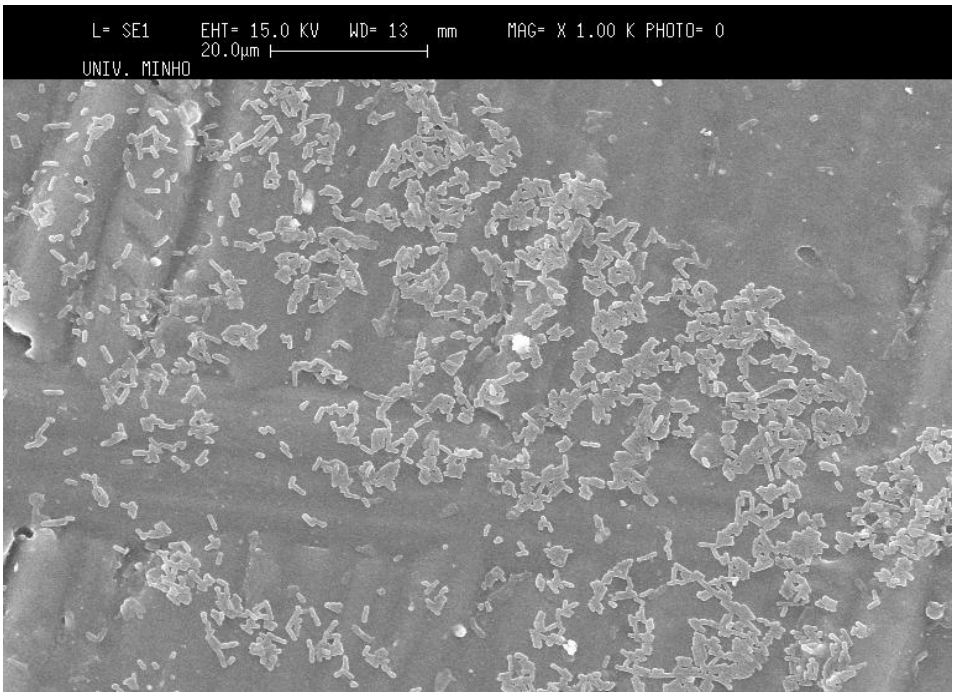


Name: P2RPP1 (1000 X)

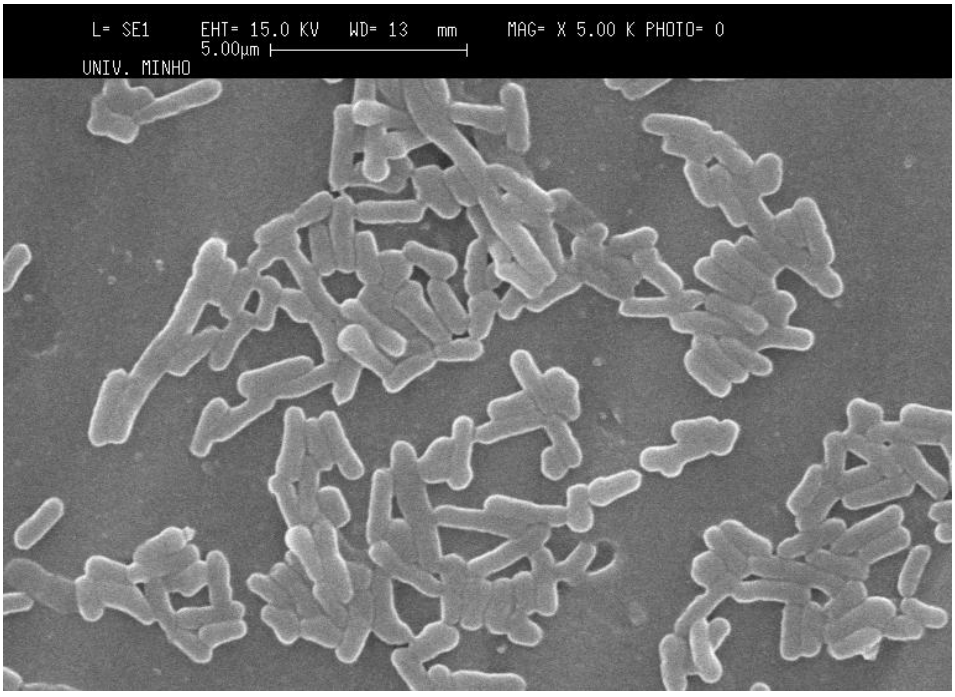


Name: P2RPP2 (5000 X)

Figure 5.13: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 37 °C on polypropylene. As like earlier cases, polypropylene surface was found to be not suitable for the cells to attach.

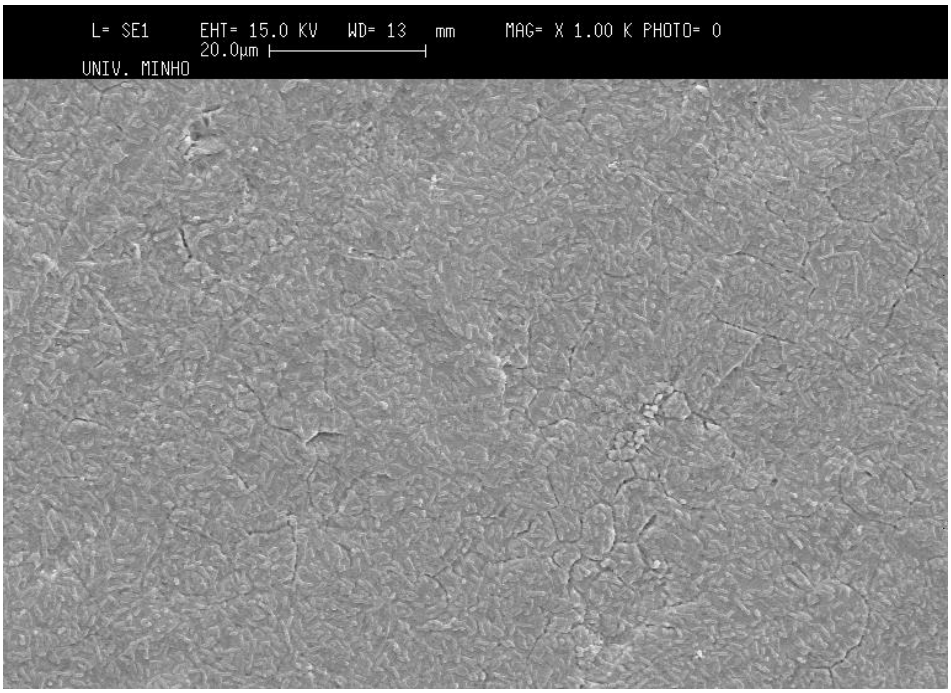


Name: P2RSI1 (1000 X)

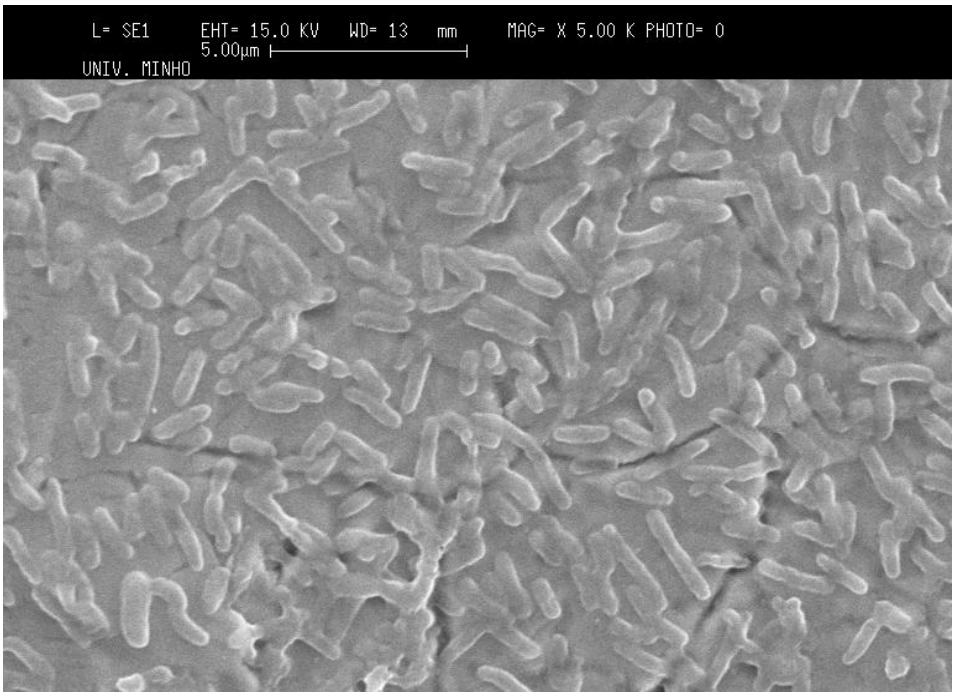


Name: P2RSI2 (5000 X)

Figure 5.14: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 37 °C on silicon. It can be seen that cells can attach well on silicon surfaces.

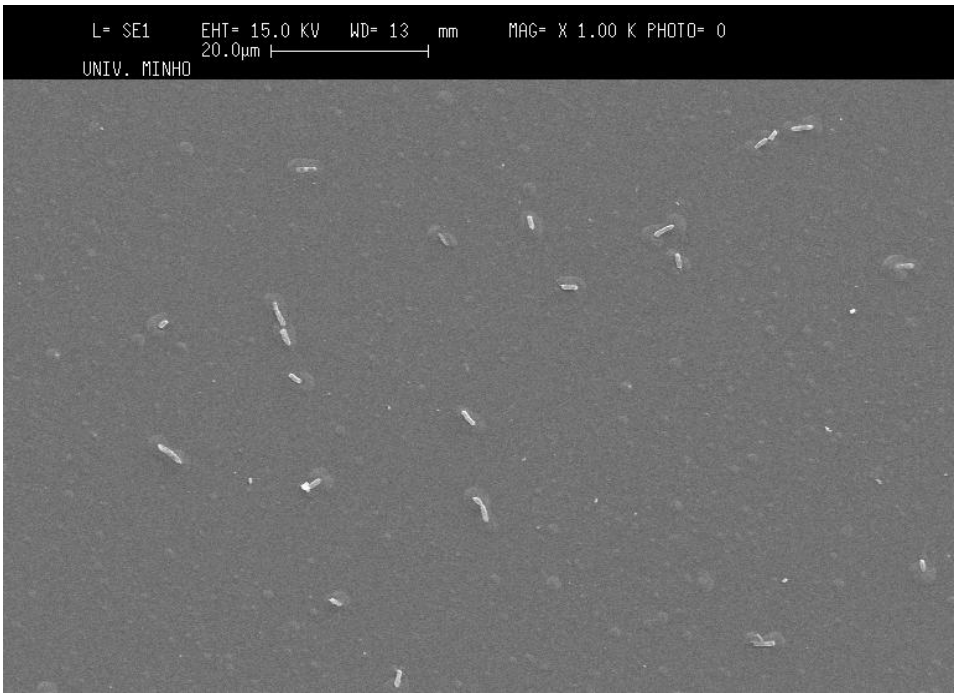


Name: P2WTACO1 (1000 X)

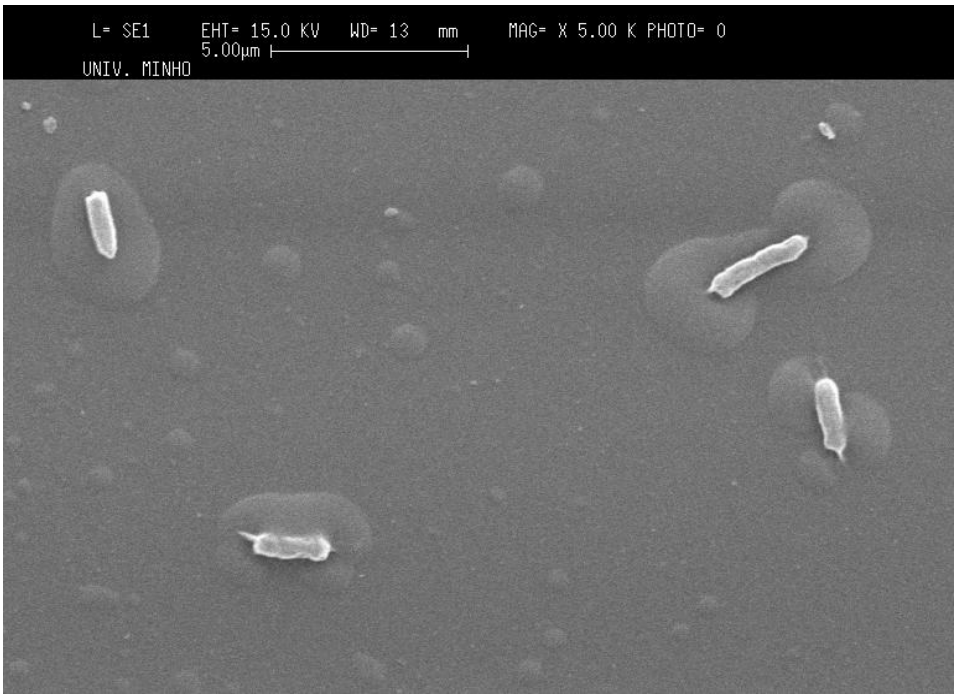


Name: P2WTACO2 (5000 X)

Figure 5.15: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 37 °C on stainless steel. Like in earlier cases, stainless steel was found to be the elite surface for cell attachment.

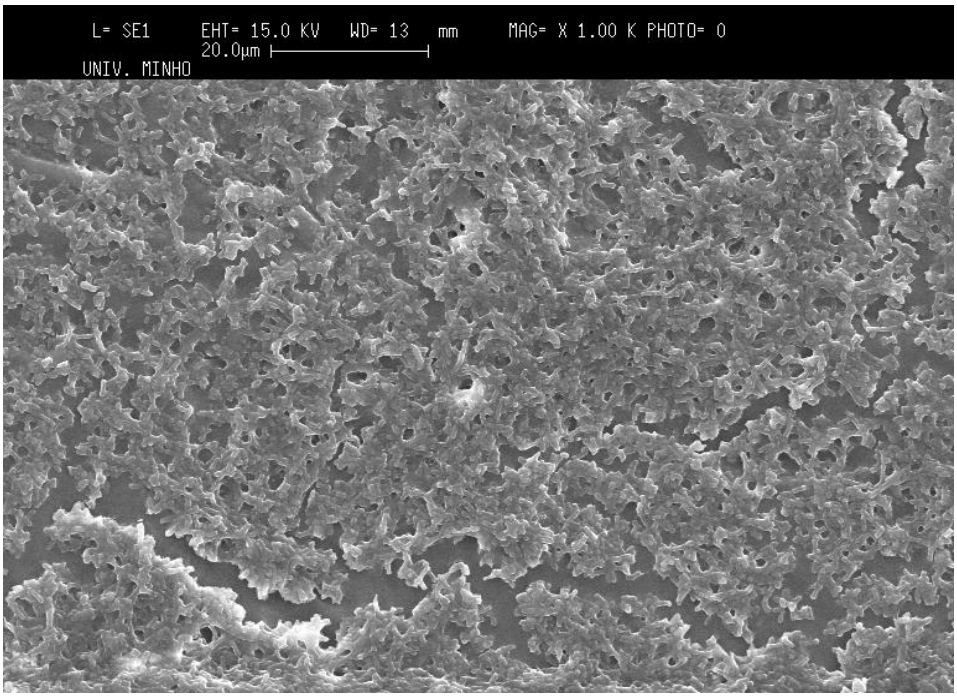


Name: P2WTPP1 (1000 X)

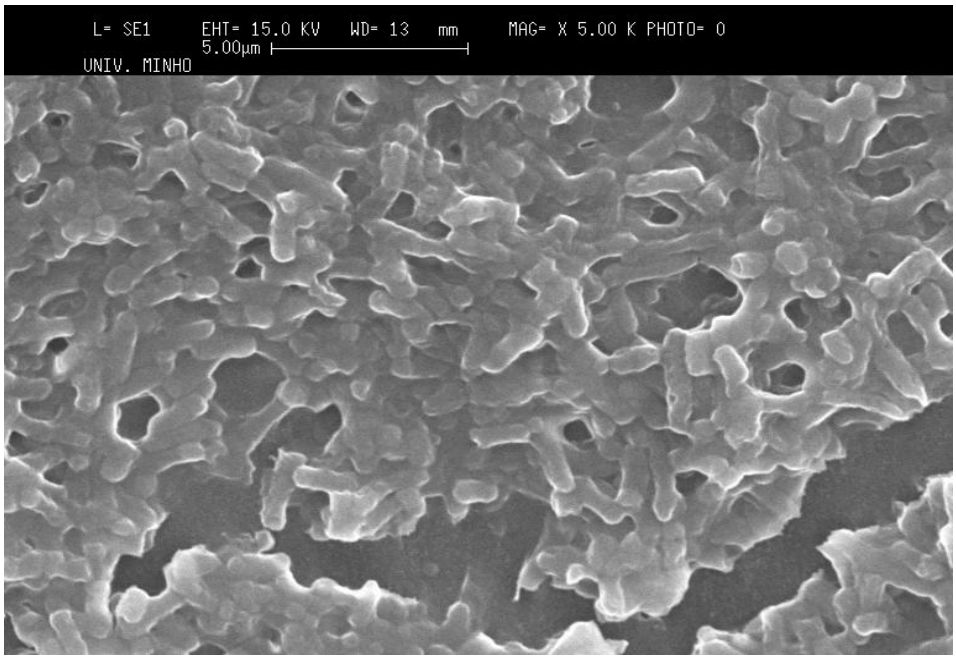


Name: P2WTPP2 (5000 X)

Figure 5.16: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 37 °C on polypropylene. The result shows that polypropylene is still not the suitable surface for cells even after 48 hours.



Name: P2WTSI1 (1000 X)



Name: P2WTSI2 (5000 X)

Figure 5.17: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 37 °C on silicon. Thick biofilm was seen in presence of *rpoS* and *bolA* genes on silicon surface after 48 hours without any stress condition.

5.6.3 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (72 h @ 37 °C).

The conditions used in this study are listed in Table 5.4. For the images presented, the following designations are used. For example, P6WTSI 1/2 should read as,

P6 - Plate 6 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

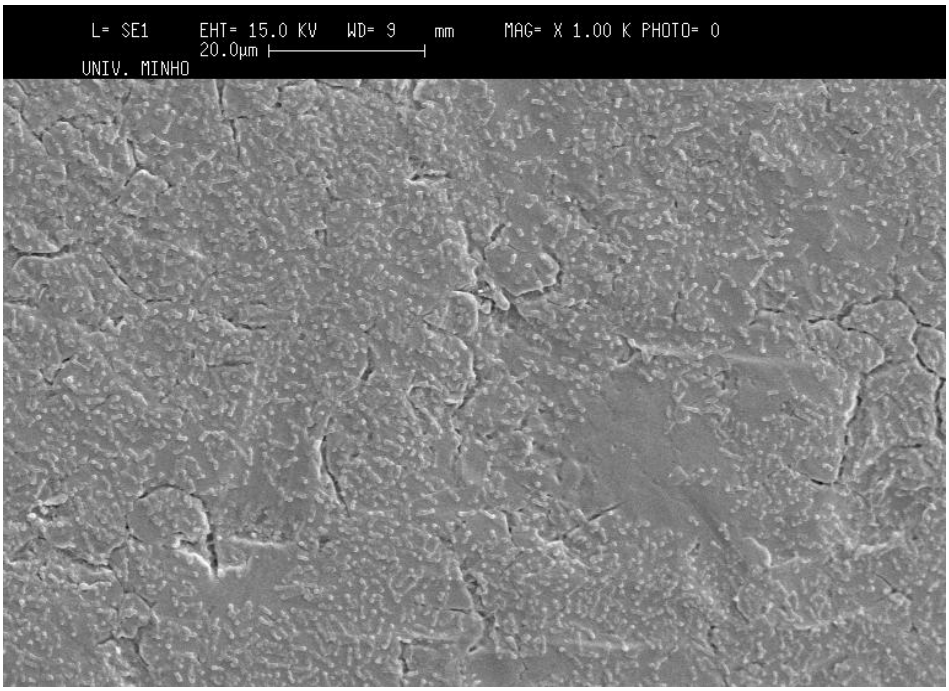
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.4: Condition used in this study with the designated names and remarks

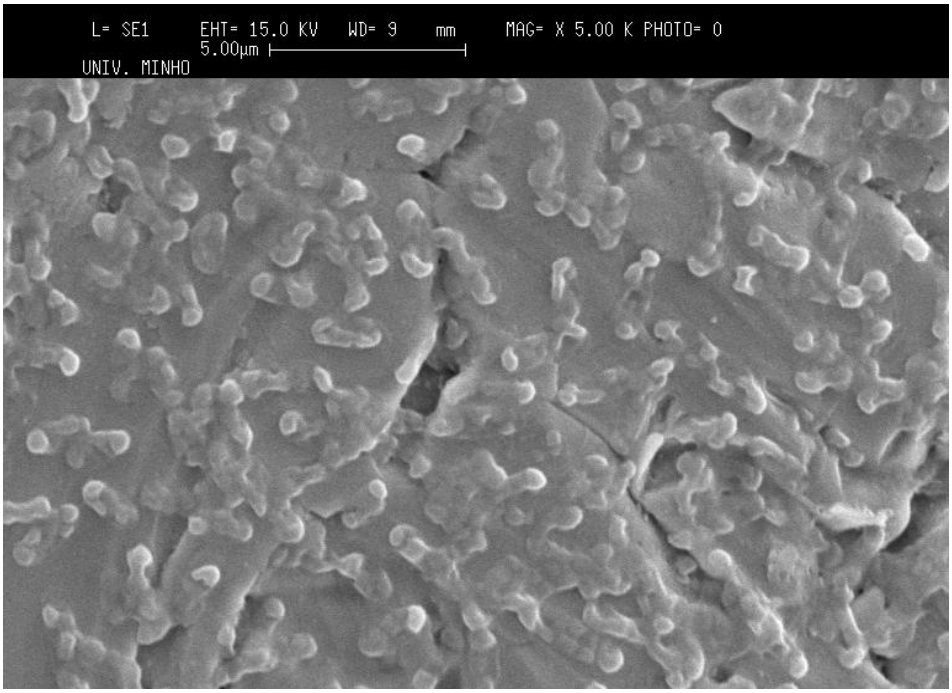
Plate	Strain	Material	Name	Figure	Remarks
Plate 6 **72 h @ 37 °C**	*WT*	Stainless Steel	P6WTACO 1/2	5.24	Thick biofilm
		Silicone	P6WTSI 1/2	5.26	Well attached
		Polypropylene	P6WTPP 1/2	5.25	Thick biofilm
	bolA+/rpoS-	Stainless Steel	P6BACO 1/2	5.18	Well attached
		Silicone	P6BSI 1/2	5.20	Well attached
		Polypropylene	P6BPP 1/2	5.19	Less attachment
	rpoS+/bolA-	Stainless Steel	P6RACO 1/2	5.21	Round morphology
		Silicone	P6RSI 1/2	5.23	Very less attachment
		Polypropylene	P6RPP 1/2	5.22	Very less attachment

Biofilm formation by *E. coli* was clearly observed on both stainless steel and silicon surfaces by all the three stains after a 3 day incubation period at 37 ºC, as shown in Figure 5.18, 5.20, 5.21, 5.23, 5.24, 5.25 and 5.26. Stainless steel and silicon surfaces provided a higher intensity of biofilm formation by WT, compared to polypropylene surfaces (Fig. 5.24 and 5.25). After 72 hours of growth, morphological changes were seen. As it is shown in figure 5.20 and 5.21, cells became significantly smaller and

more globular. These changes cannot be caused by the over-expression of recombinant proteins, but are caused by high cell density. The shape of cells changed from rod-shaped to spherical. It was found that WT (Fig. 5.24) could attach and grow into biofilms on stainless steel at a faster rate when compared to *rpoS* and *bolA* (Fig. 5.18 and 5.21) mutated cells. This shows the importance of these two genes in attachment to stainless steel and their ability to grow as biofilms. It is almost impossible to quantify surface microorganisms since they may occur in groups and the cells may be arranged in overlapping layers, but it is confirmed that mutation in *bolA* and *rpoS* reduces the attachment of cells to stainless steel and silicon.



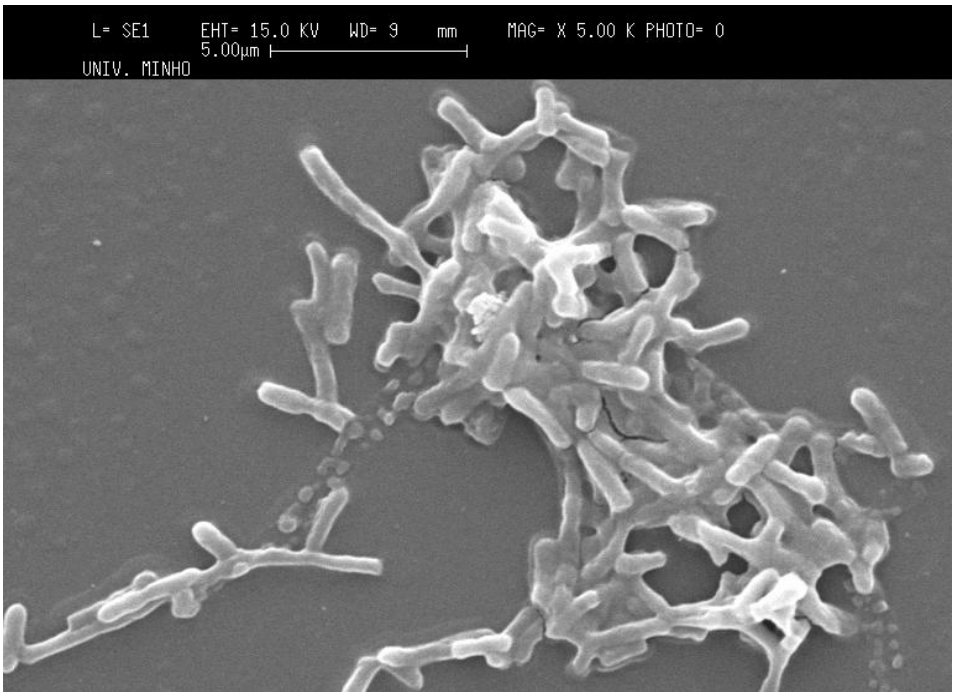
Name: P6BACO1 (1000 X)



Name: P6BACO2 (5000 X)

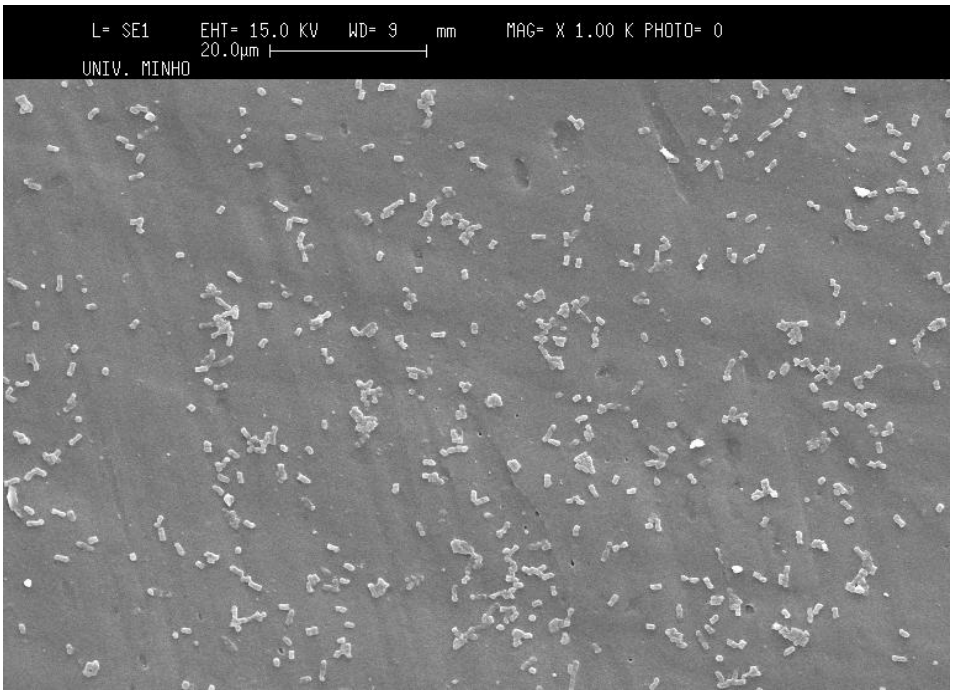
Figure 5.18: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 37 °C on stainless steel. High cell density can be seen on surface. Mutation in *rpoS* changes the morphology of cell.

Name: P6BPP1 (1000 X)

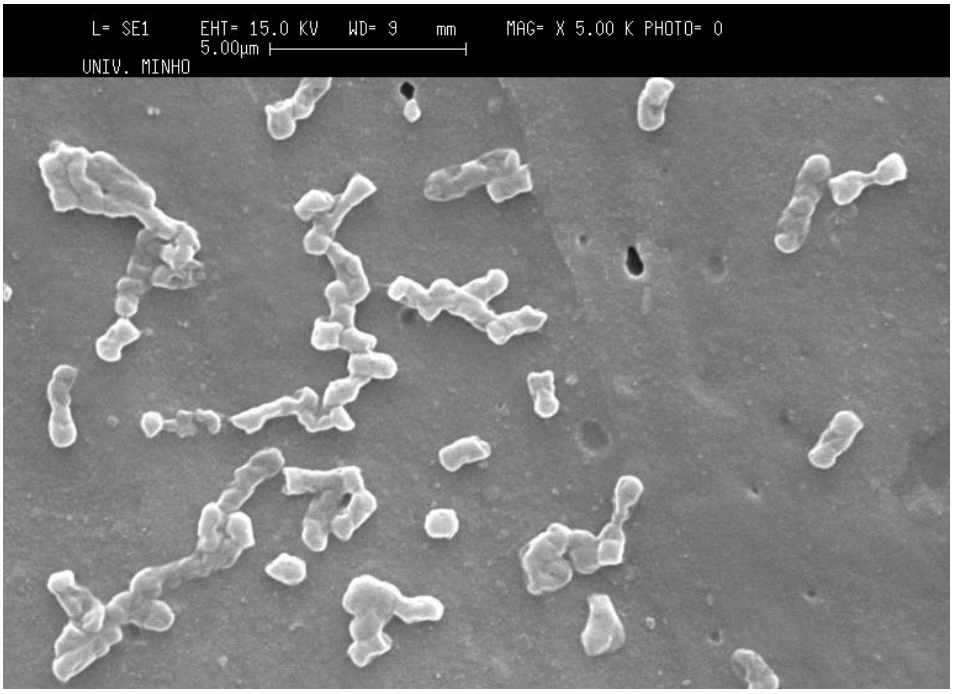


Name: P6BPP2 (5000 X)

Figure 5.19: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 37 °C on polypropylene. Very less number of cells can be seen attached on polypropylene surface.

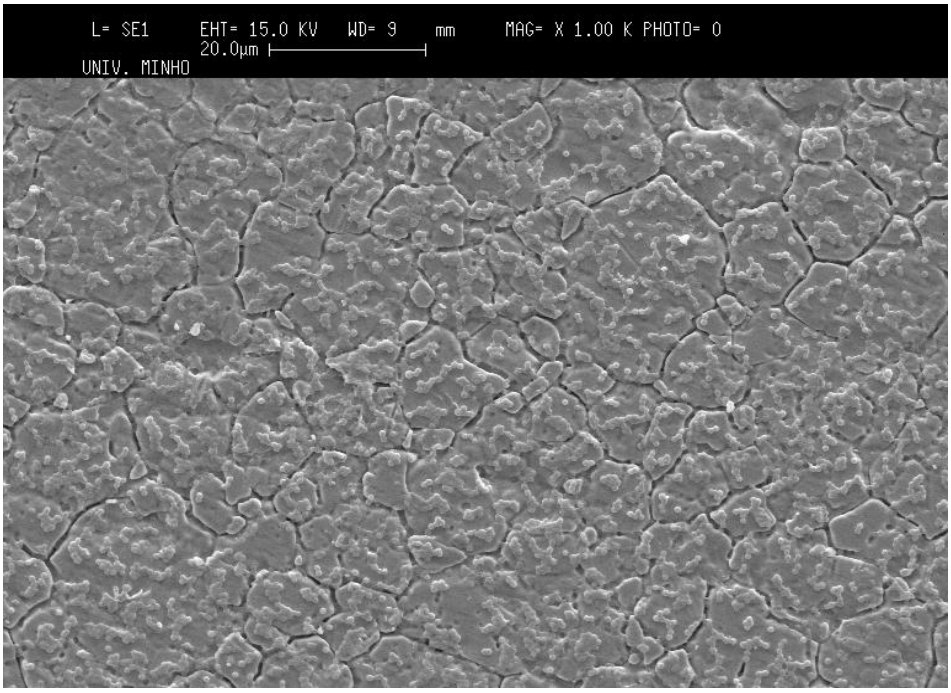


Name: P6BSI1 (1000 X)

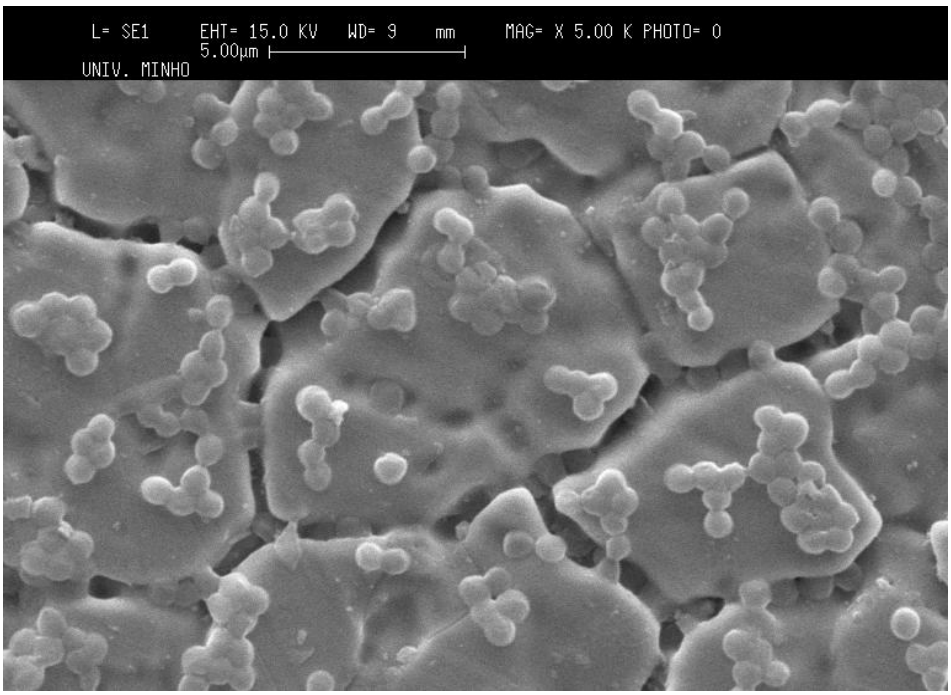


Name: P6BSI2 (5000 X)

Figure 5.20: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 37 °C on silicon. Very less number of cells were found to be attached and changes in the morphology was seen.

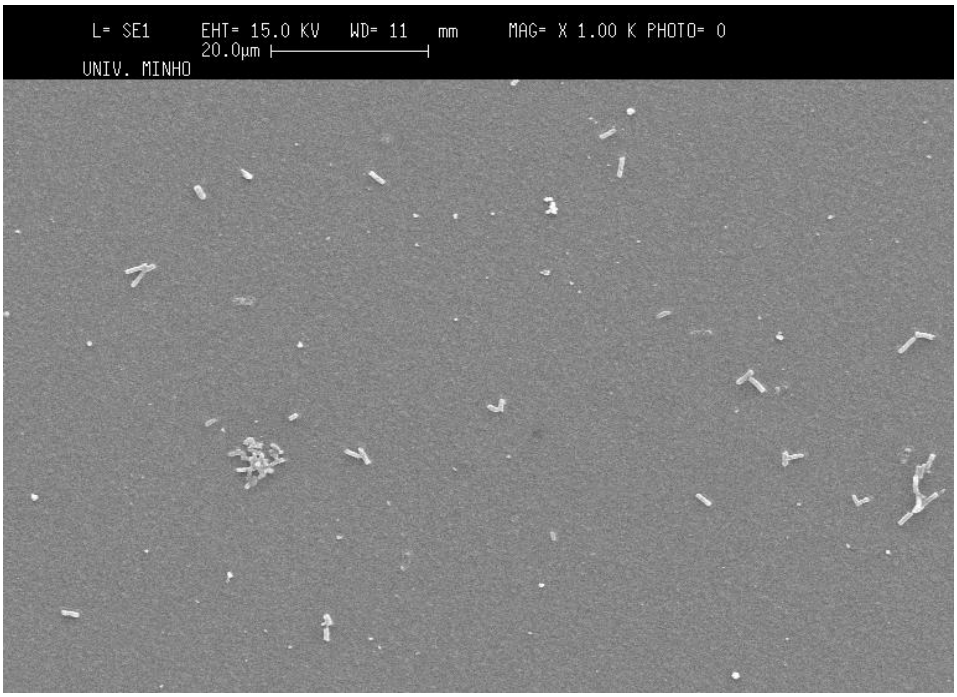


Name: P6RACO1 (1000 X)

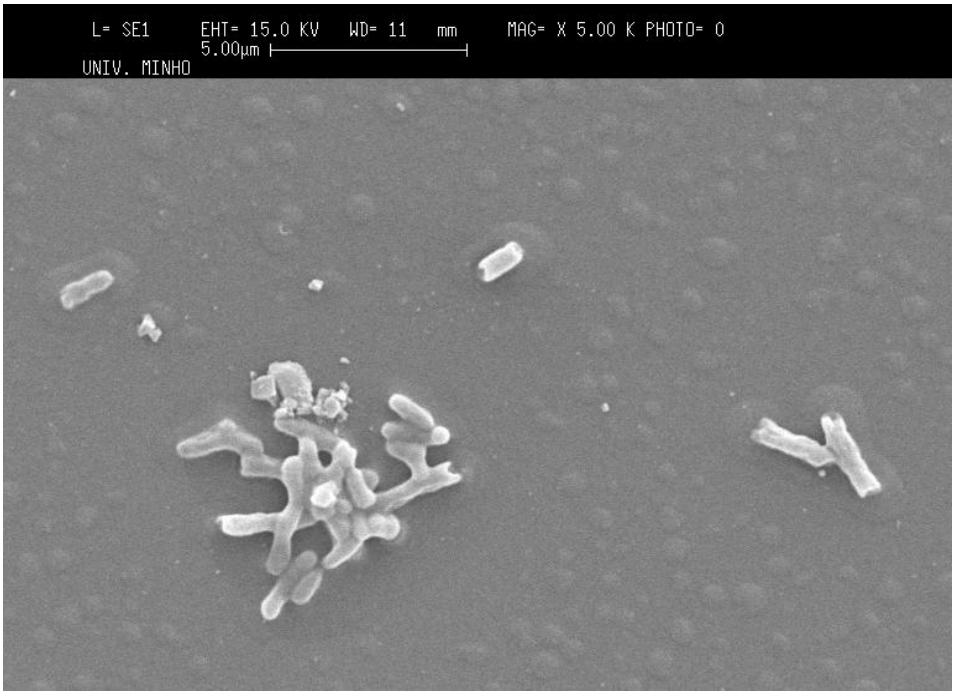


Name: P6RACO2 (5000 X)

Figure 5.21: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 37 °C on stainless steel.

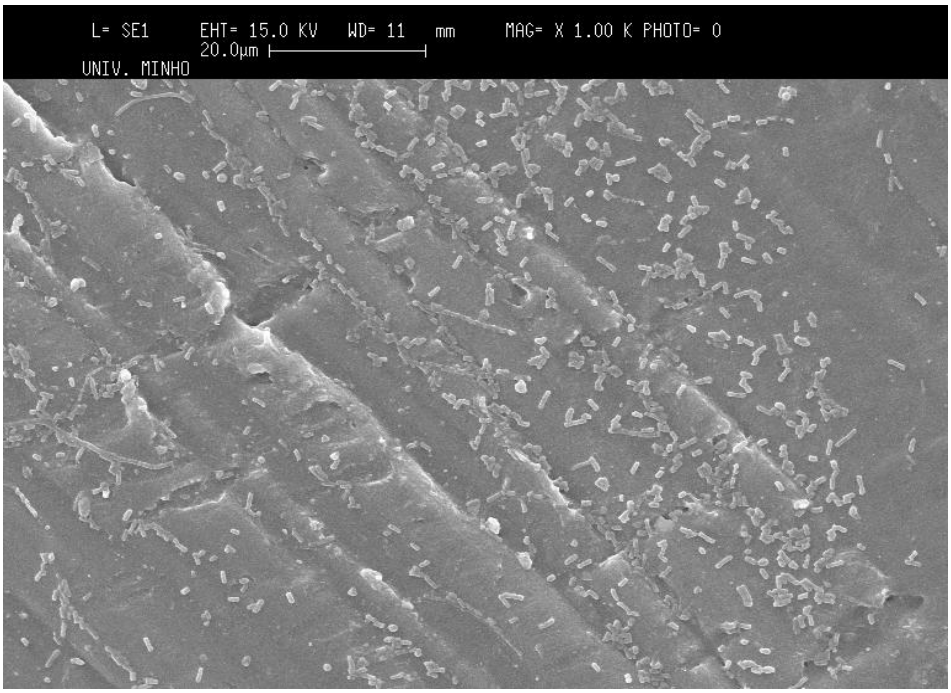


Name: P6RPP1 (1000 X)

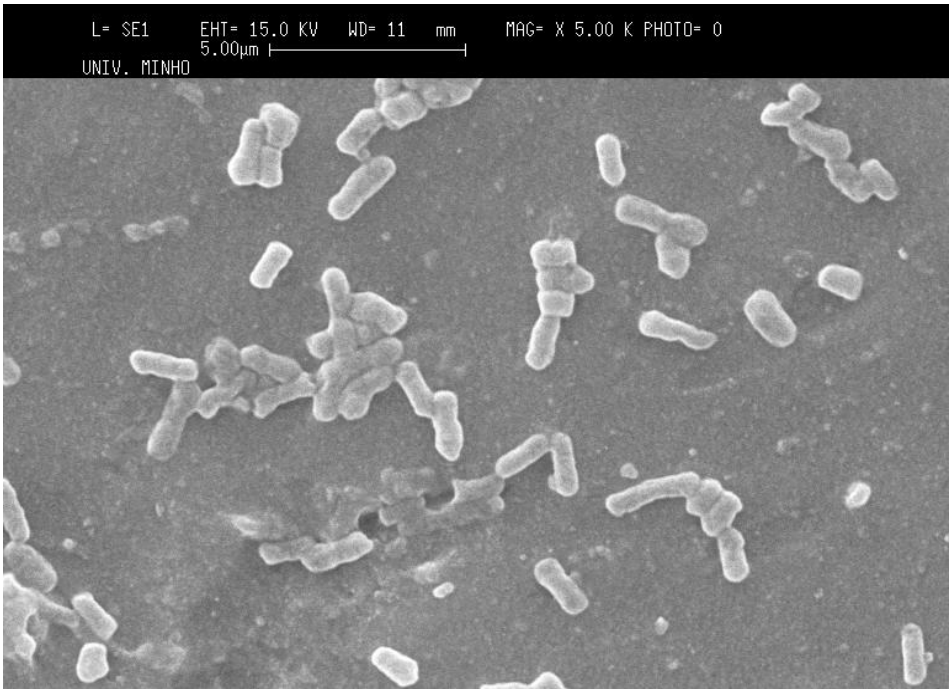


Name: P6RPP2 (5000 X)

Figure 5.22: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 37 °C on polypropylene.

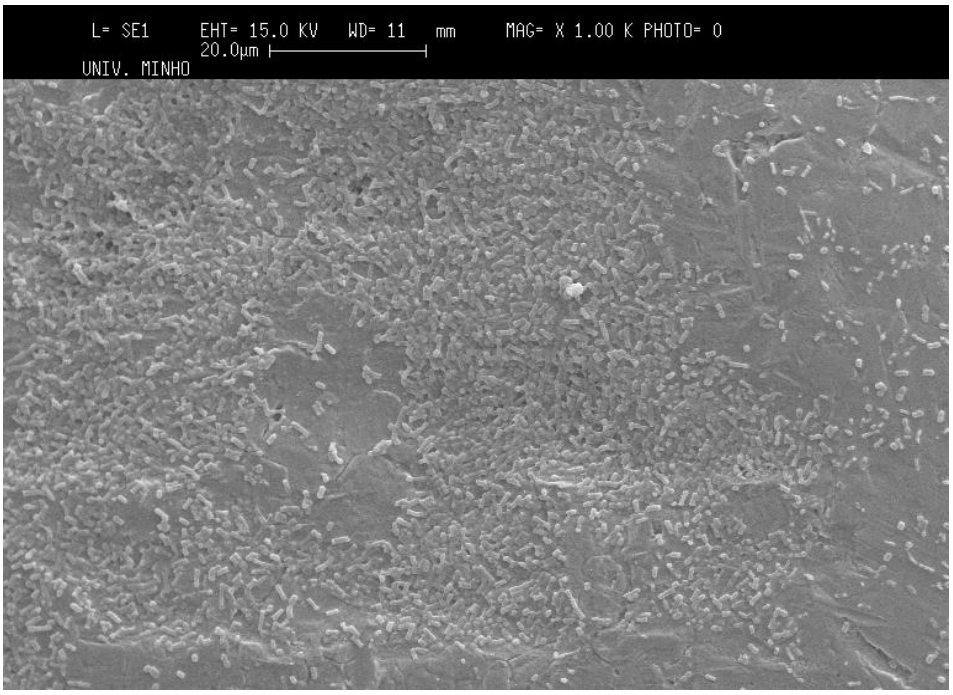


Name: P6RSI1 (1000 X)

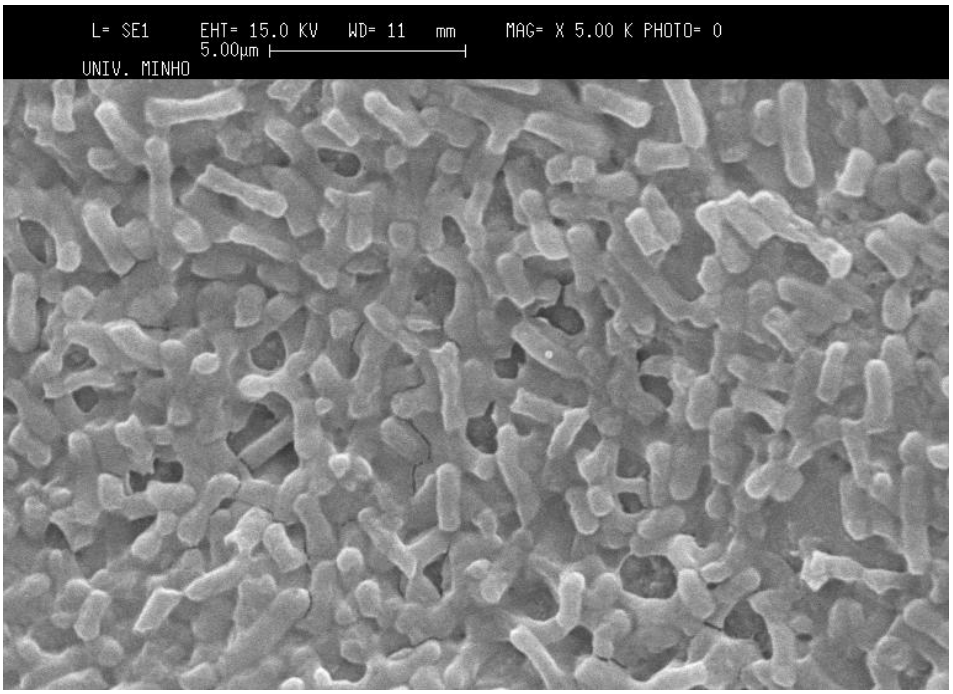


Name: P6RSI2 (5000 X)

Figure 5.23: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 37 °C on silicon.

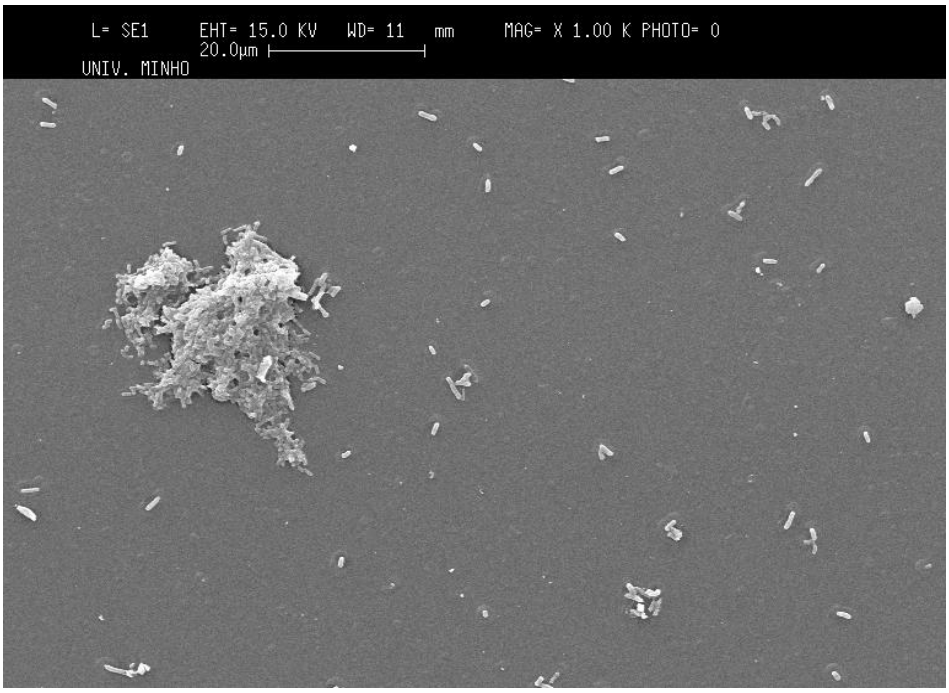


Name: P6WTACO1 (1000 X)

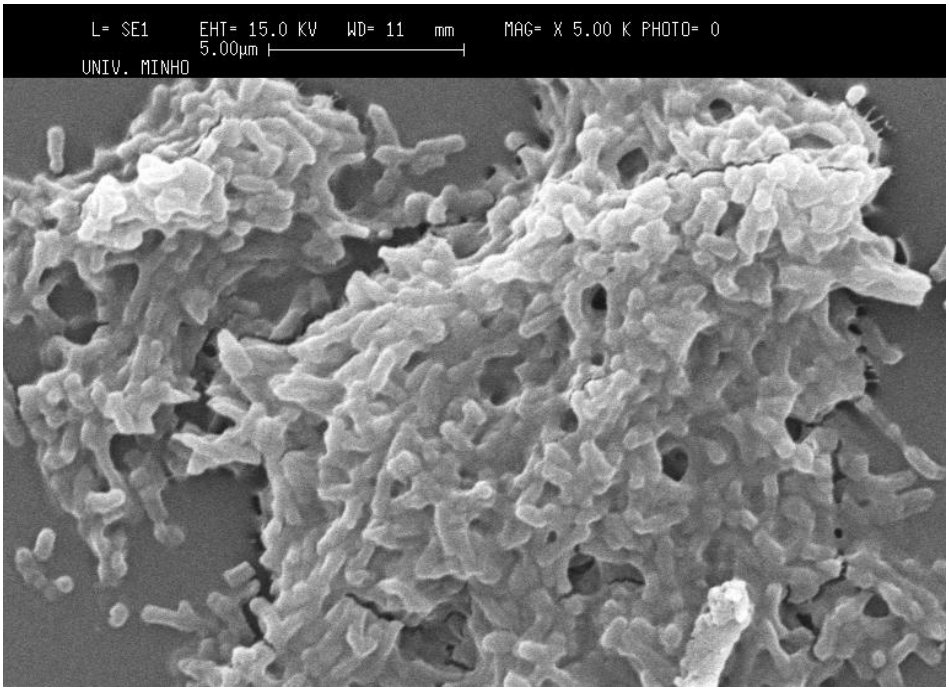


Name: P6WTACO2 (5000 X)

Figure 5.24: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 37 °C on stainless steel.

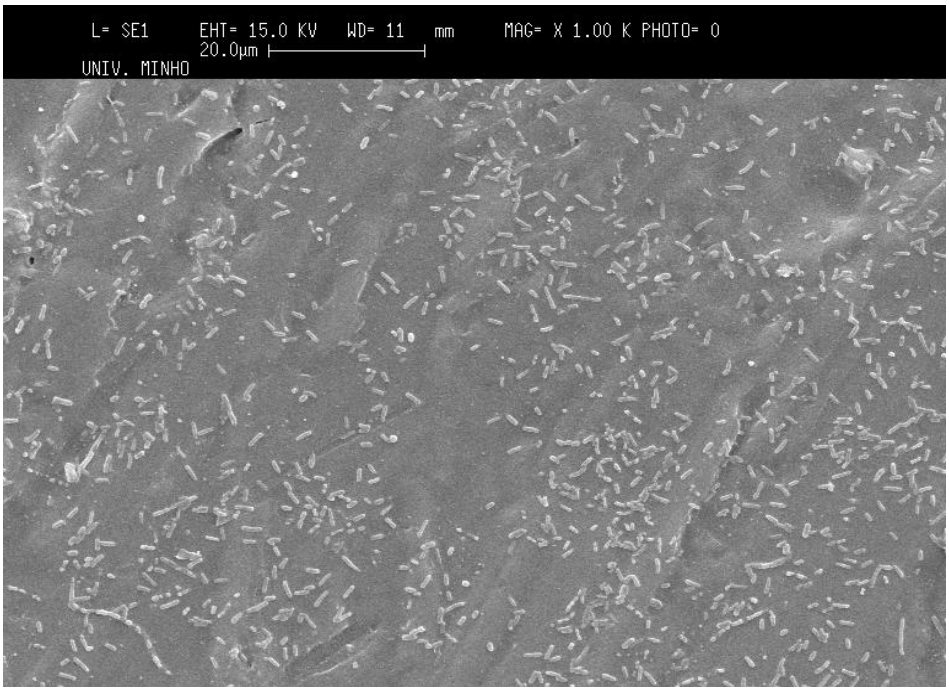


Name: P6WTPP1 (1000 X)

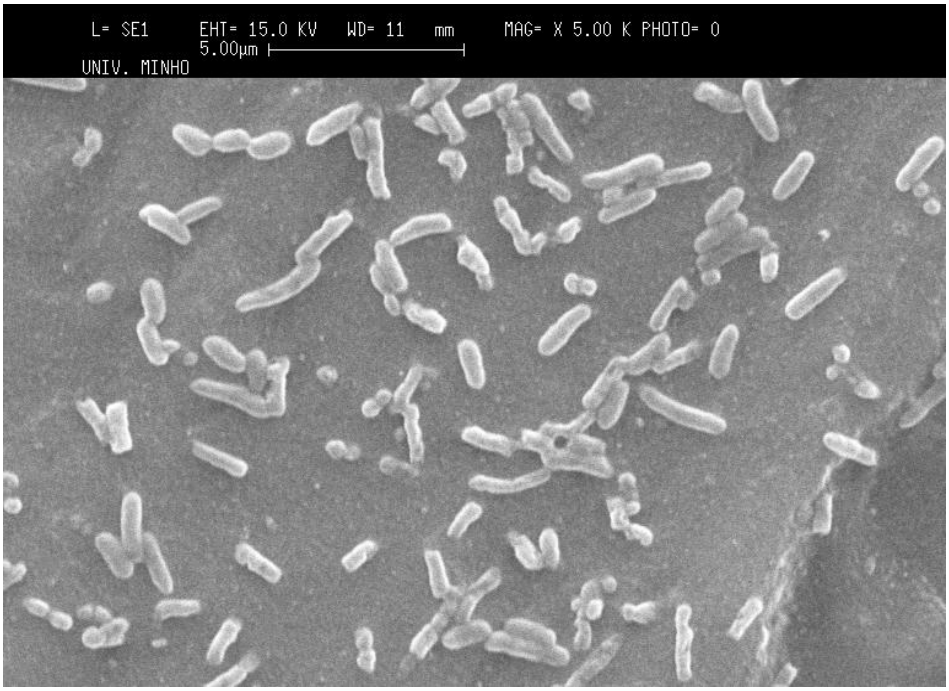


Name: P6WTPP2 (5000 X)

Figure 5.25: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 37 °C on polypropylene.



Name: P6WTSI1 (1000 X)



Name: P6WTSI2 (5000 X)

Figure 5.26: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 37 °C on silicon.

5.6.4 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (48 h stress @ 46 °C).

The conditions used in this study are listed in Table 5.5. For the images presented, the following designations are used. For example, P3WTSI 1/2 should read as,

P3 - Plate 3 (condition)

WT - Wild Type (strain), **B** - *bolA*+/*rpoS*-, **R** - *rpoS*+/*bolA*-

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

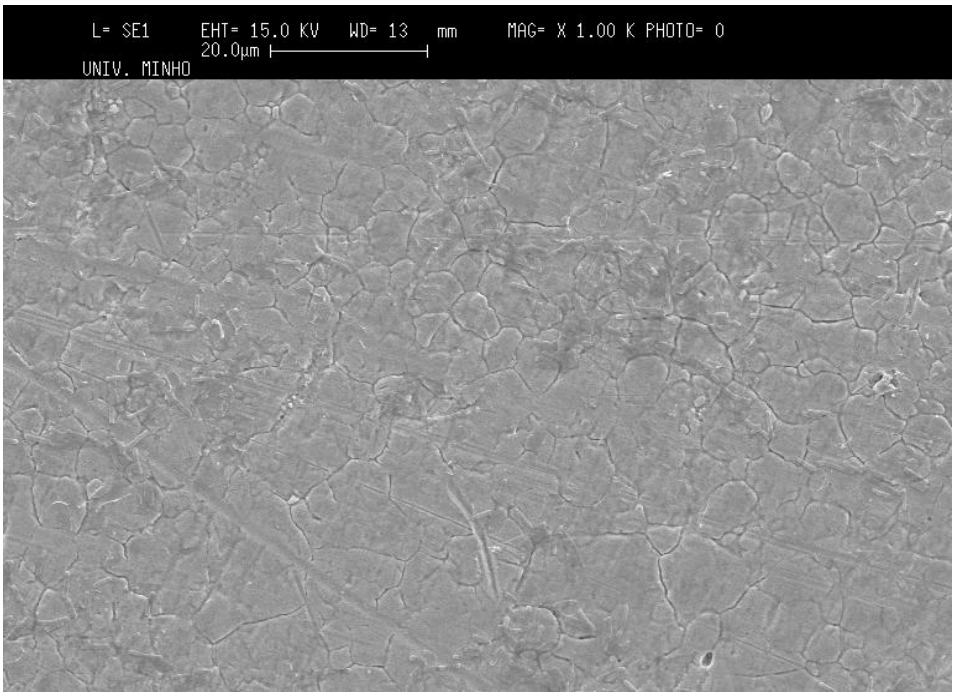
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.5: Condition used in this study with the designated names and remarks

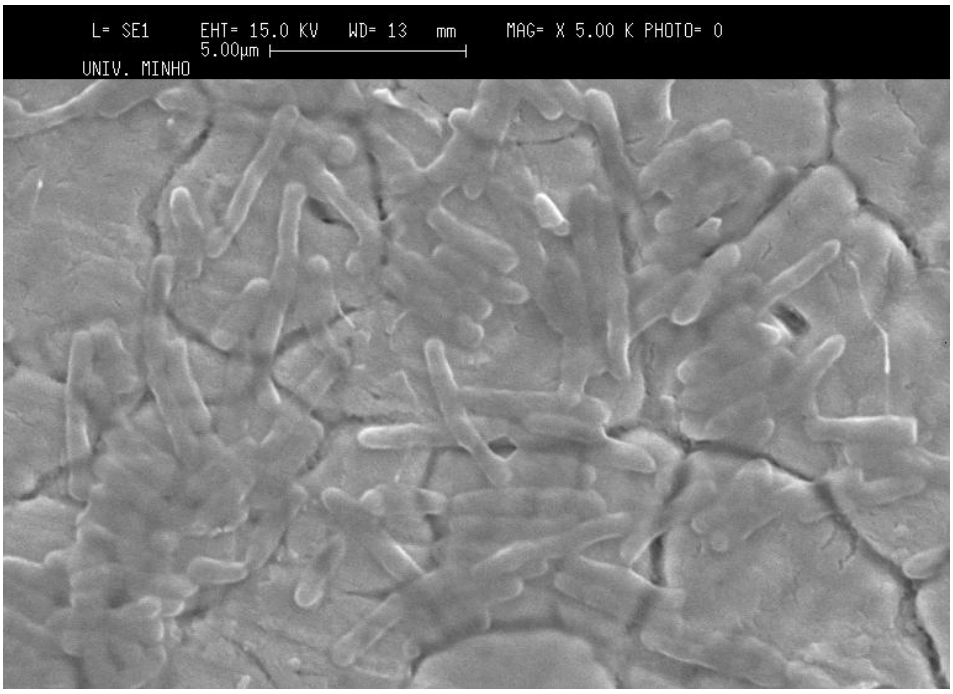
Plate	Strain	Material	Name	Figure	Remarks
Plate 3 **48 h stress @ 46 °C (24 h/37 °C + 24 h/46 °C)**	*WT*	Stainless Steel	P3WTACO 1/2	5.32	Very less attachment
		Silicone	P3WTSI 1/2	5.34	Less attachment
		Polypropylene	P3WTPP 1/2	5.33	L-form
	bolA+/*rpoS*-	Stainless Steel	P3BACO 1/2	5.27	Elongated cells
		Silicone	P3BSI 1/2	5.29	Well attached
		Polypropylene	P3BPP 1/2	5.28	Less attachment/elongated cells
	rpoS+/*bolA*-	Stainless Steel	-		Nothing visible
		Silicone	P3RSI 1/2	5.31	Round morphology
		Polypropylene	P3RPP 1/2	5.30	Very less attachment

Only WT (Fig. 5.33) and *bolA*+ (Fig. 5.28) cells were able to attach to polypropylene surfaces under the heat shock condition (46 °C), while in absence of *bolA* and in presence of *rpoS* the cells did not attach to polypropylene. Unusual results were seen under heat shock condition. It was found that the WT strain underwent drastic morphological changes (Fig. 5.32, 5.33 and 5.34), possibly

leading to L-form conversion, i.e. lacking rigid walls. Transition of cells to polymorphic L-forms in response to stress factors has been considered as a potential mechanism for the survival of microbes in unfavourable environments. While in presence of *rpoS* and *bolA* genes it is unclear and incorrect to speculate whether cells are undergoing conversion to the L-form or not. In absence of *bolA*, cells did not attach to stainless steel under heat shock condition (image not shown here).

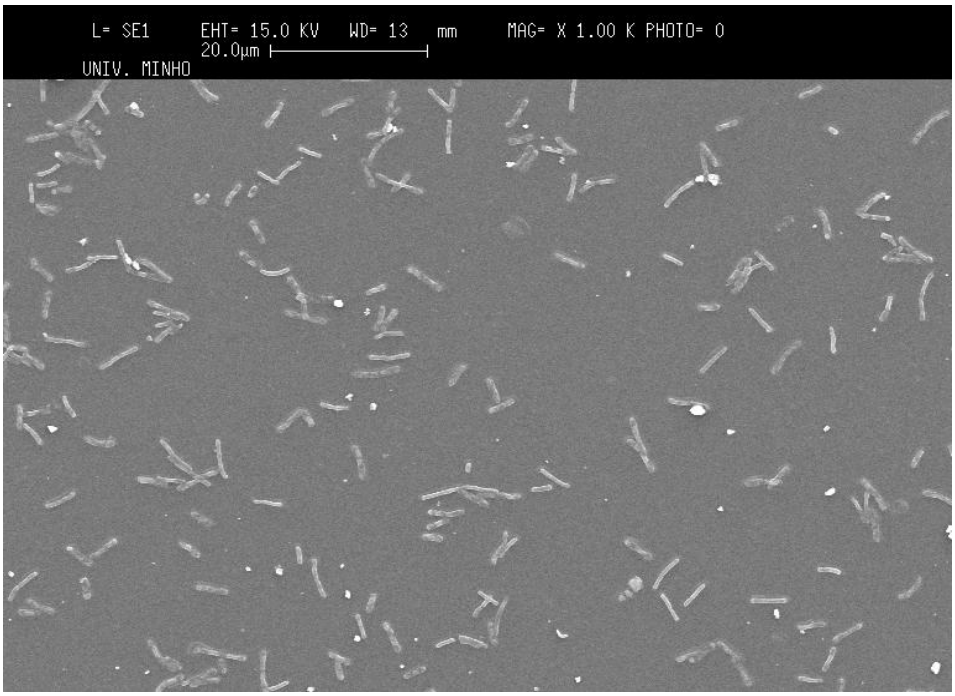


Name: P3BACO1 (1000 X)



Name: P3BACO2 (5000 X)

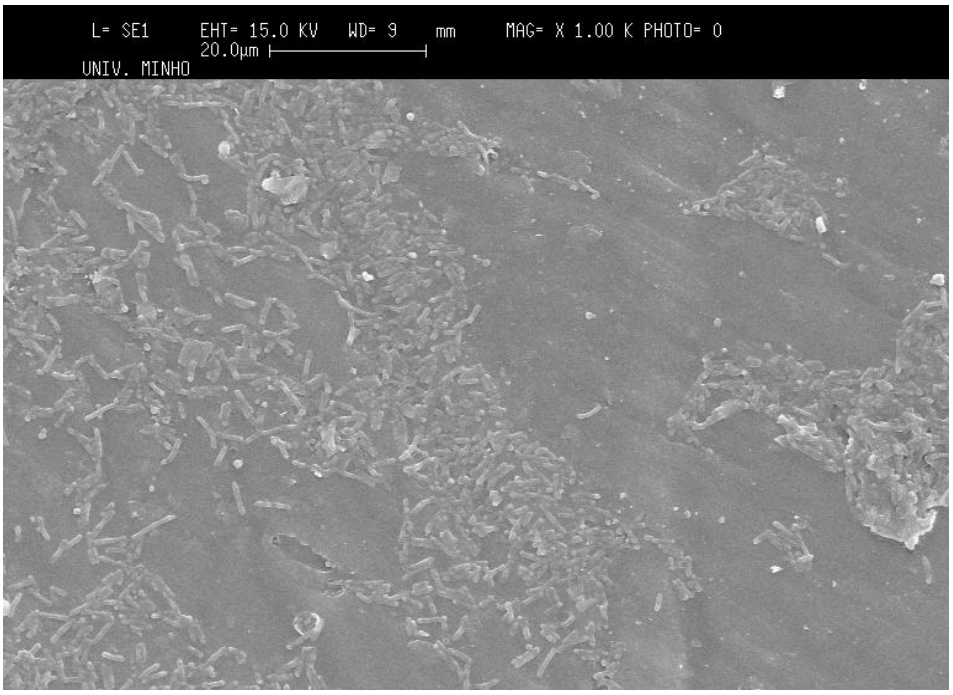
Figure 5.27: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 46 °C on stainless steel.



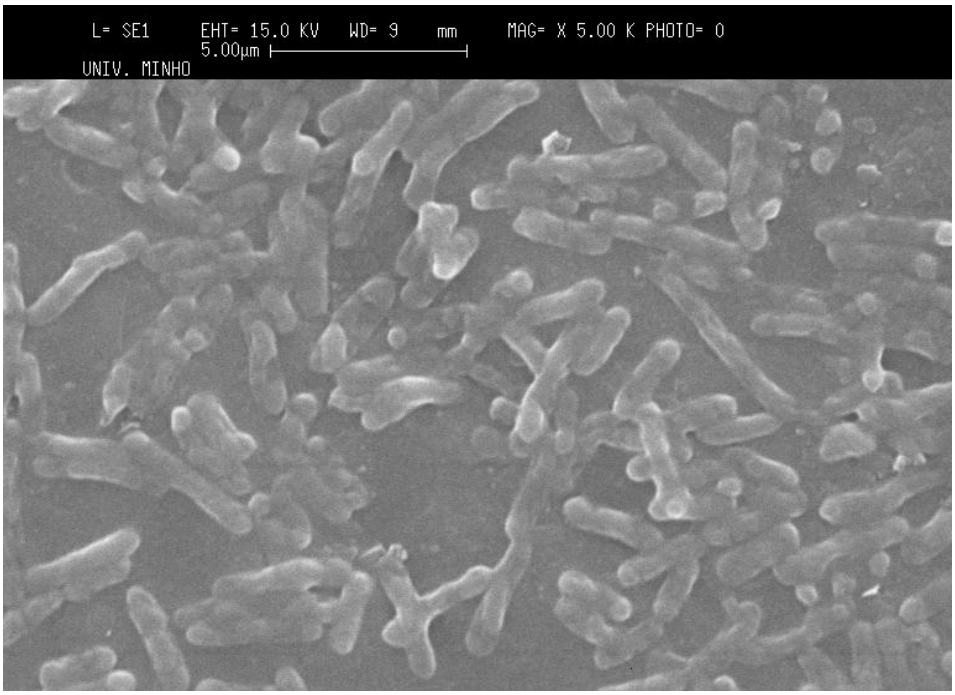
Name: P3BPP1 (1000 X)

Name: P3BPP2 (5000 X)

Figure 5.28: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 46 °C on polypropylene.

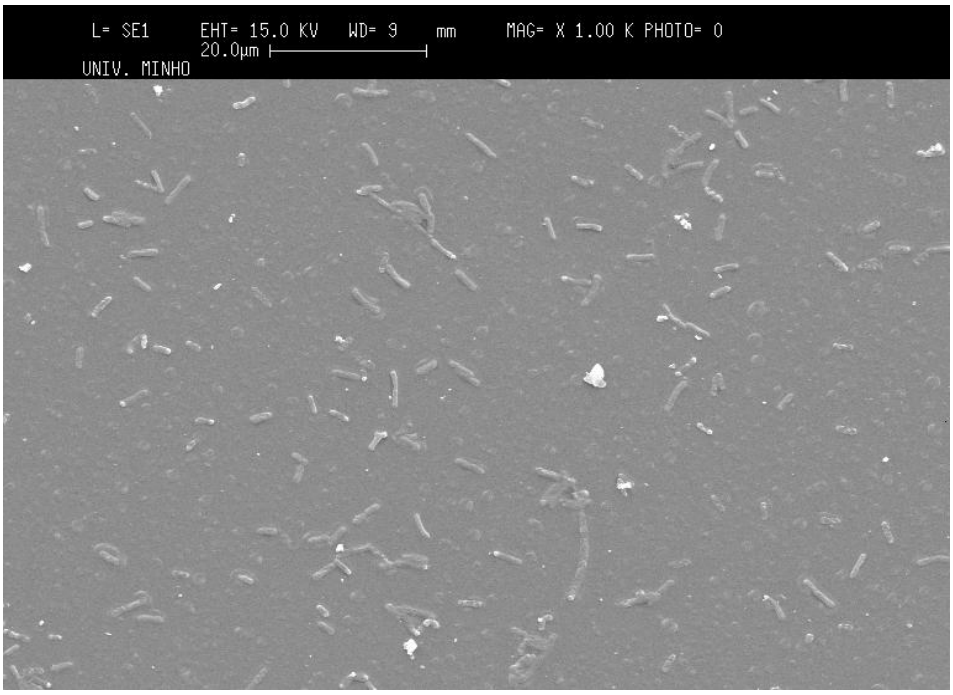


Name: P3BSI1 (1000 X)

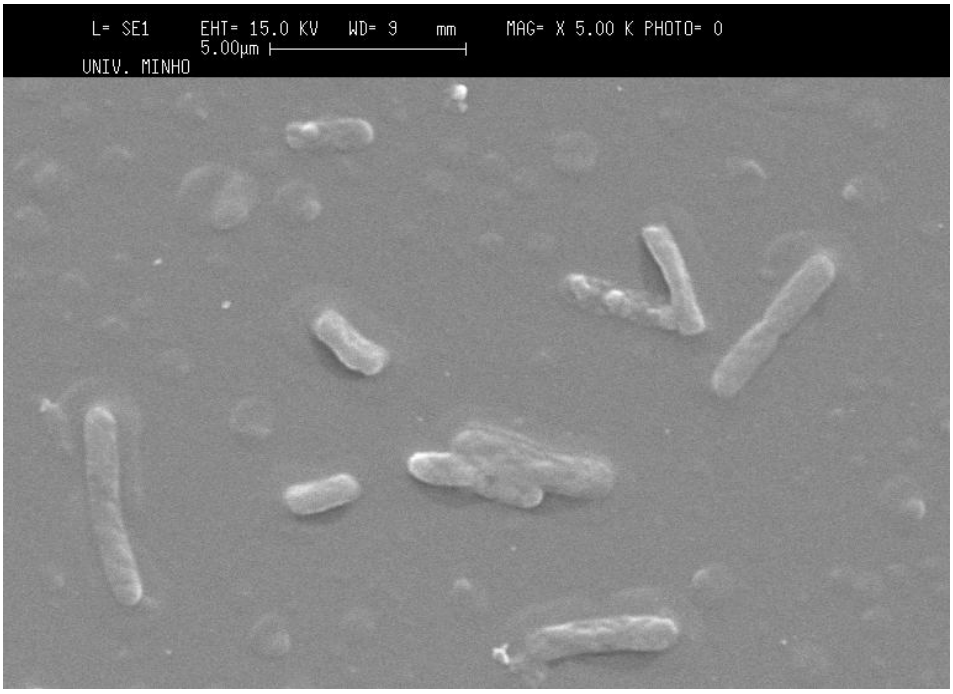


Name: P3BSI2 (5000 X)

Figure 5.29: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 46 °C on silicon.

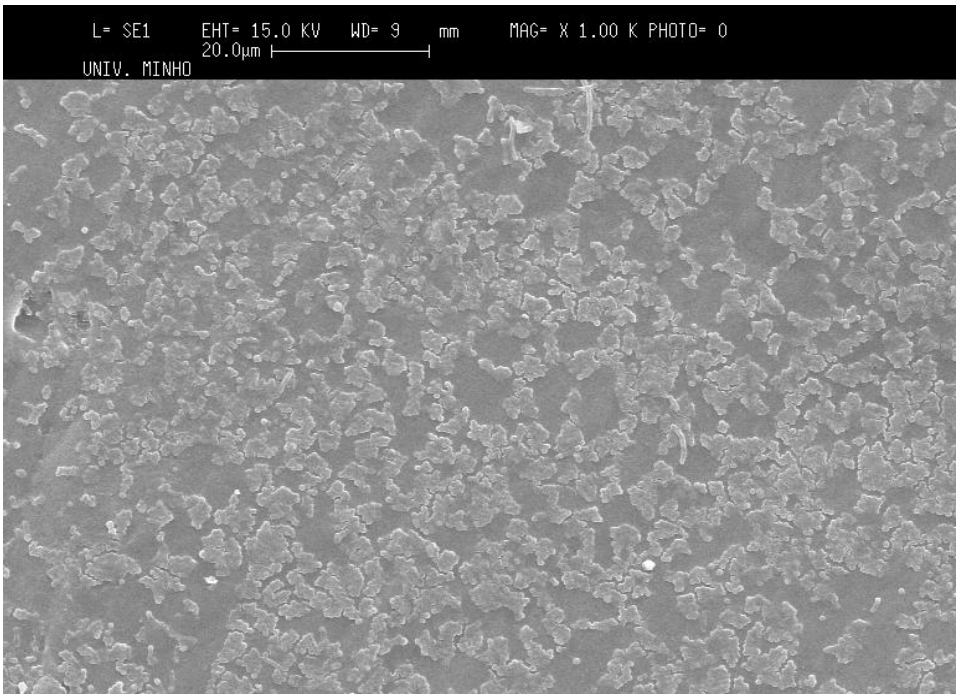


Name: P3RPP1 (1000 X)

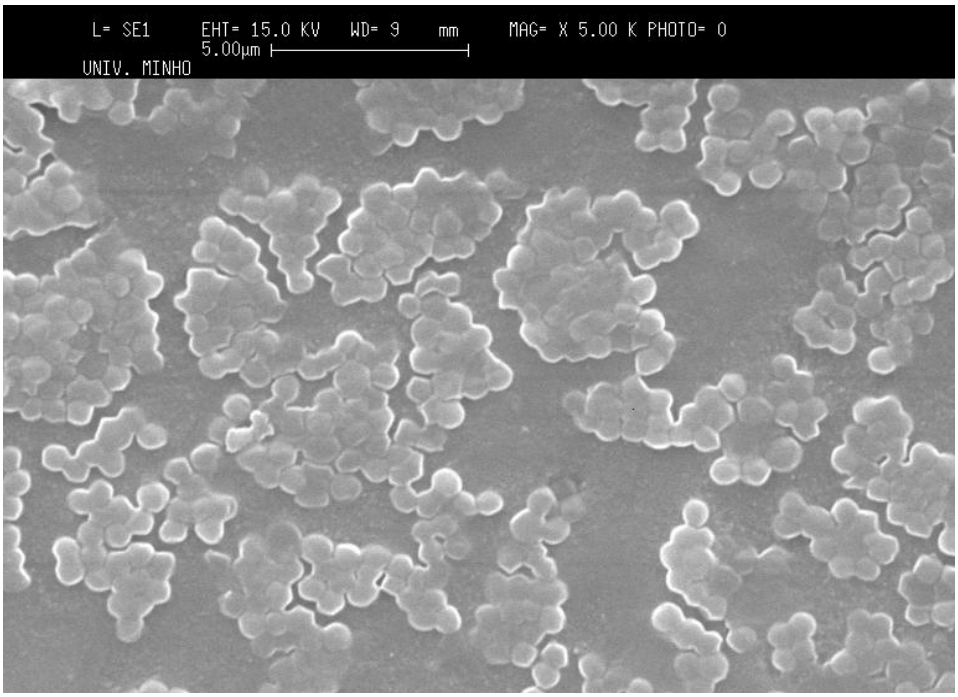


Name: P3RPP2 (5000 X)

Figure 5.30: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 46 °C on polypropylene.

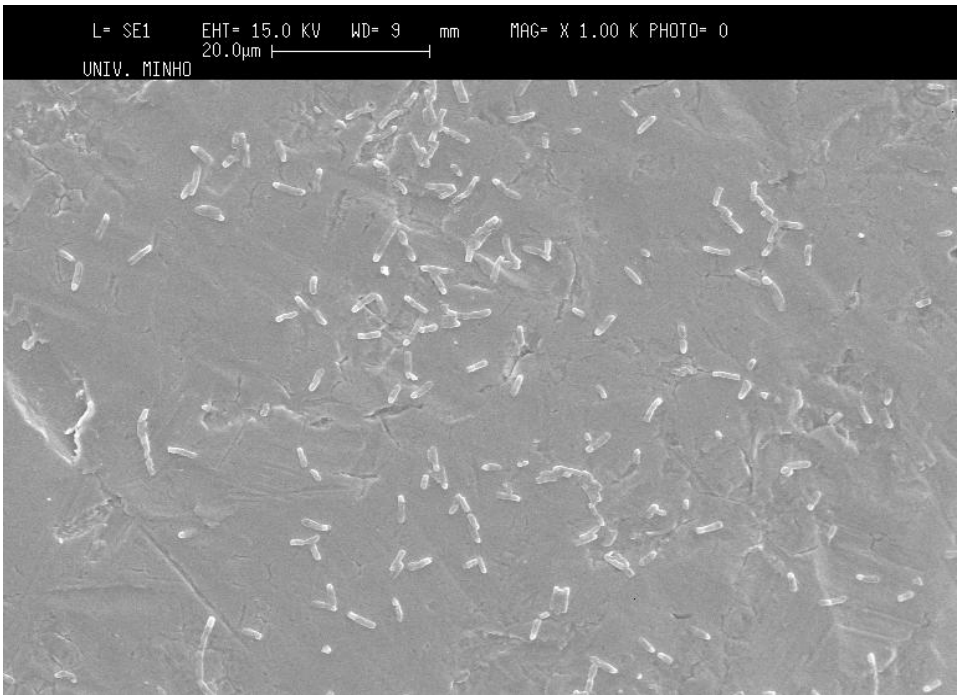


Name: P3RSI1 (1000 X)

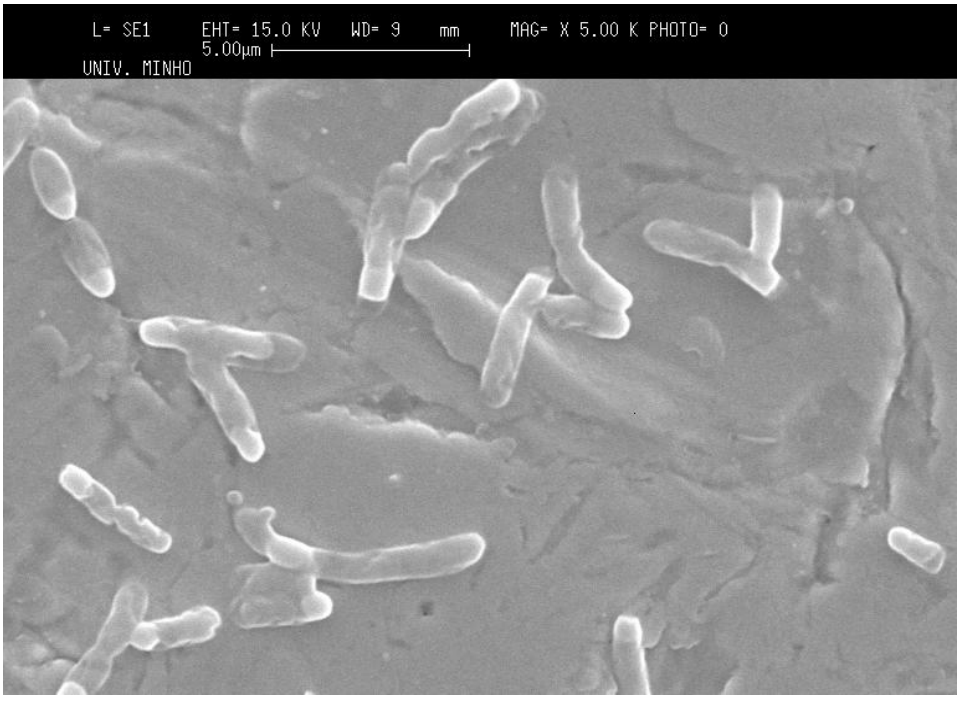


Name: P3RSI2 (5000 X)

Figure 5.31: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 46 °C on silicon.

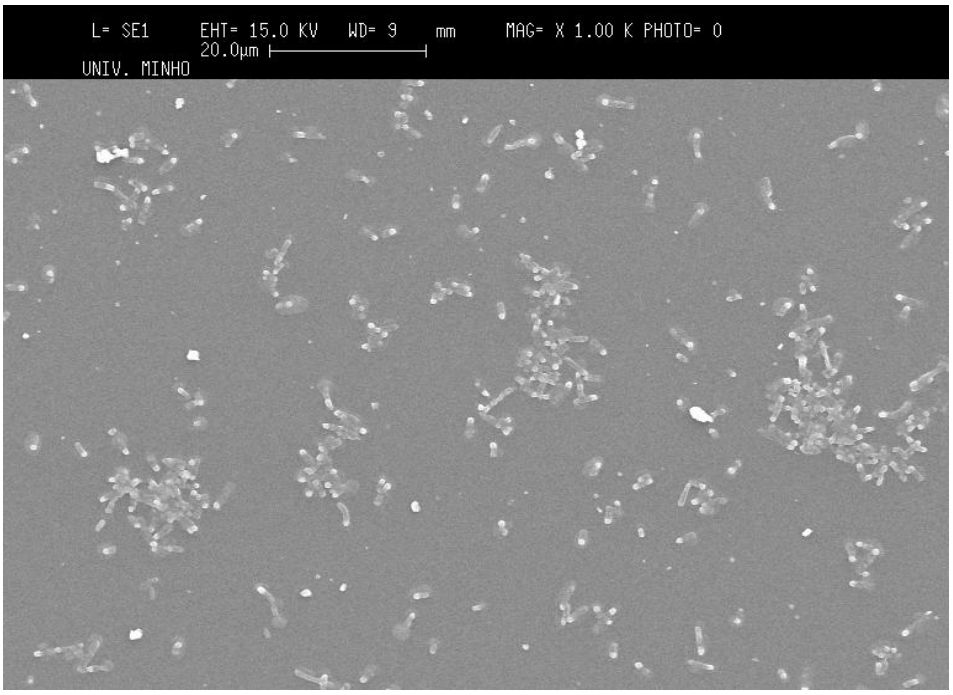


Name: P3WTACO1 (1000 X)

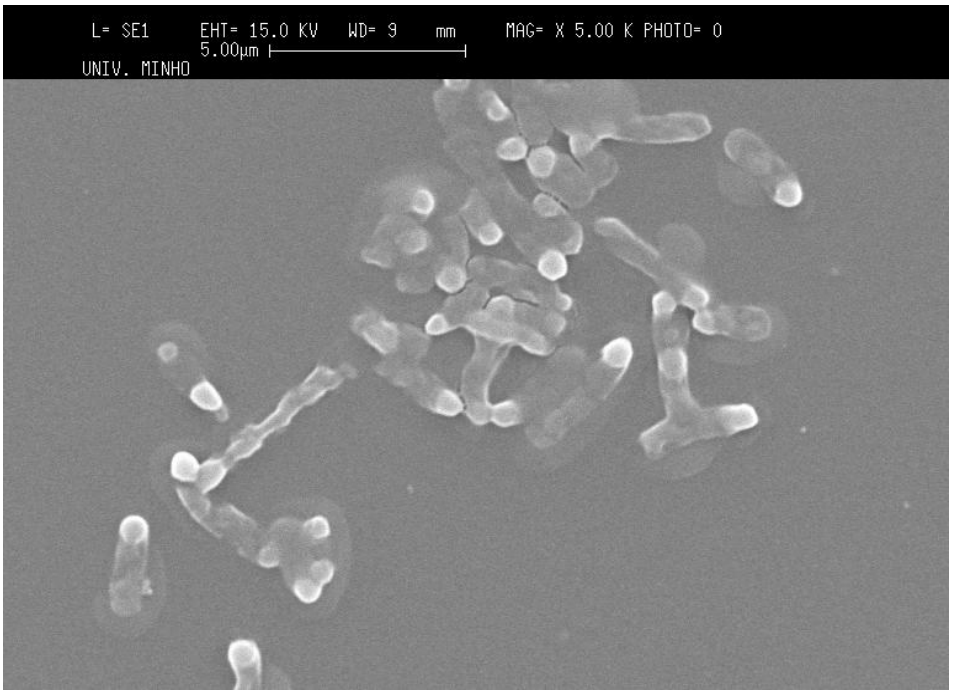


Name: P3WTACO2 (5000 X)

Figure 5.32: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 46 °C on stainless steel.

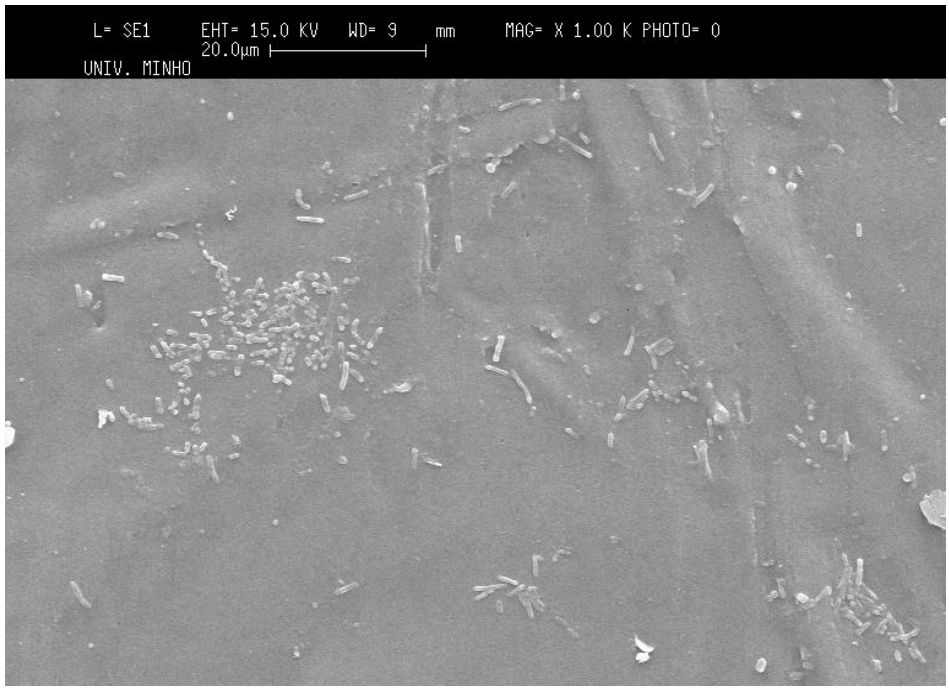


Name: P3WTPP1 (1000 X)

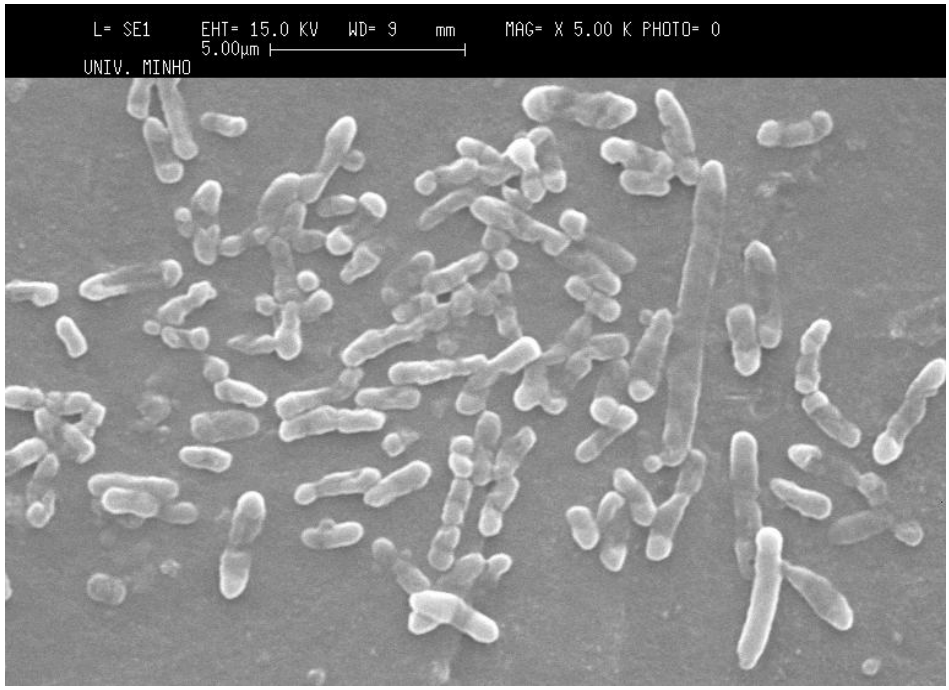


Name: P3WTPP2 (5000 X)

Figure 5.33: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 46 °C on polypropylene.



Name: P3WTSI1 (1000 X)



Name: P3WTSI2 (5000 X)

Figure 5.34: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 46 °C on silicon.

5.6.5 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (72 h stress @ 46 °C).

The conditions used in this study are listed in Table 5.6. For the images presented, the following designations are used. For example, P7WTSI 1/2 should read as,

P7 - Plate 7 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

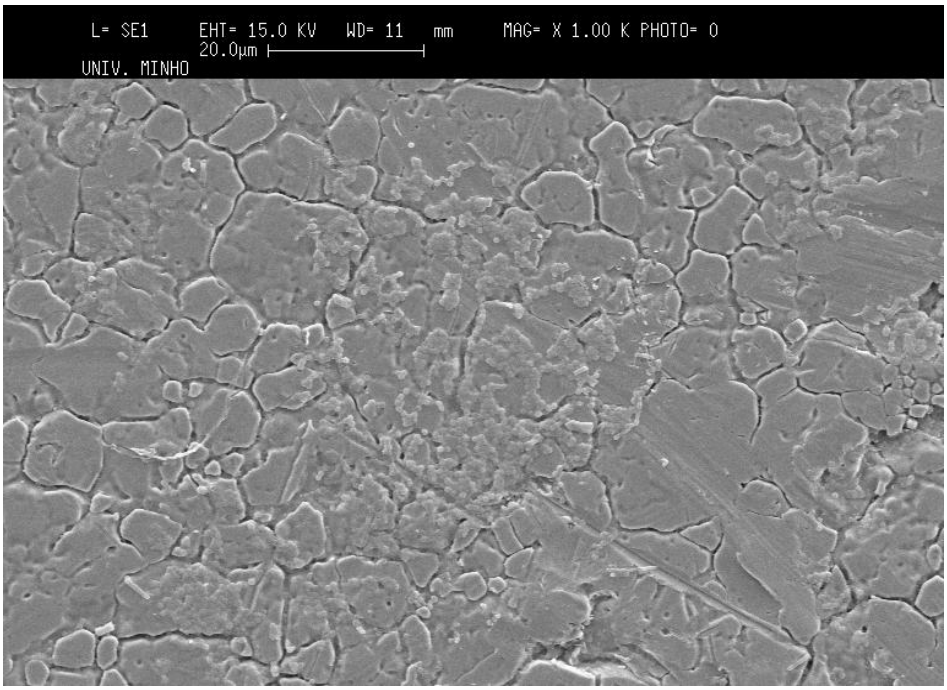
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.6: Condition used in this study with the designated names and remarks

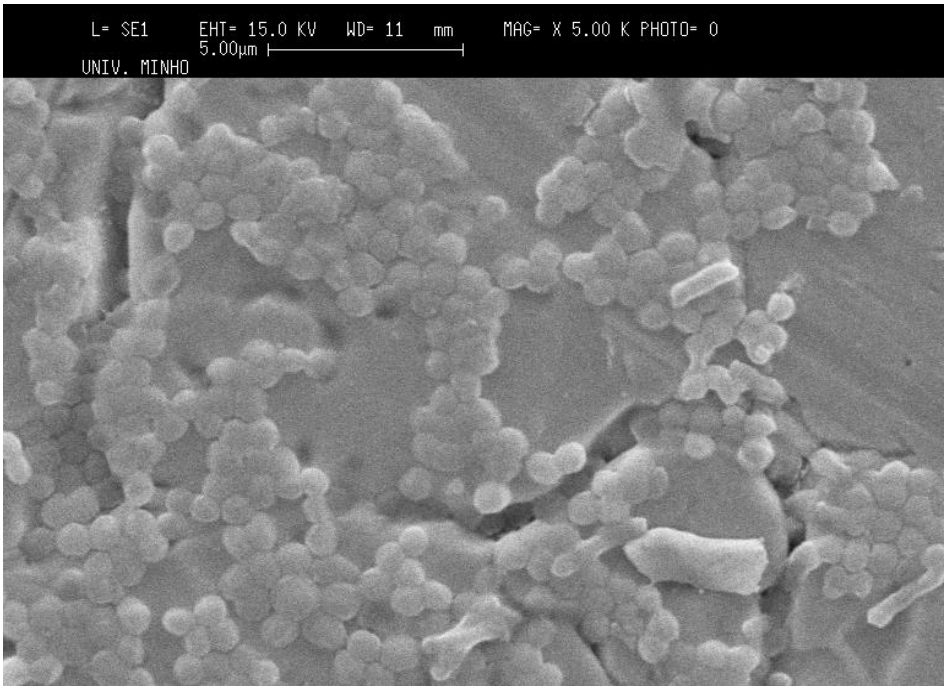
Plate	Strain	Material	Name	Figure	Remarks
Plate 7 **72 h stress @ 46 °C (48 h/37 °C + 24 h/46 °C)**	*WT*	Stainless Steel	P7WTACO 1/2	5.41	Very less attachment
		Silicone	P7WTSI 1/2	5.43	Well attached
		Polypropylene	P7WTPP 1/2	5.42	Elongated cells
	bolA+/rpoS-	Stainless Steel	P7BACO 1/2	5.35	Round morphology
		Silicone	P7BSI 1/2	5.36	Thick biofilm
		Polypropylene	P7BPP 1/2	5.37	No attachment/artifact
	rpoS+/bolA-	Stainless Steel	P7RACO 1/2	5.38	Well attached
		Silicone	P7RSI 1/2	5.40	Well attached
		Polypropylene	P7RPP 1/2	5.39	Very less attachment

A high cell density biofilm was seen on the surface silicon under the heat shock condition after 72 hour of growth (Fig. 5.36). In this case the attachment of cells in the absence of *bolA* was seen on stainless steel after 3 days of cell cultivation, After 48 hours, cells were unable to attach to stainless steel. These results are similar to those obtained after 48 hours under heat shock. Fig. 5.36 shows thick biofilm formation under heat shock condition in presence of *bolA* on silicon surface,

whereas, attachment of cells were seen in absence of *bolA*, but without biofilm formation (Fig.5.40). High number of cells was seen with a round/spherical morphology at 46 °C after 72 hours on the surface stainless steel in presence of *bolA* (Fig.5.35).

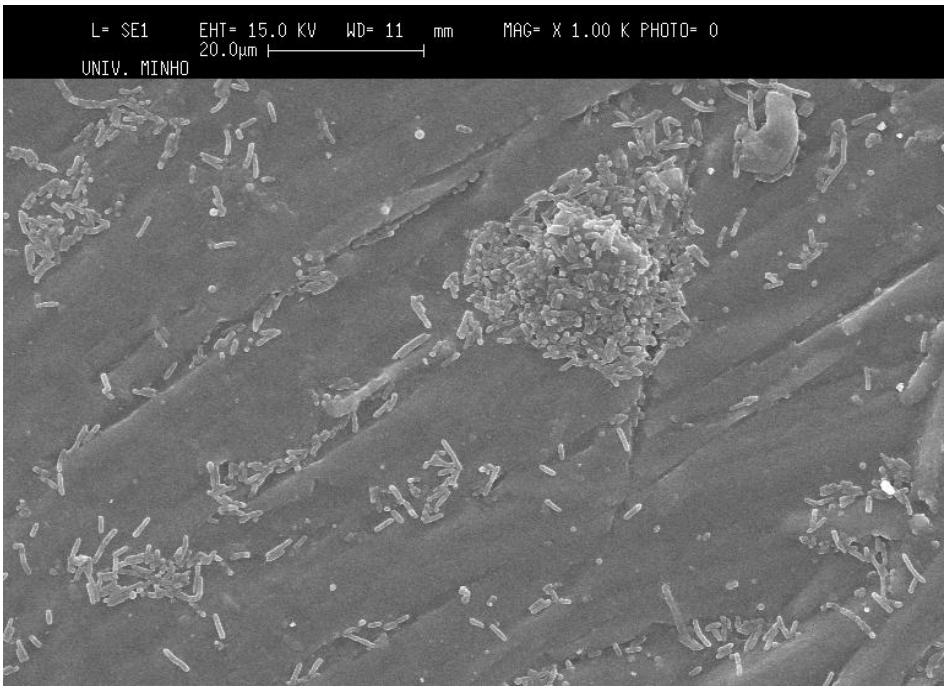


Name: P7BACO1 (1000 X)

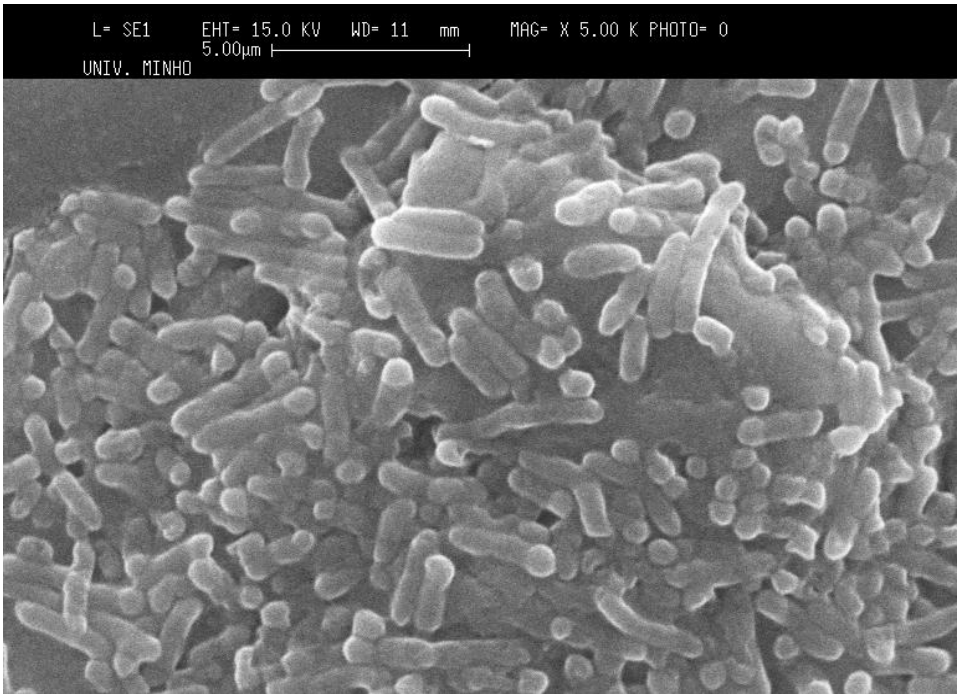


Name: P7BACO2 (5000 X)

Figure 5.35: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 46 °C on stainless steel.

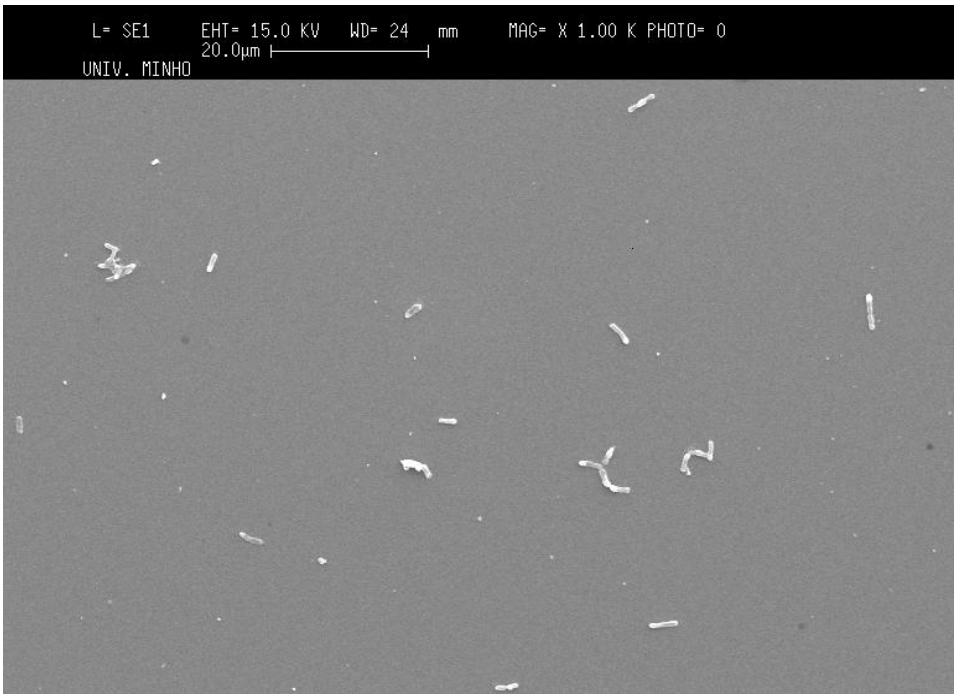


Name: P7BSI1 (1000 X)



Name: P7BSI2 (5000 X)

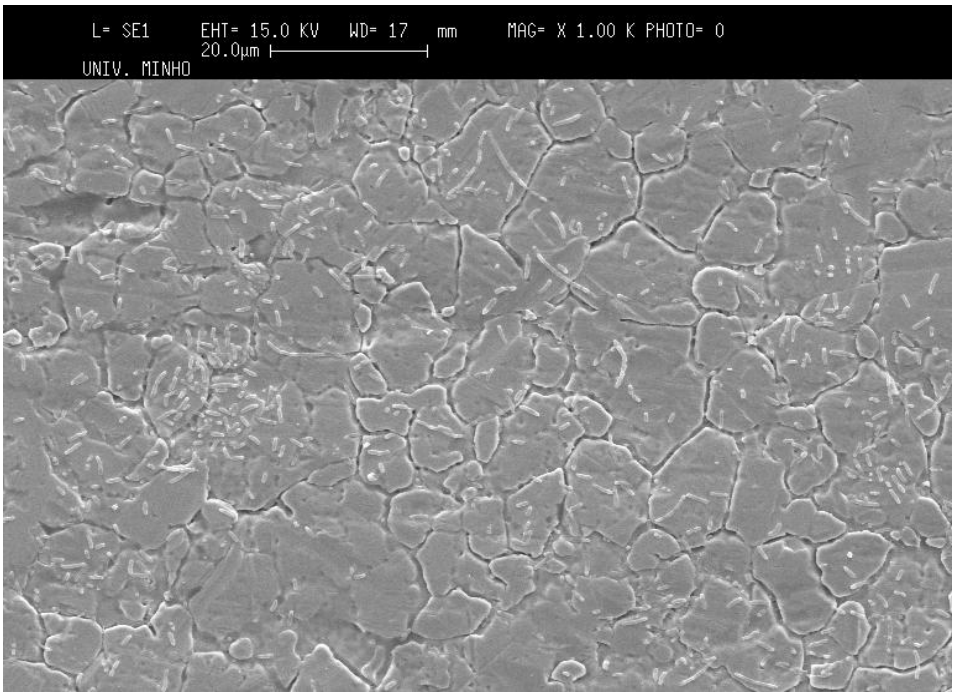
Figure 5.36: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 46 °C on silicon.



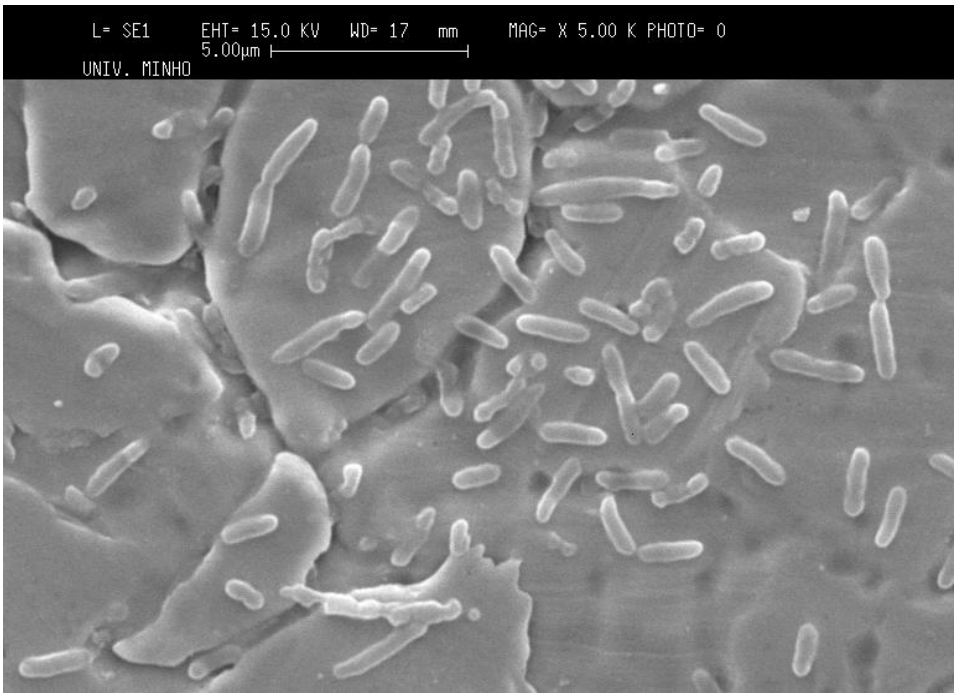
Name: P7BPP1 (1000 X)

Name: P7BPP2 (5000 X)

Figure 5.37: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 46 °C on polypropylene.

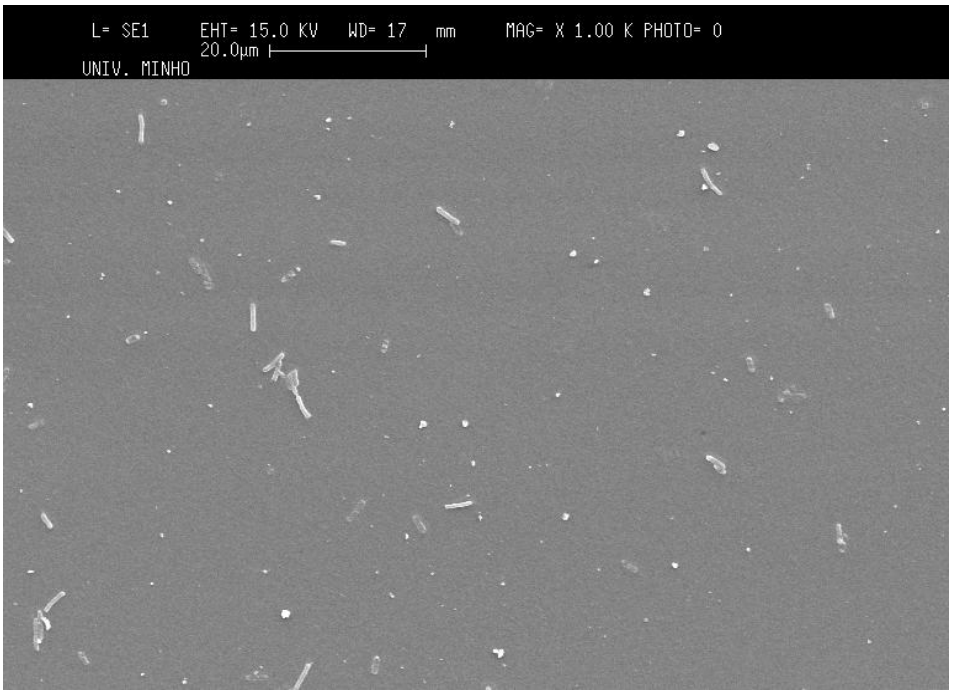


Name: P7RACO1 (1000 X)

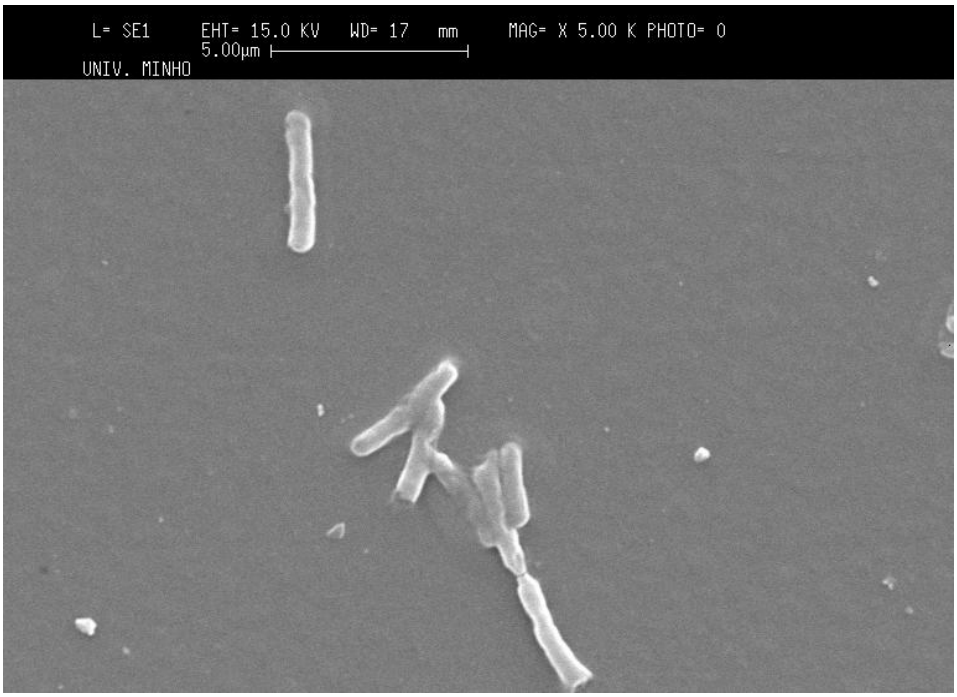


Name: P7RACO2 (5000 X)

Figure 5.38: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 46 °C on stainless steel.

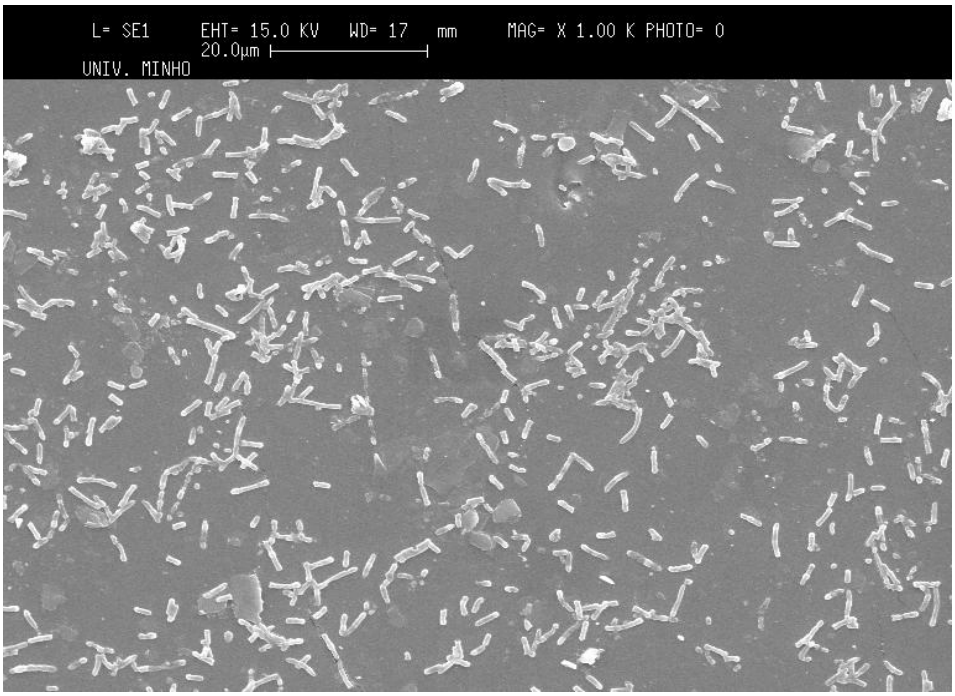


Name: P7RPP1 (1000 X)

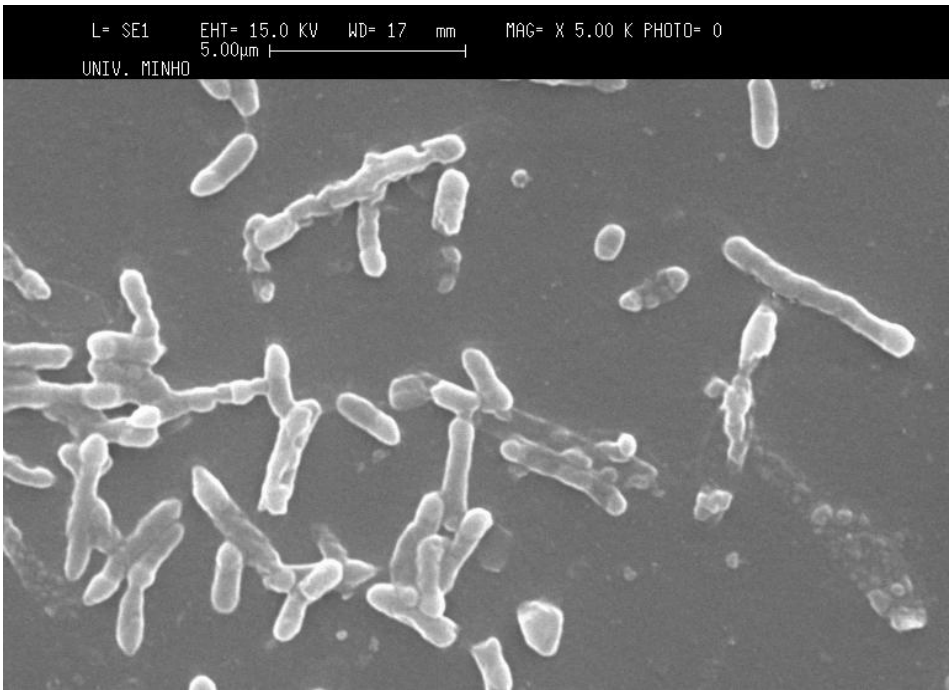


Name: P7RPP2 (5000 X)

Figure 5.39: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 46 °C on polypropylene.

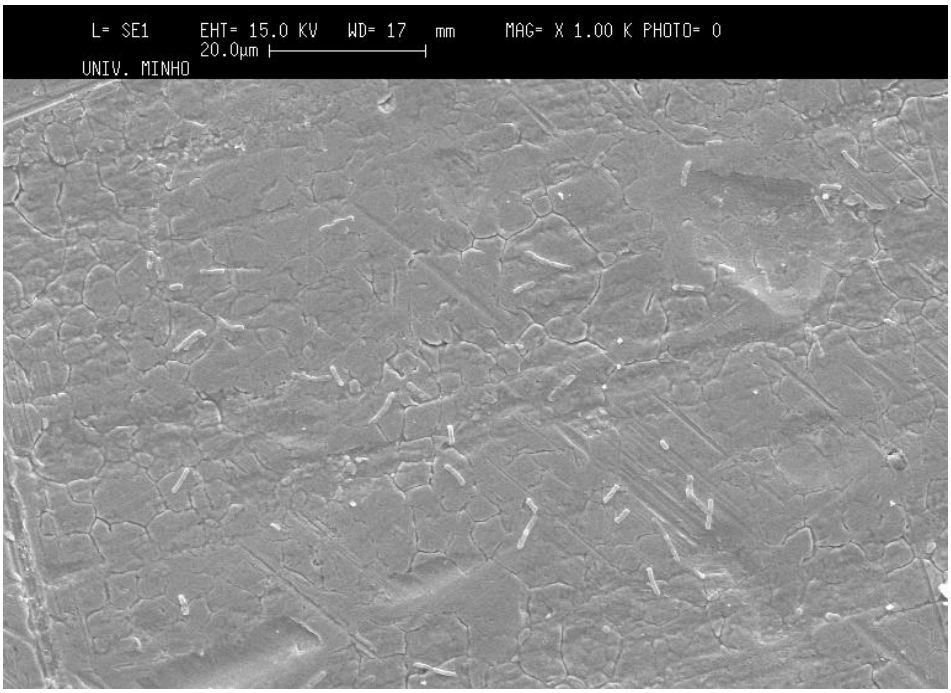


Name: P7RSI1 (1000 X)

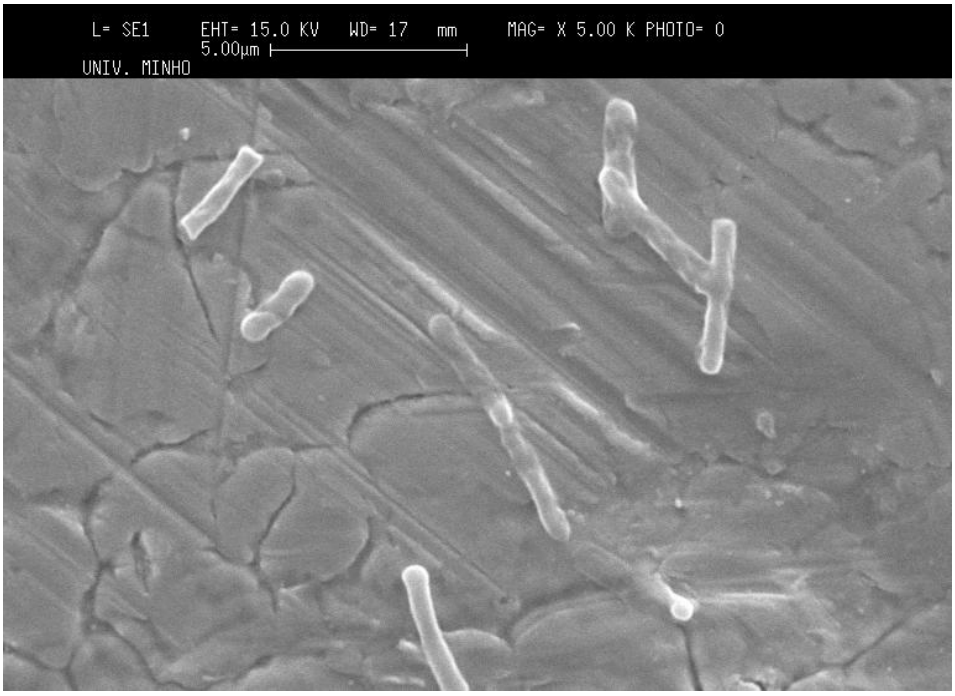


Name: P7RSI2 (5000 X)

Figure 5.40: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 46 °C on silicon.

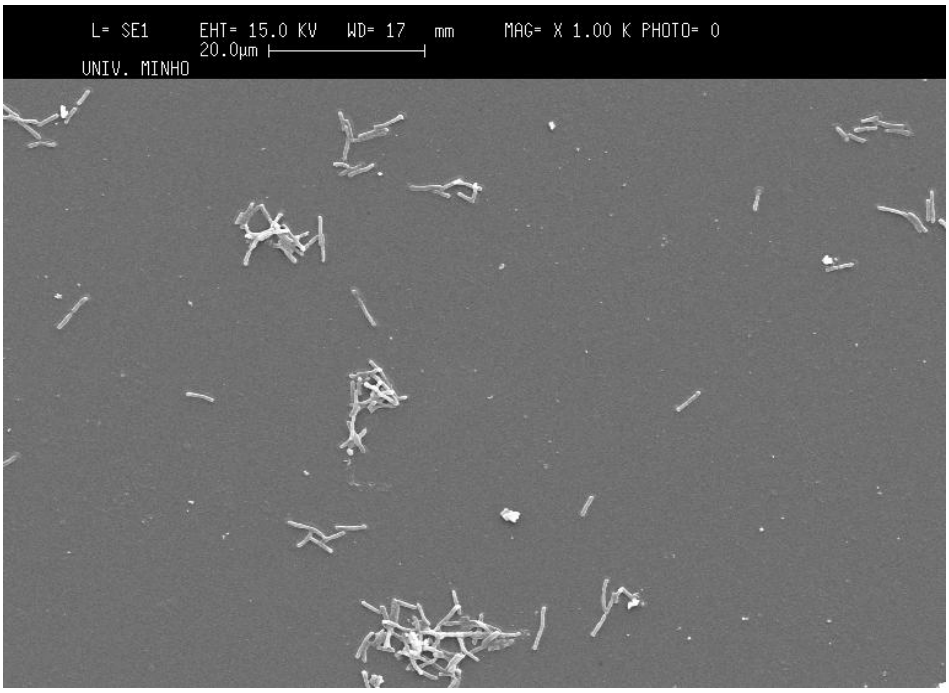


Name: P7WTACO1 (1000 X)

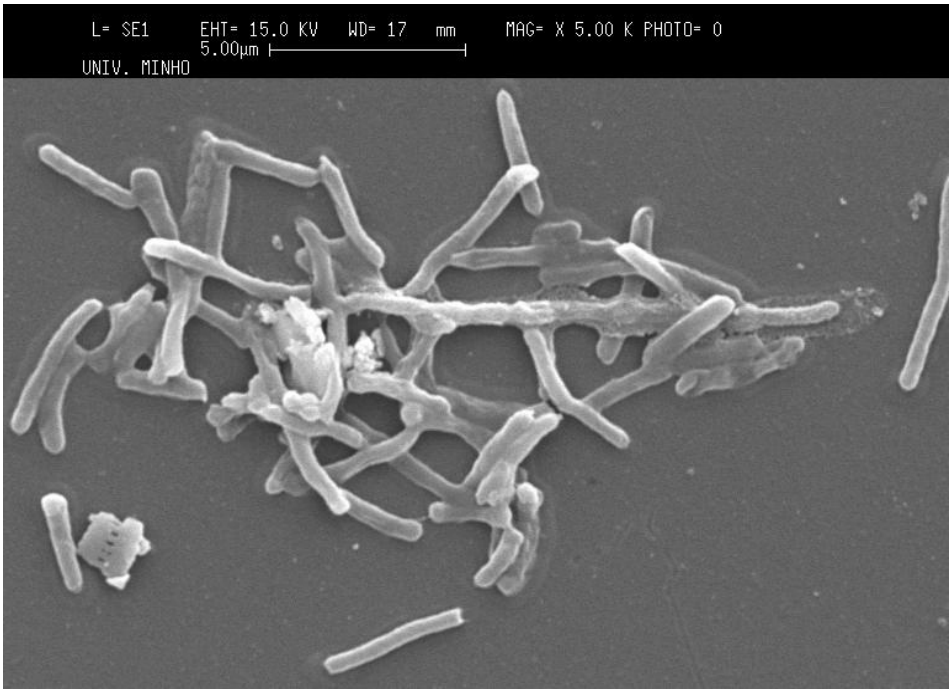


Name: P7WTACO2 (5000 X)

Figure 5.41: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 46 °C on stainless steel.

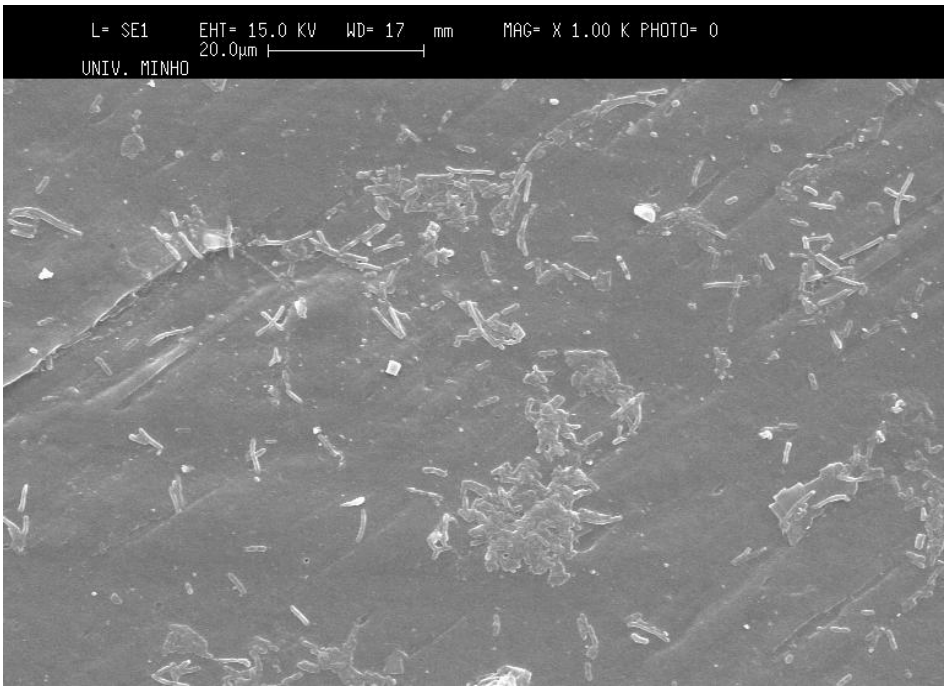


Name: P7WTPP1 (1000 X)

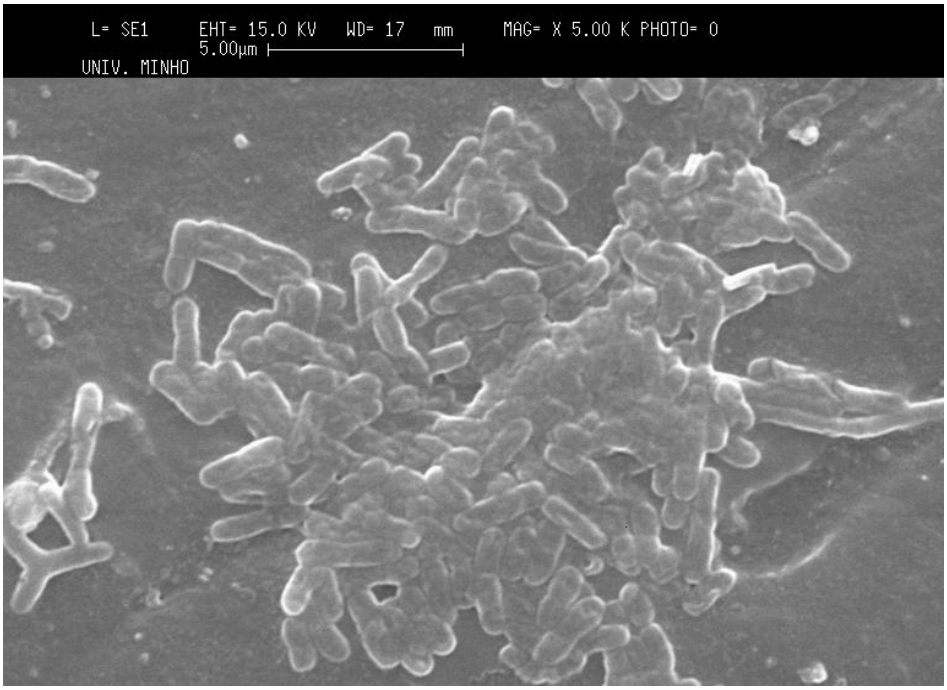


Name: P7WTPP2 (5000 X)

Figure 5.42: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 46 °C on polypropylene.



Name: P7WTSI1 (1000 X)



Name: P7WTSI2 (5000 X)

Figure 5.43: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 46 °C on silicon.

5.6.6 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (48 h stress @ 5 °C).

The conditions used in this study are listed in Table 5.7. For the images presented, the following designations are used. For example, P4WTSI 1/2 should read as,

P4 - Plate 4 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

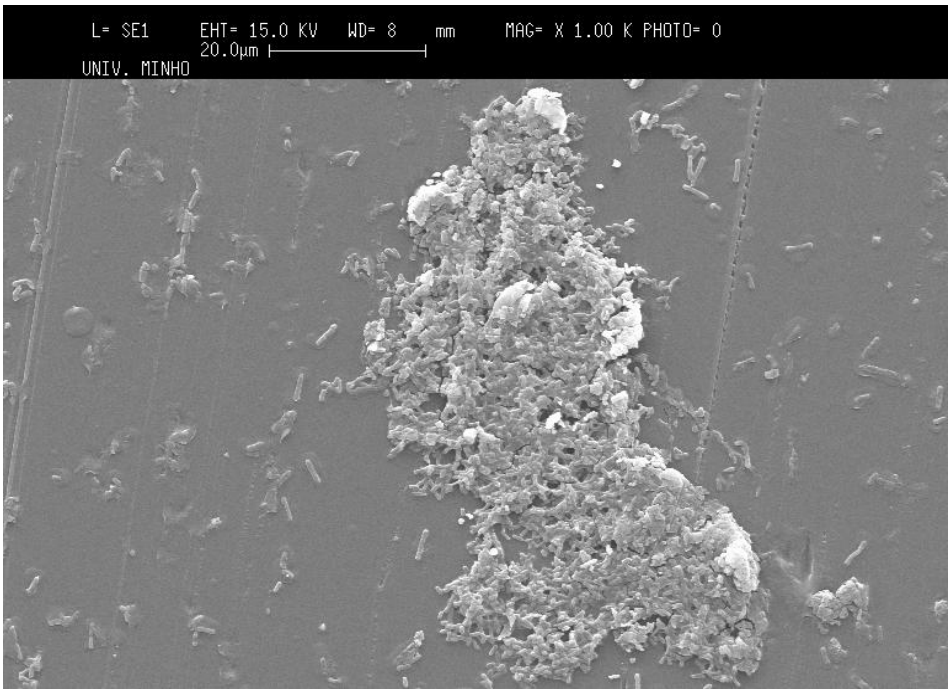
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.7: Condition used in this study with the designated names and remarks

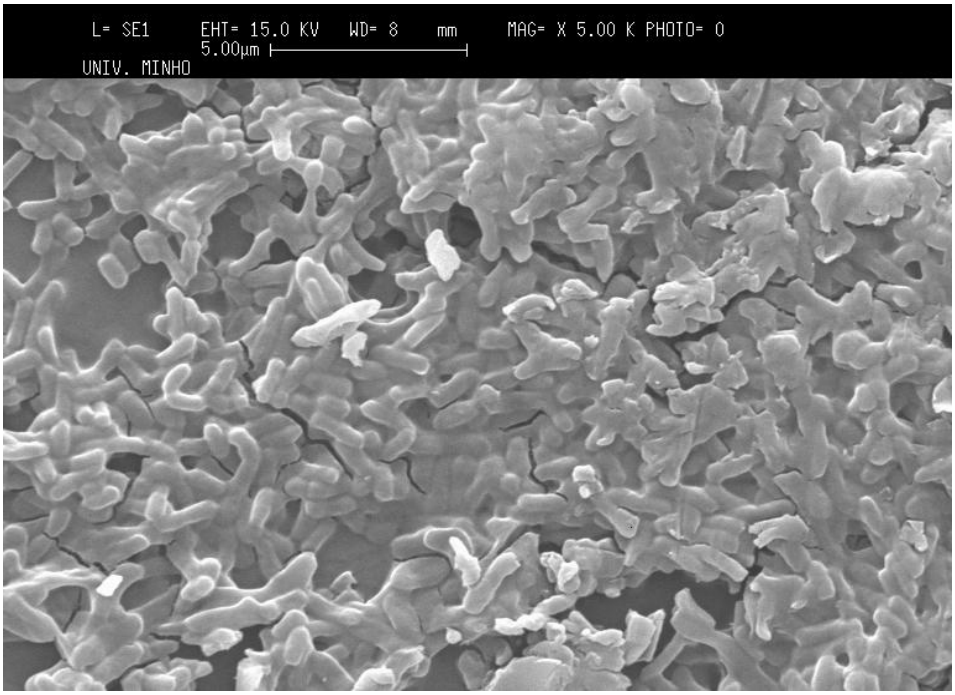
Plate	Strain	Material	Name	Figure	Remarks
Plate 4 **48 h stress @ 5 °C (24 h/37 °C + 24 h/5 °C)**	*WT*	Stainless Steel	P4WTACO 1/2	5.50	Less attachment
		Silicone	P4WTSI 1/2	5.51	Thick biofilm
		Polypropylene	P4WTPP 1/2	5.44	Very thick biofilm
	bolA+/rpoS-	Stainless Steel	P4BACO 1/2	5.45	Well attached
		Silicone	P4BSI 1/2	5.46	Less attachment
		Polypropylene	-	NA	Nothing visible
	rpoS+/bolA-	Stainless Steel	P4RACO 1/2	5.47	Round morphology
		Silicone	P4RSI 1/2	5.49	Well attached
		Polypropylene	P4RPP 1/2	5.48	Very well attached

Under cold shock condition (5 °C) the surface of polypropylene was found for the first time to be suitable for the attachment and growth of *E. coli* cells as biofilms after 48 hours. A thick and dense biofilm of WT was seen on the surface of polypropylene (Fig. 5.44). *RpoS* gene was found to be more important under cold shock environment for *E. coli* than *bolA*. Mutation in *rpoS* didn't allow the cells to attach and grow in biofilms (image not shown here), while mutation in *bolA* i.e. in

presence of *rpoS* the cells were able to attach to the surface and grow (Fig. 5.48). A high density of cells was seen in presence of *rpoS* on the surface of propylene (Fig. 5.48). Even on silicon, thick and matured biofilm was seen under cold shock conditions by WT (Fig. 5.51). This shows that under cold shock environment cells can attach to stainless steel, polypropylene and silicon surfaces and grow as thick and dense biofilms in 48 hours. *RpoS* gene was found to be important under this environment for the attachment of *E. coli* cells to these three surfaces.

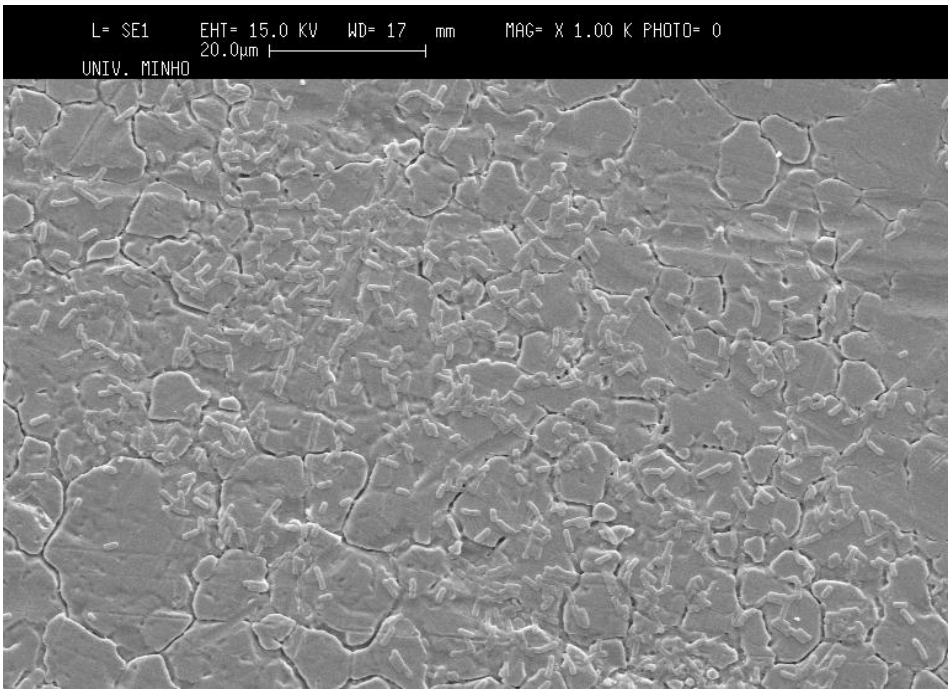


Name: P4WTPP1 (1000 X)

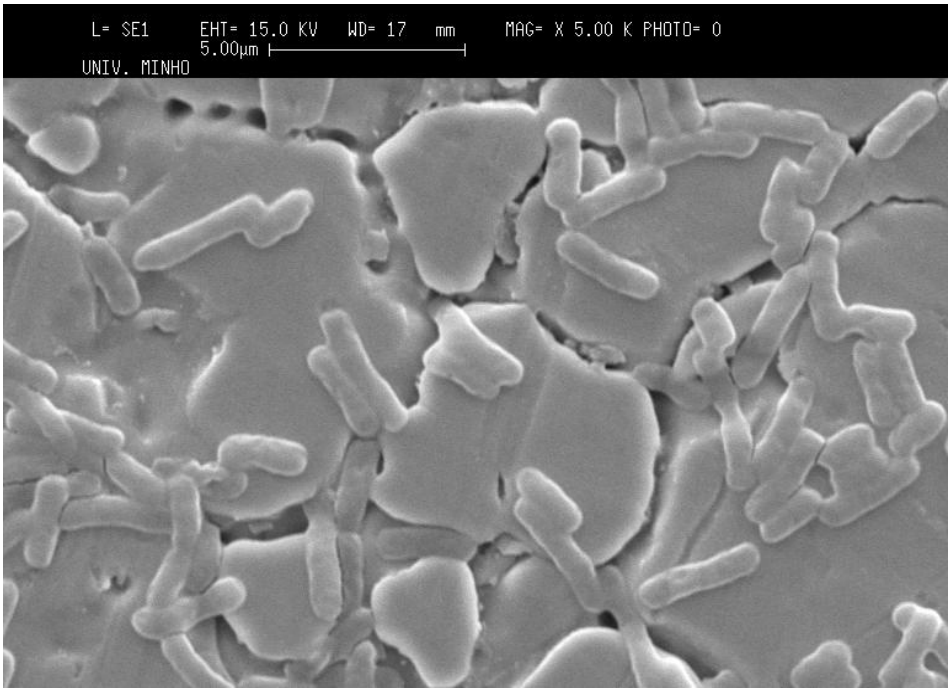


Name: P4WTPP2 (5000 X)

Figure 5.44: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 5 °C on polypropylene.

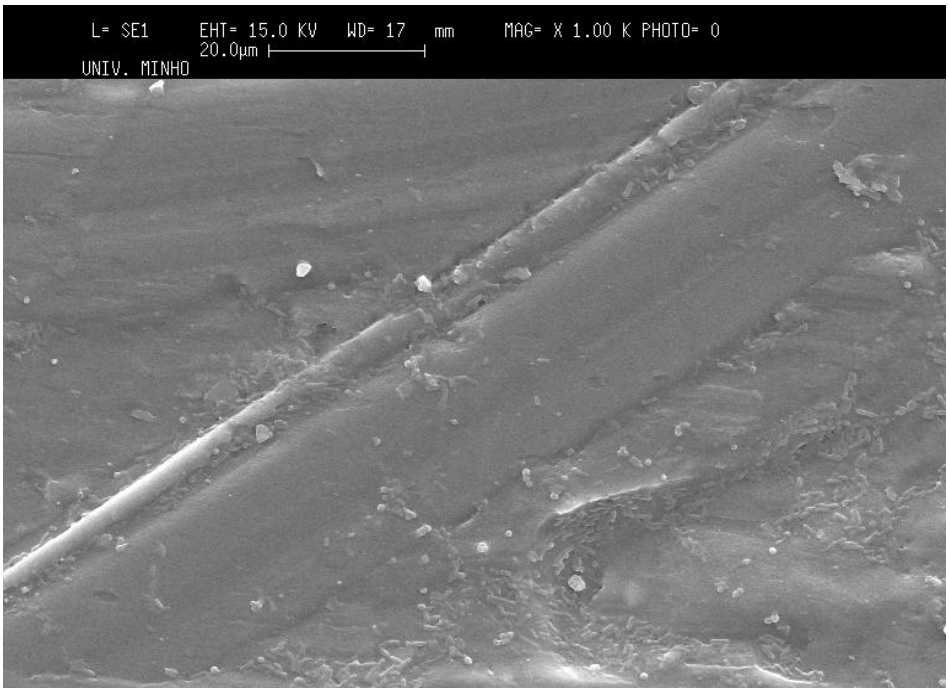


Name: P4BACO1 (1000 X)

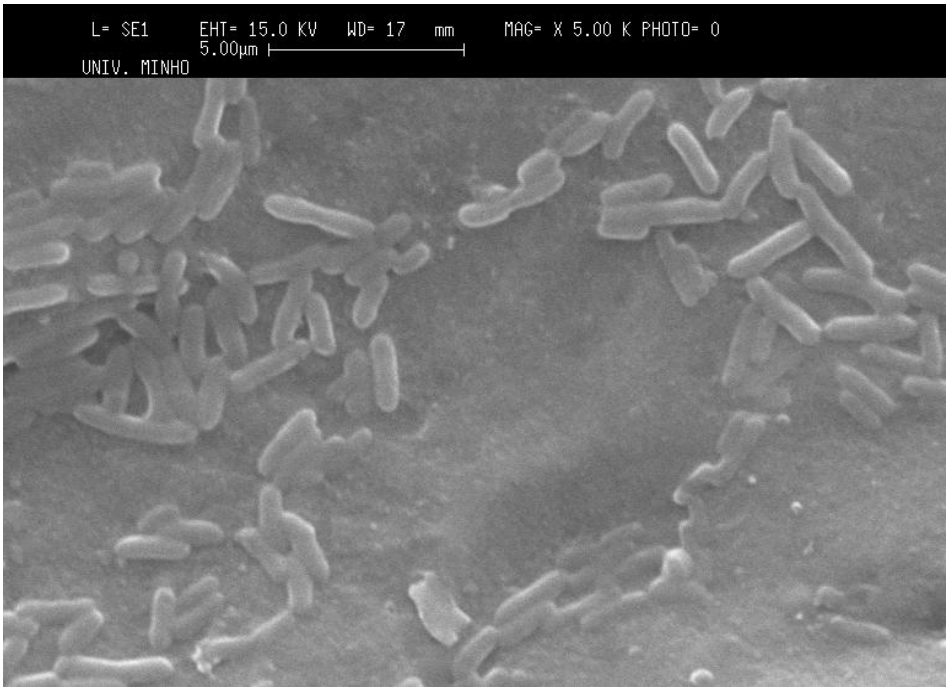


Name: P4BACO2 (5000 X)

Figure 5.45: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 5 °C on stainless steel.

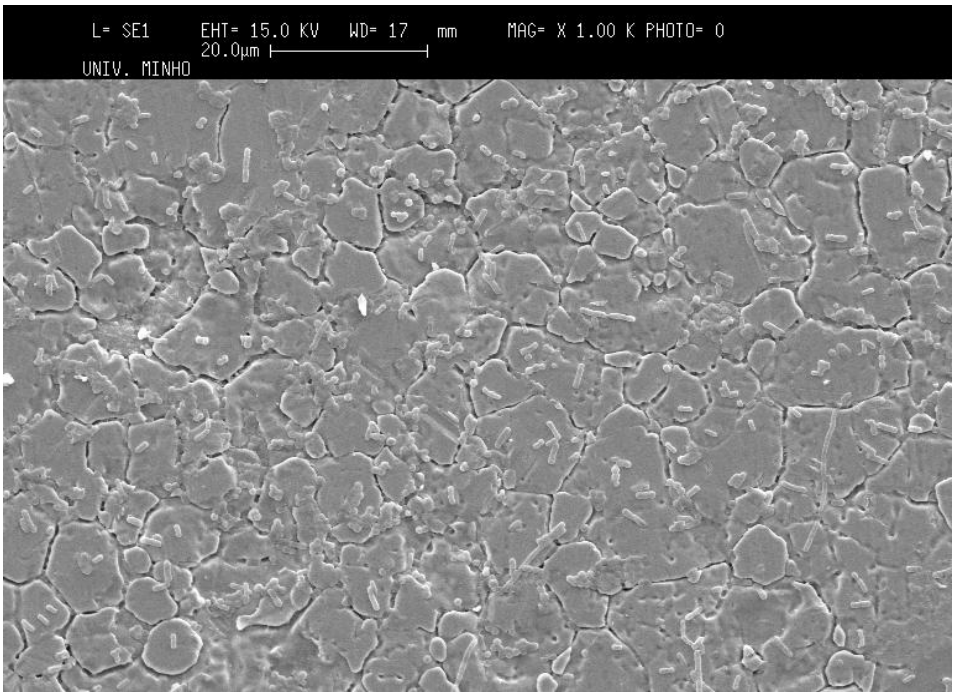


Name: P4BSI1 (1000 X)

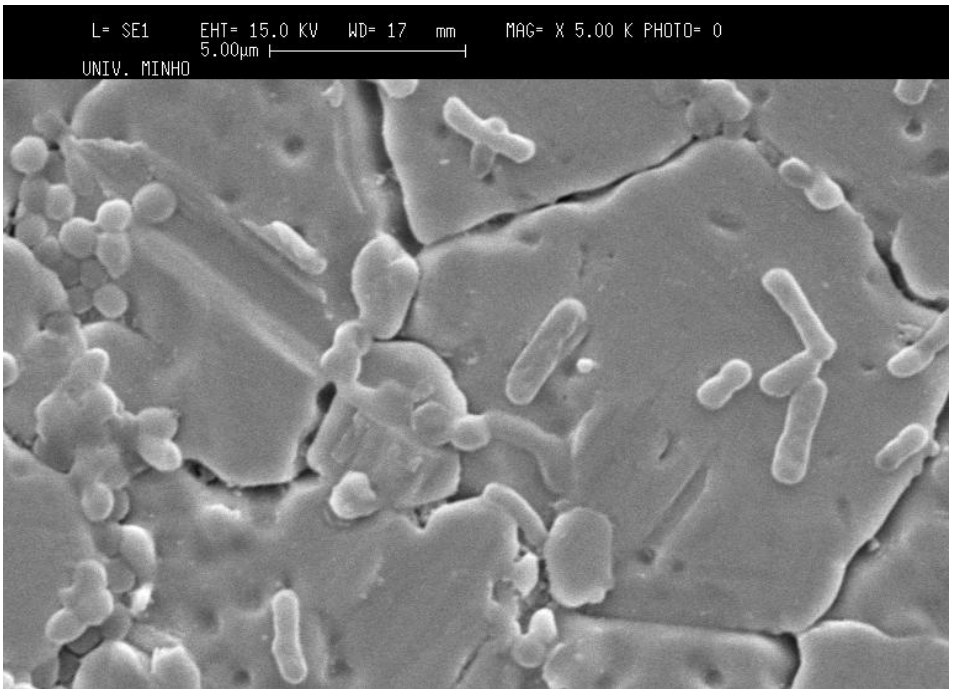


Name: P4BSI2 (5000 X)

Figure 5.46: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 5 °C on silicon.

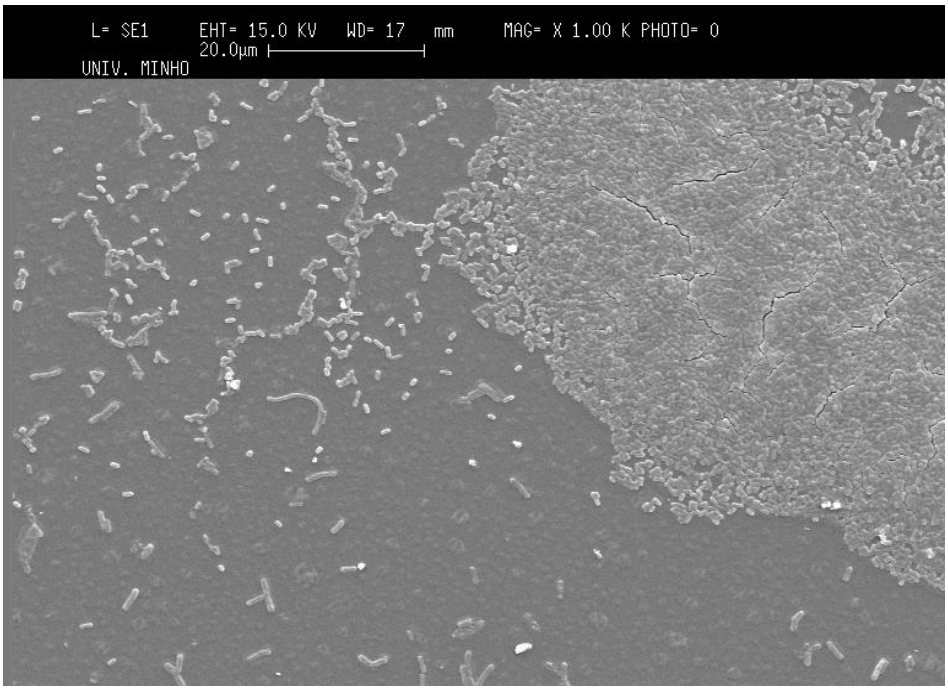


Name: P4RACO1 (1000 X)

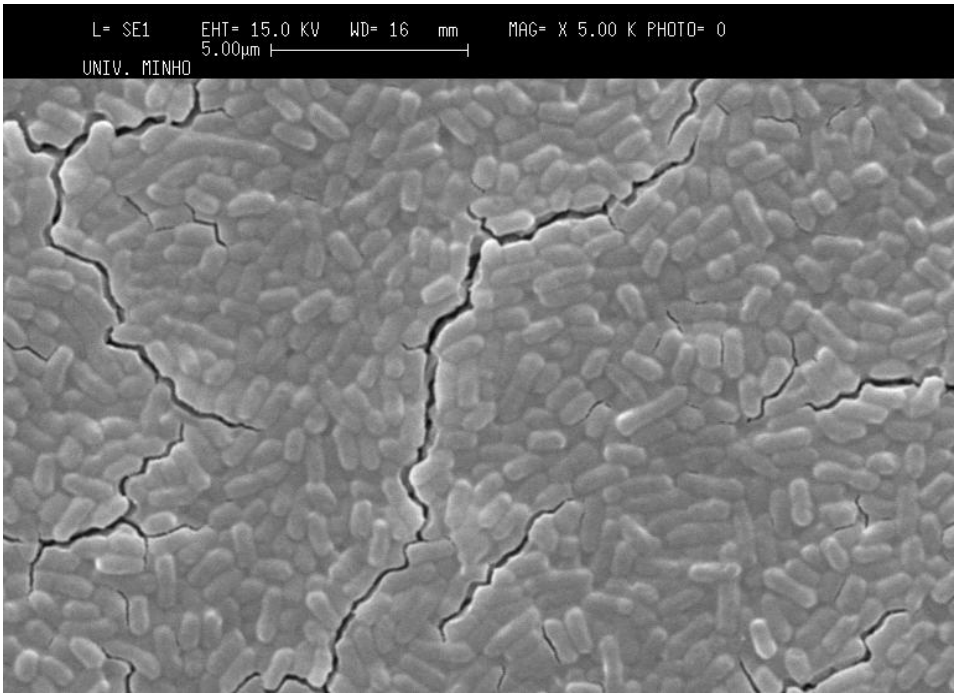


Name: P4RACO2 (5000 X)

Figure 5.47: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 5 °C on stainless steel.

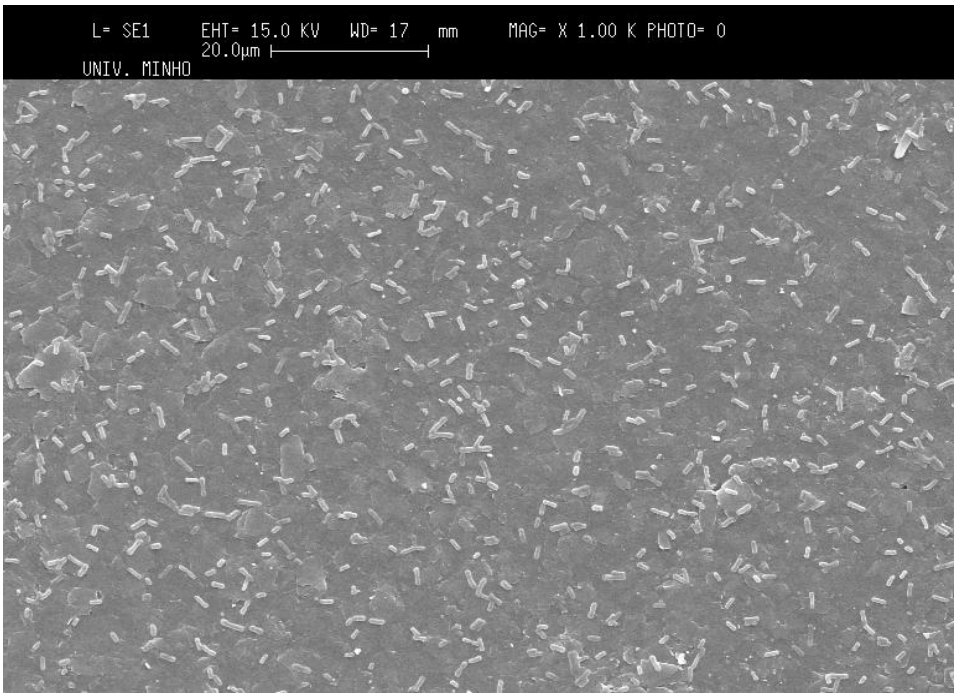


Name: P4RPP1 (1000 X)

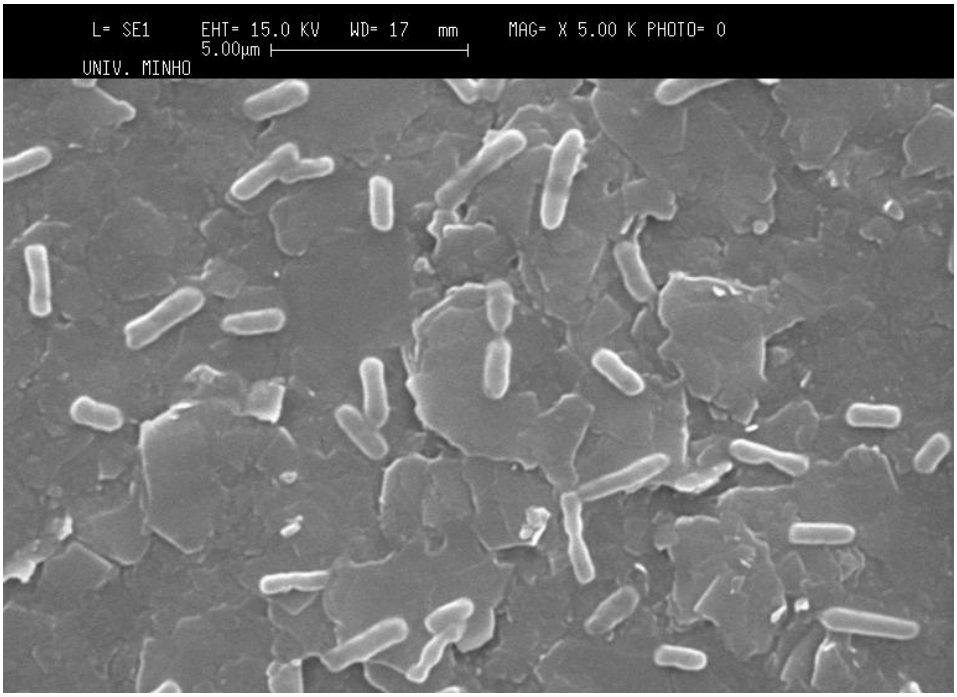


Name: P4RPP2 (5000 X)

Figure 5.48: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 5 °C on polypropylene.

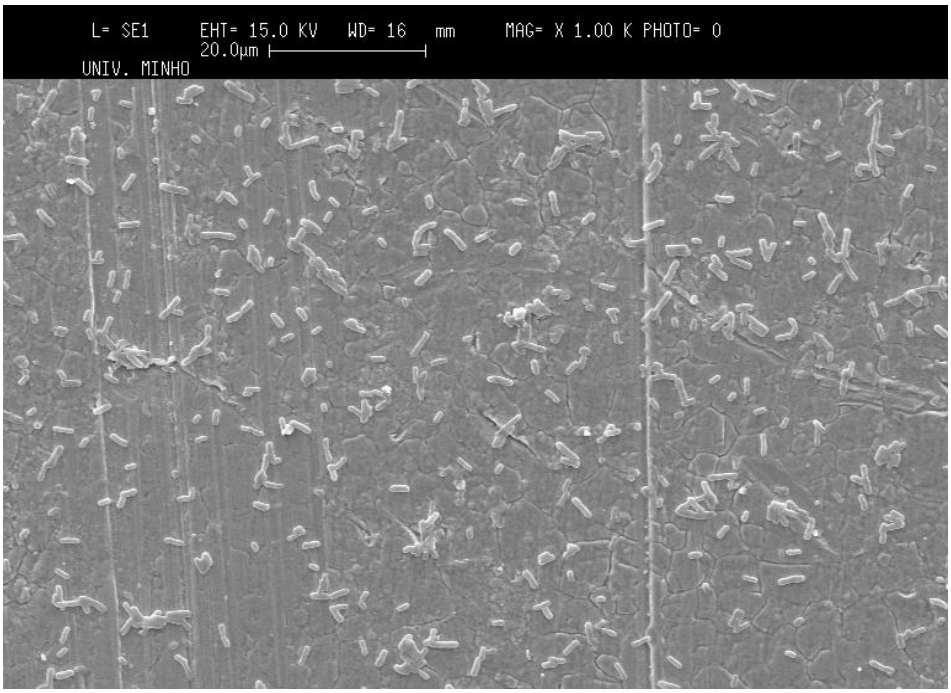


Name: P4RSI1 (1000 X)

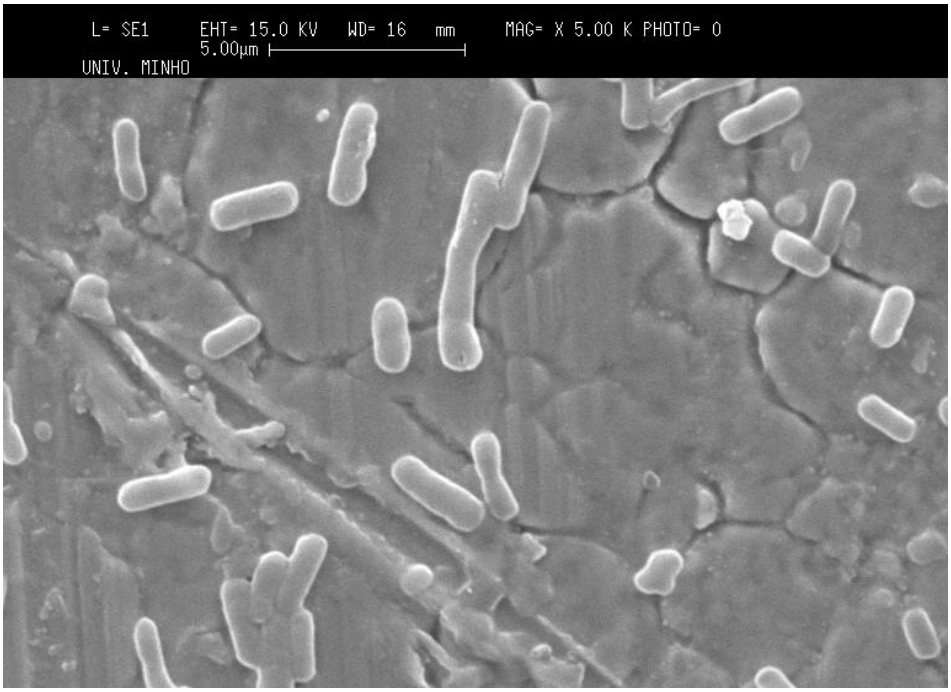


Name: P4RSI2 (5000 X)

Figure 5.49: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 5 °C on silicon.

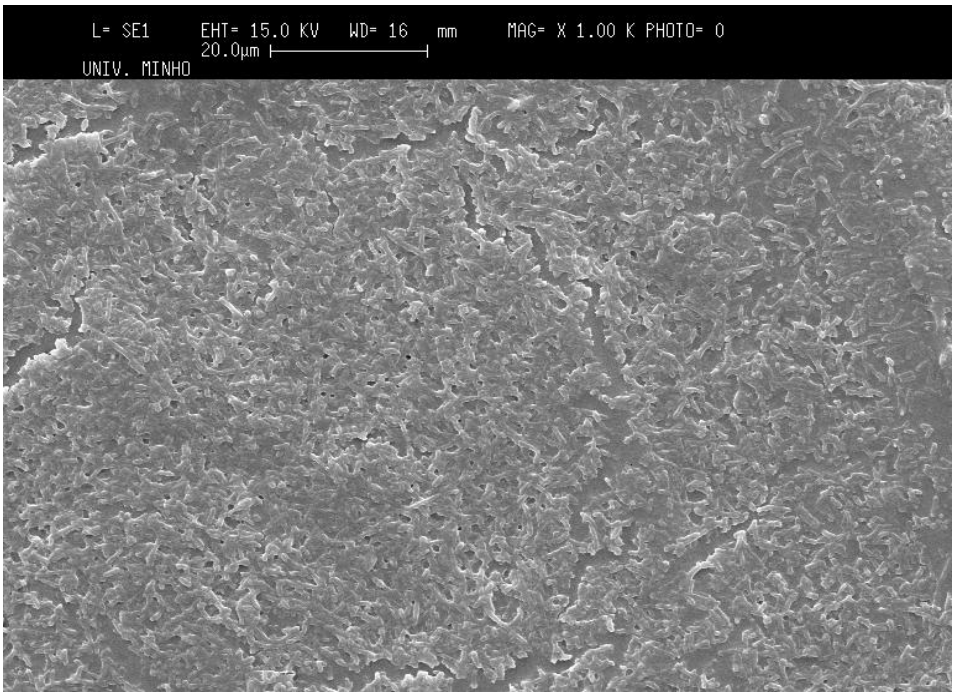


Name: P4WTACO1 (1000 X)

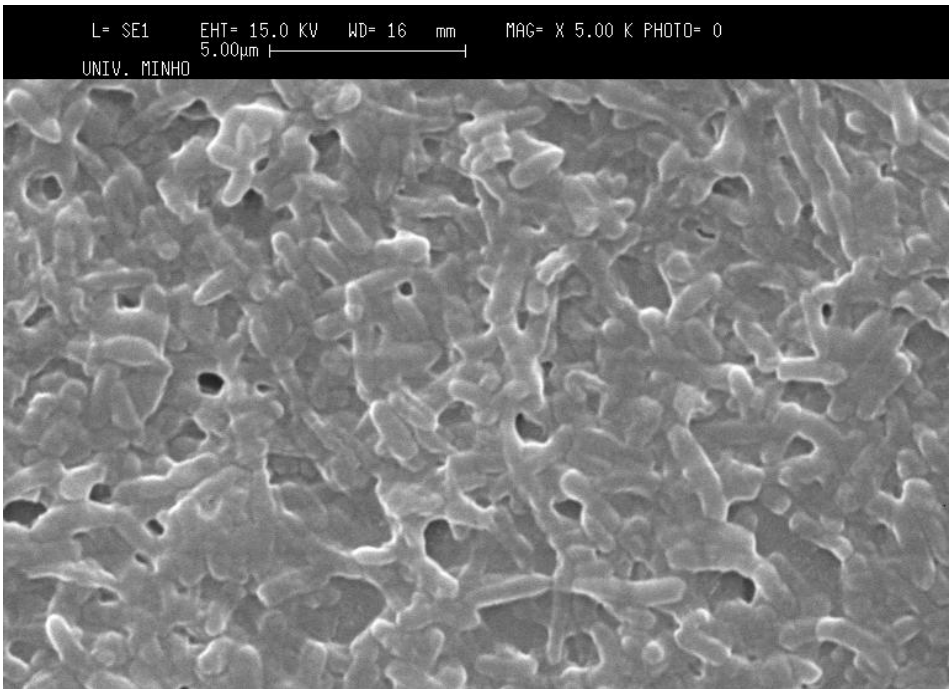


Name: P4WTACO2 (5000 X)

Figure 5.50: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 5 °C on stainless steel.



Name: P4WTSI1 (1000 X)



Name: P4WTSI2 (5000 X)

Figure 5.51: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 5 °C on silicon.

5.6.7 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (72 h stress @ 5 °C).

The conditions used in this study are listed in Table 5.8. For the images presented, the following designations are used. For example, P8WTSI 1/2 should read as,

P8 - Plate 8 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

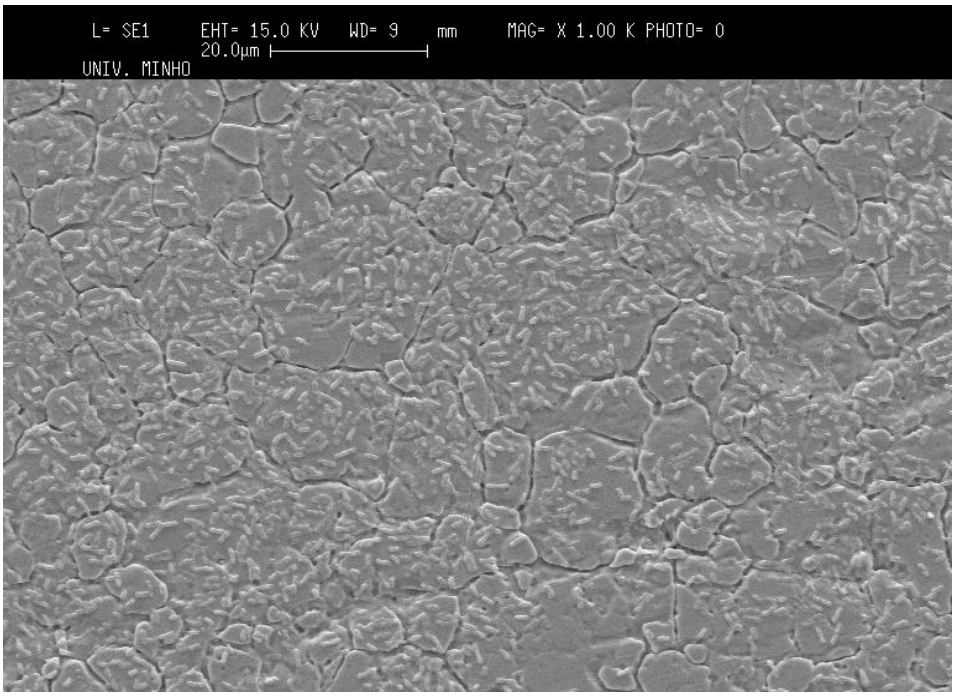
1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.8: Condition used in this study with the designated names and remarks

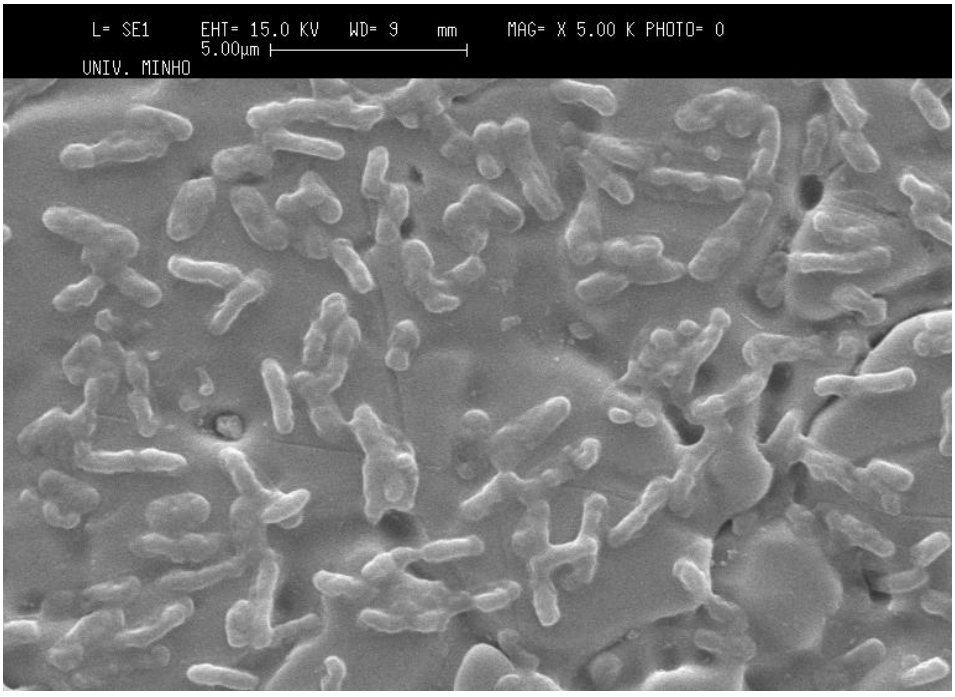
Plate	Strain	Material	Name	Figure	Remarks
Plate 8 **72 h stress @ 5 °C (48 h/37 °C + 24h/5 °C)**	*WT*	Stainless Steel	P8WTACO 1/2	5.58	Very well attachment
		Silicone	P8WTSI 1/2	5.60	Well attached
		Polypropylene	P8WTPP 1/2	5.59	Very well attached
	bolA+/rpoS-	Stainless Steel	P8BACO 1/2	5.52	Very well attached
		Silicone	P8BSI 1/2	5.54	Very well attached
		Polypropylene	P8BPP 1/2	5.53	Well attached
	rpoS+/bolA-	Stainless Steel	P8RACO 1/2	5.55	Well attached
		Silicone	P8RSI 1/2	5.57	Very less attachment
		Polypropylene	P8RPP 1/2	5.56	Thick biofilm

The results show the importance of the *bolA* gene in the stationary phase of growth and in long term survival. There was not a great deal of difference seen between the growth observed at 48 hours and 72 hours at 5 °C. Figure 5.53 showed some interesting results when *rpoS-/bolA+* cells were seen attached on polypropylene surface after 72 hours which was not the case after 48 hours (image not shown). This shows that after 48 hours, *bolA* plays a role in cell attachment especially to the

surface of polypropylene. Another interesting result was seen after 72 hours on the surface polypropylene by *rpoS*+/*bolA*- cells (Fig. 5.56). After 48 hours it was seen that cells can attach to the surface of polypropylene in the presence of *rpoS* through the quorum sensing mode (Fig. 5.48). While after 72 hours it was seen that *E. coli* cells started forming biofilm to cope with the temperature stress. Morphological changes were seen by all the three stains on the surface of silicon (Fig. 5.54, 5.57 and 5.60), but not on the surface of stainless steel and polypropylene. The importance of *bolA* and *rpoS* genes in the attachment of cells leading to biofilm formation, was clearly seen under cold sock conditions.

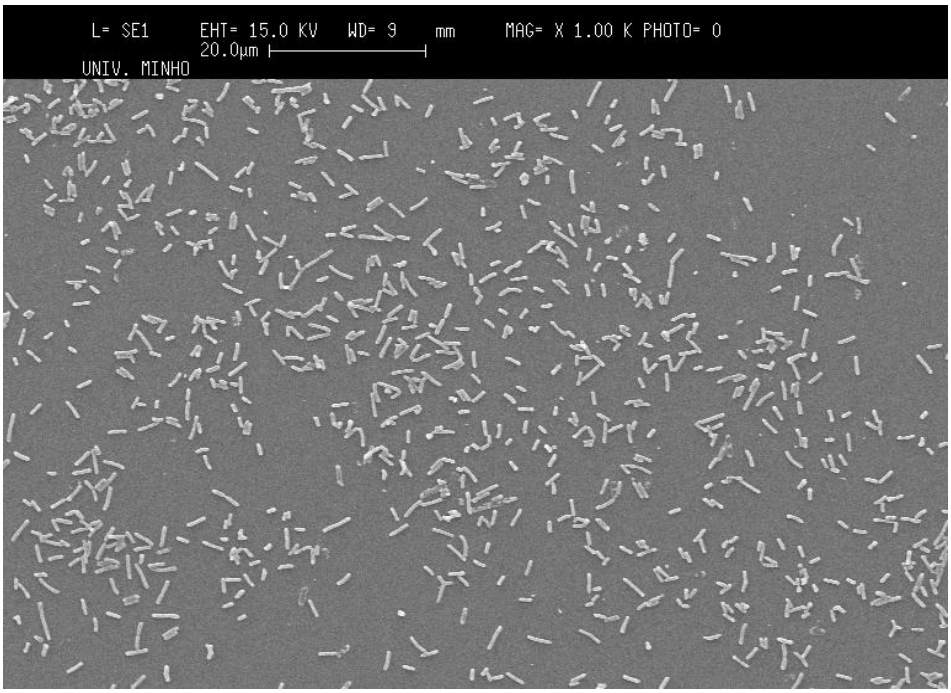


Name: P8BACO1 (1000 X)

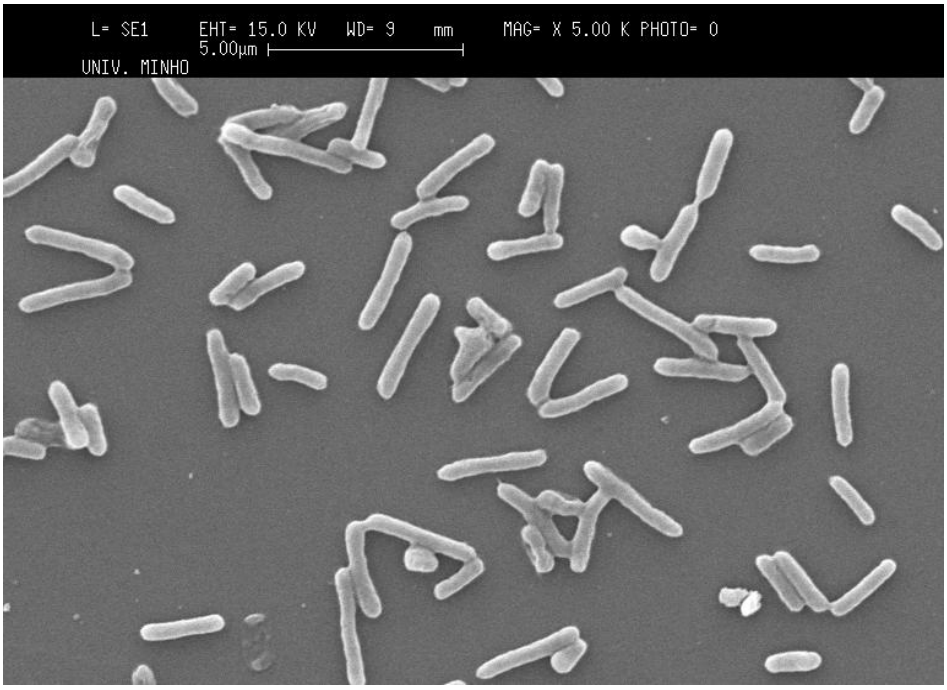


Name: P8BACO2 (5000 X)

Figure 5.52: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 5 °C on stainless steel.

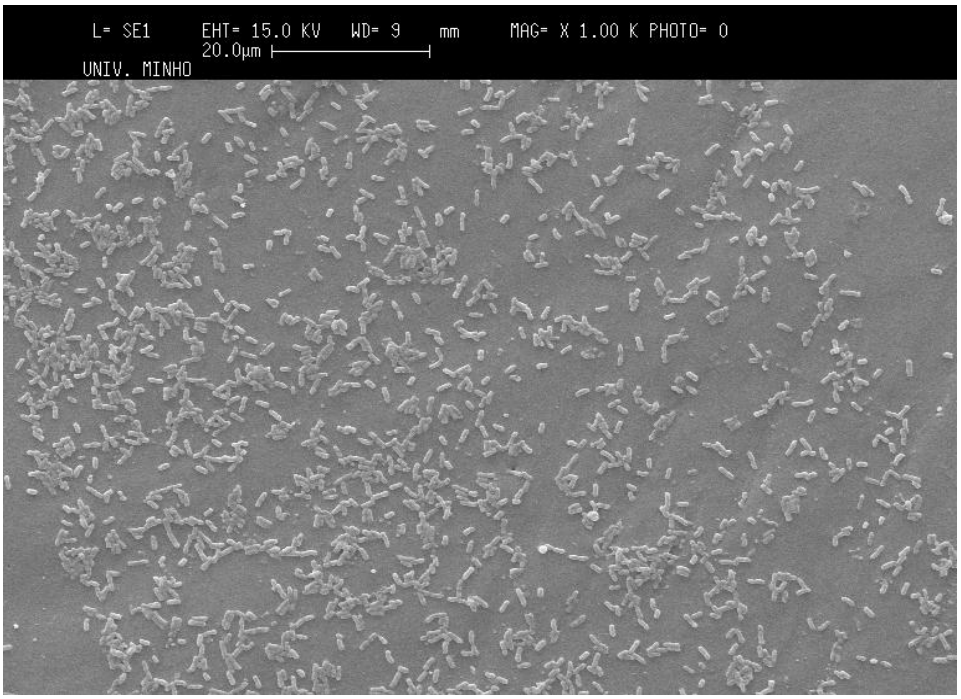


Name: P8BPP1 (1000 X)

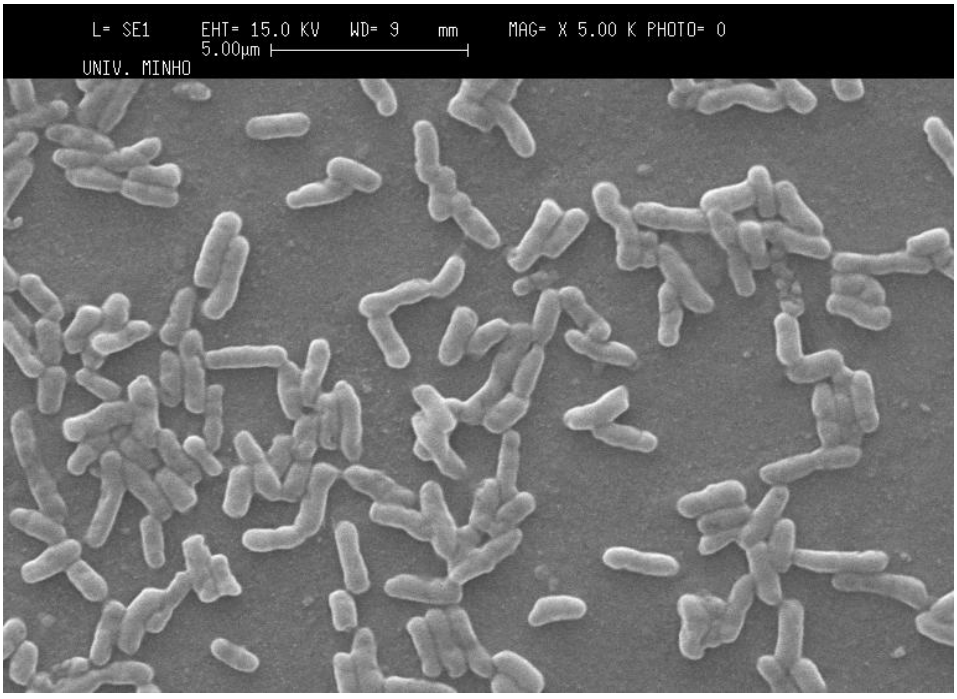


Name: P8BPP2 (5000 X)

Figure 5.53: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 5 °C on polypropylene.

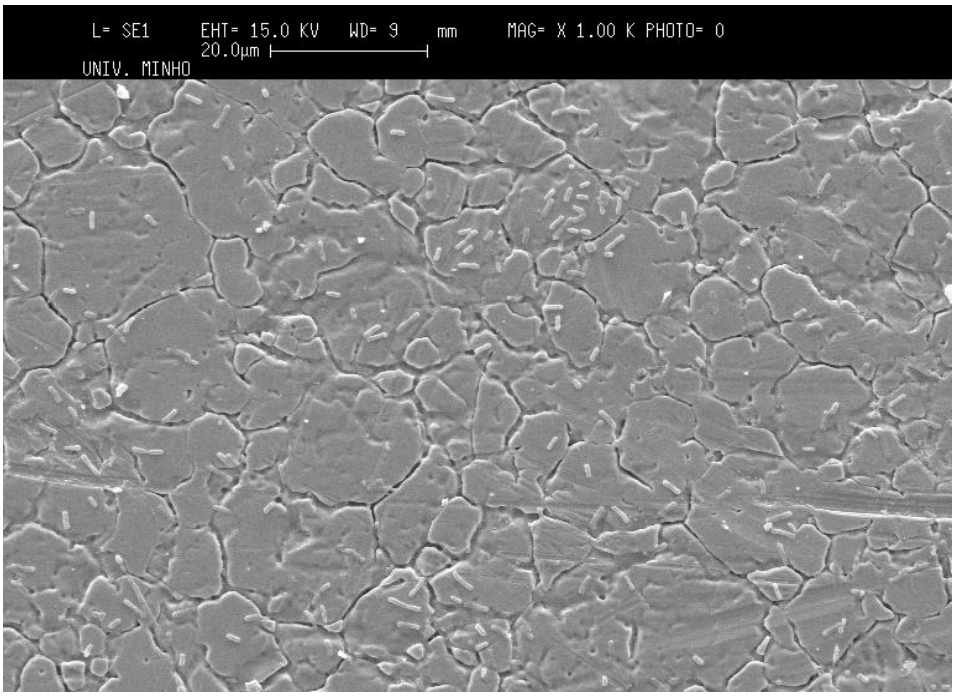


Name: P8BSI1 (1000 X)

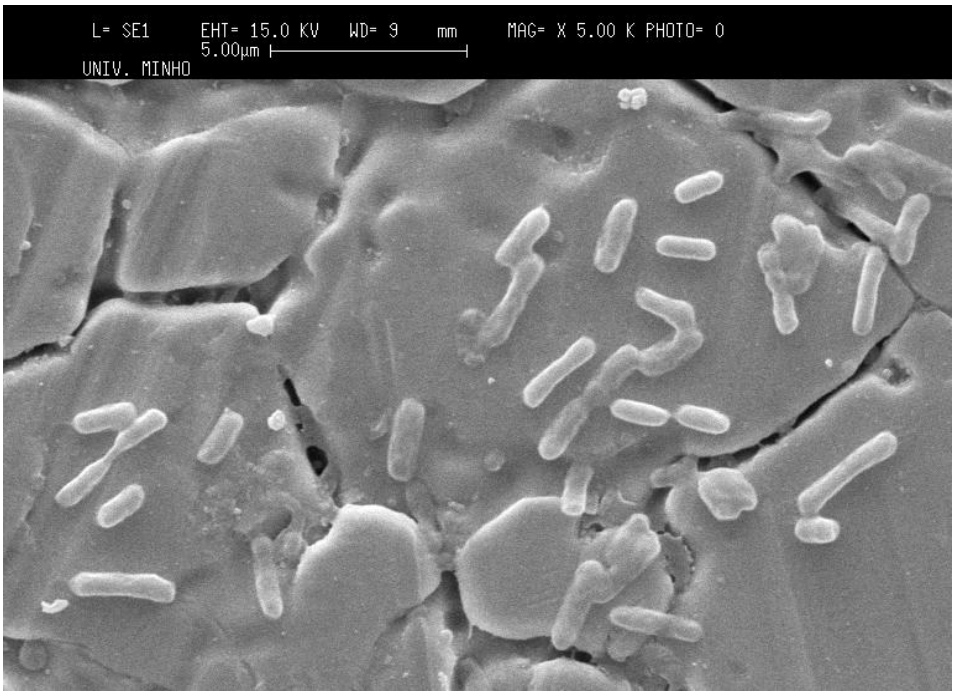


Name: P8BSI2 (5000 X)

Figure 5.54: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 5 °C on silicon.

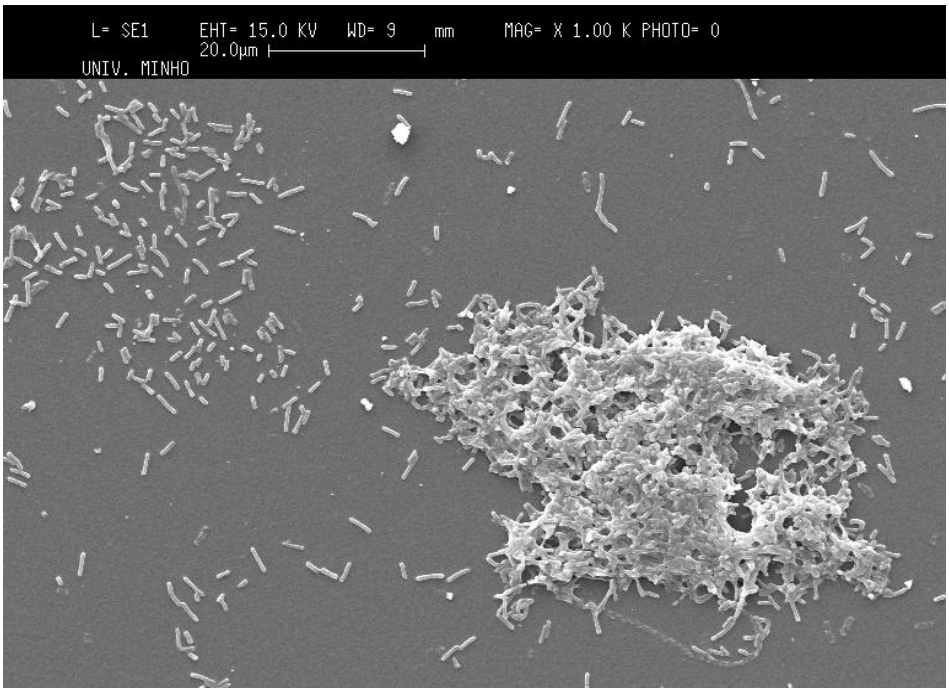


Name: P8RACO1 (1000 X)

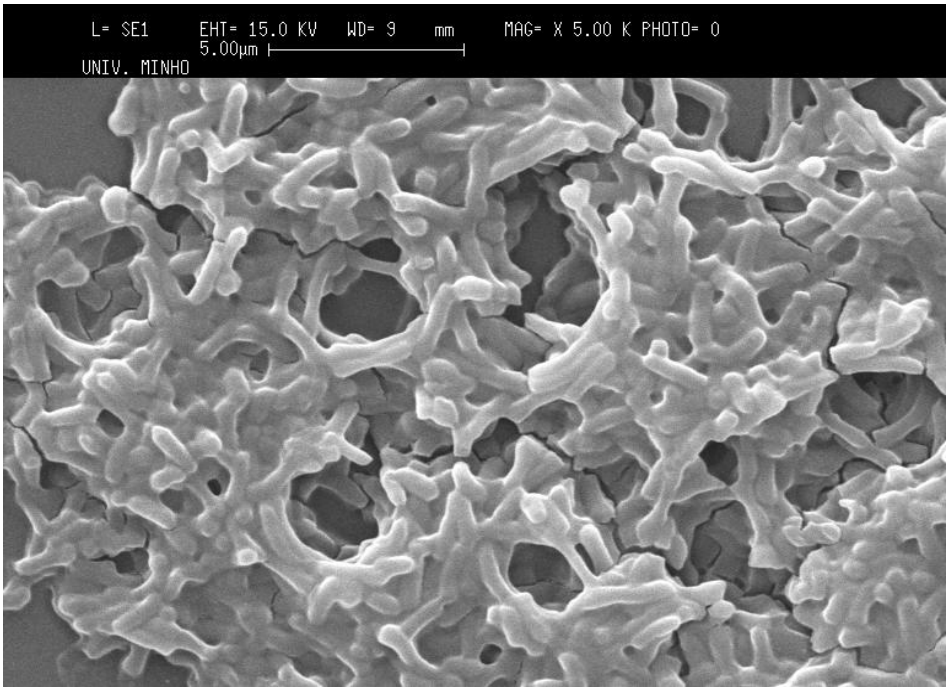


Name: P8RACO2 (5000 X)

Figure 5.55: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 5 °C on stainless steel.

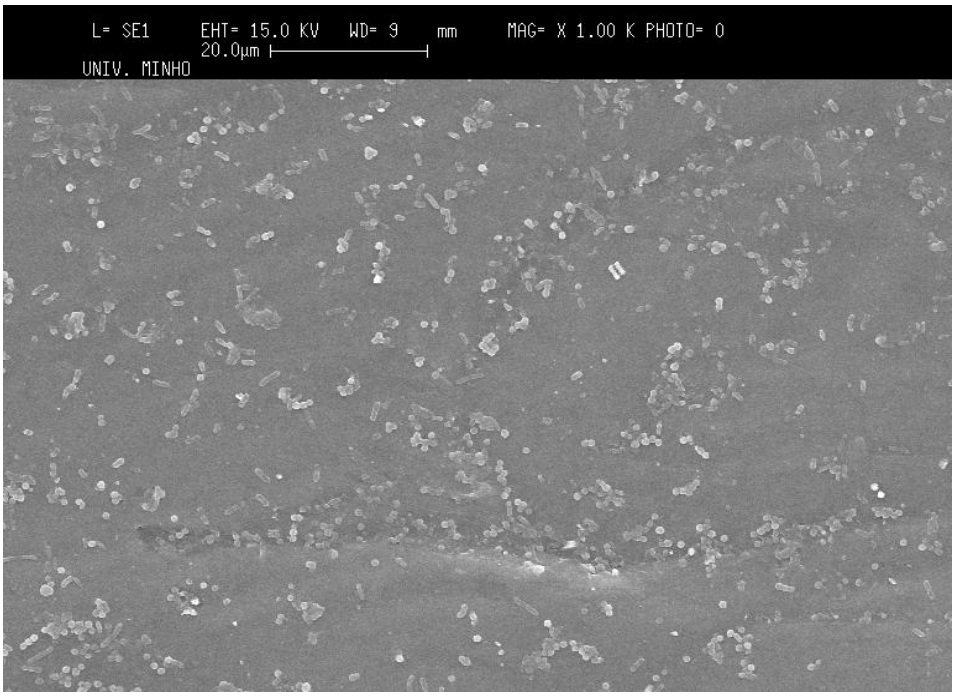


Name: P8RPP1 (1000 X)

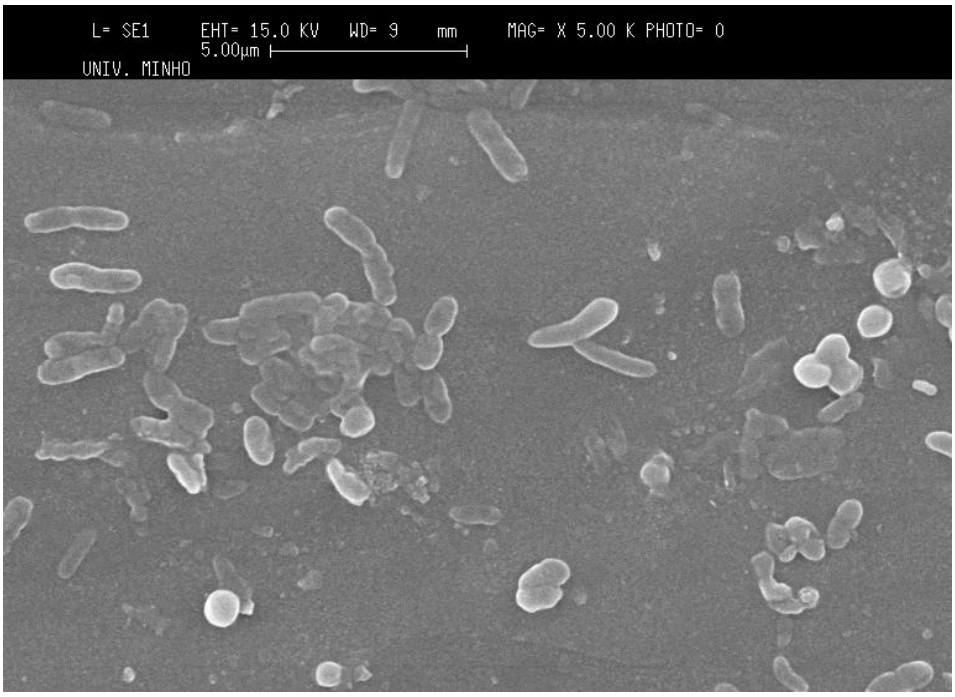


Name: P8RPP2 (5000 X)

Figure 5.56: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 5 °C on polypropylene.

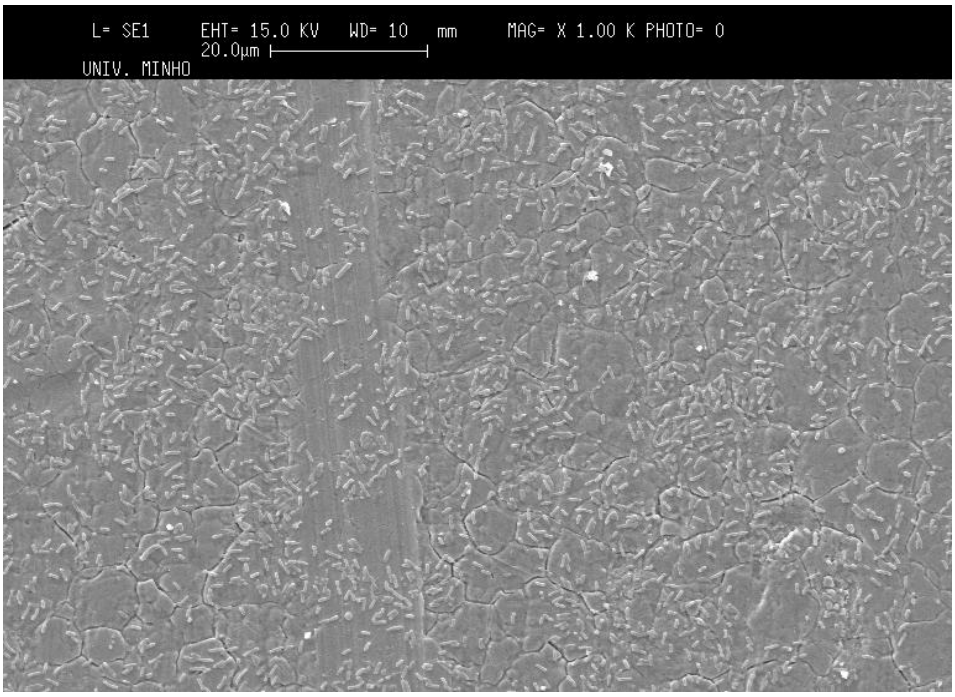


Name: P8RSI1 (1000 X)

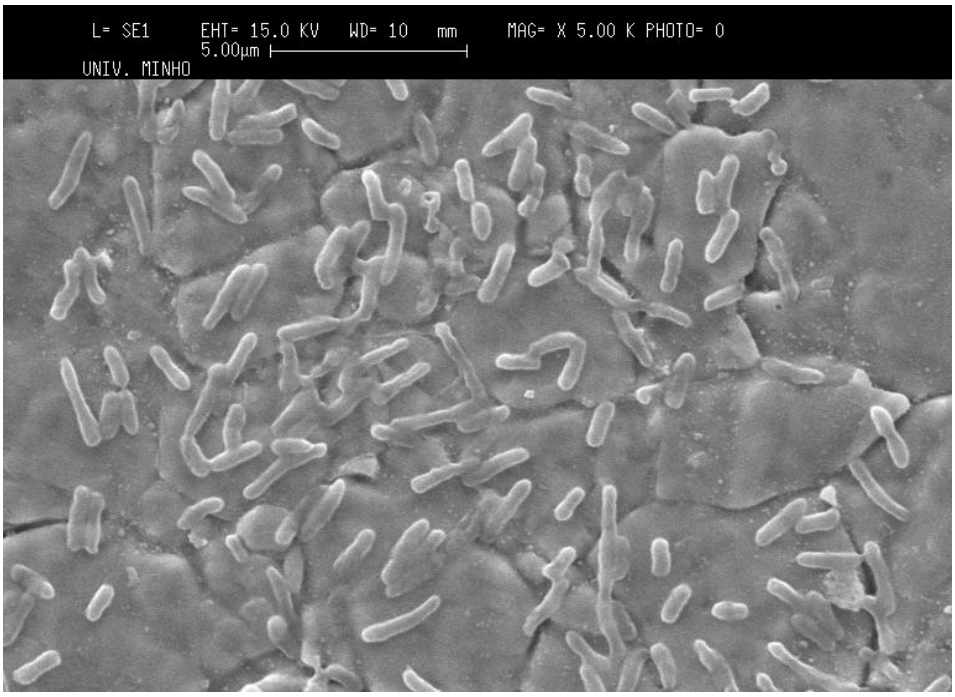


Name: P8RSI2 (5000 X)

Figure 5.57: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 5 °C on silicon.

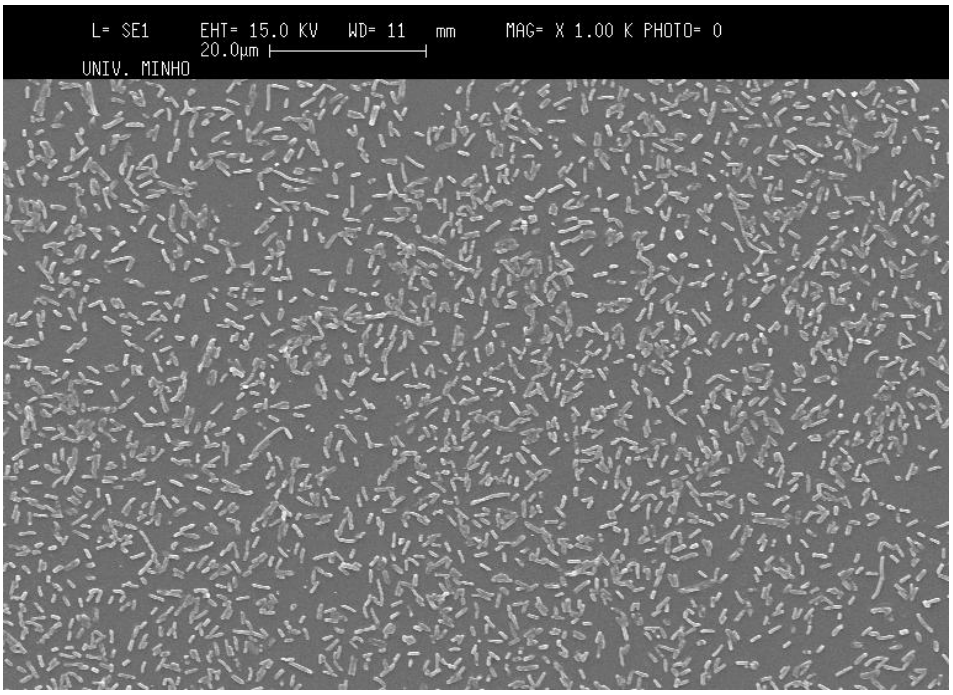


Name: P8WTACO1 (1000 X)

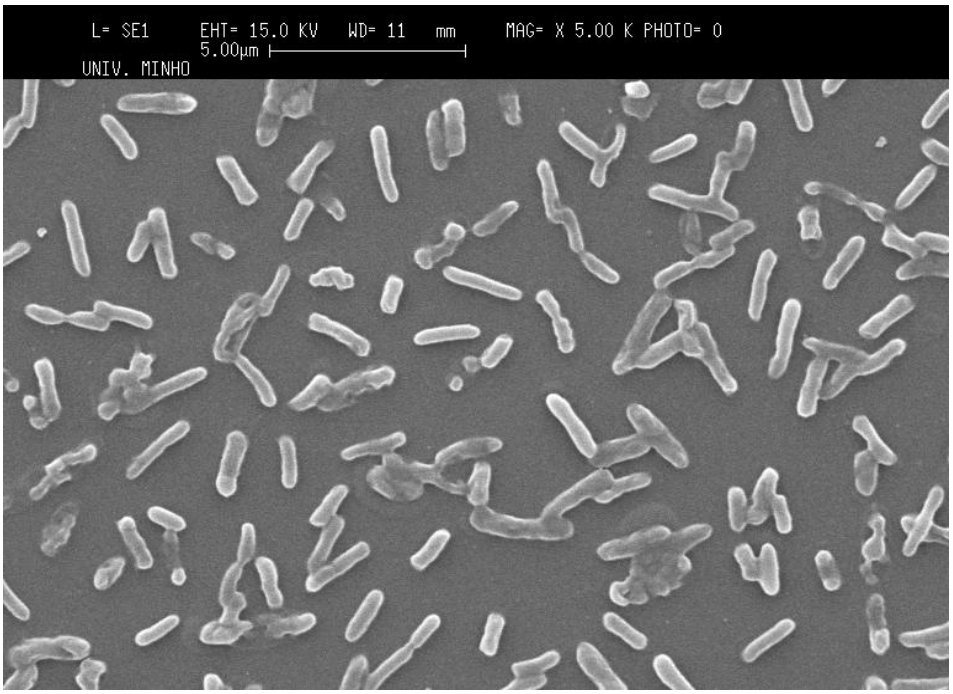


Name: P8WTACO2 (5000 X)

Figure 5.58: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 5 °C on stainless steel.

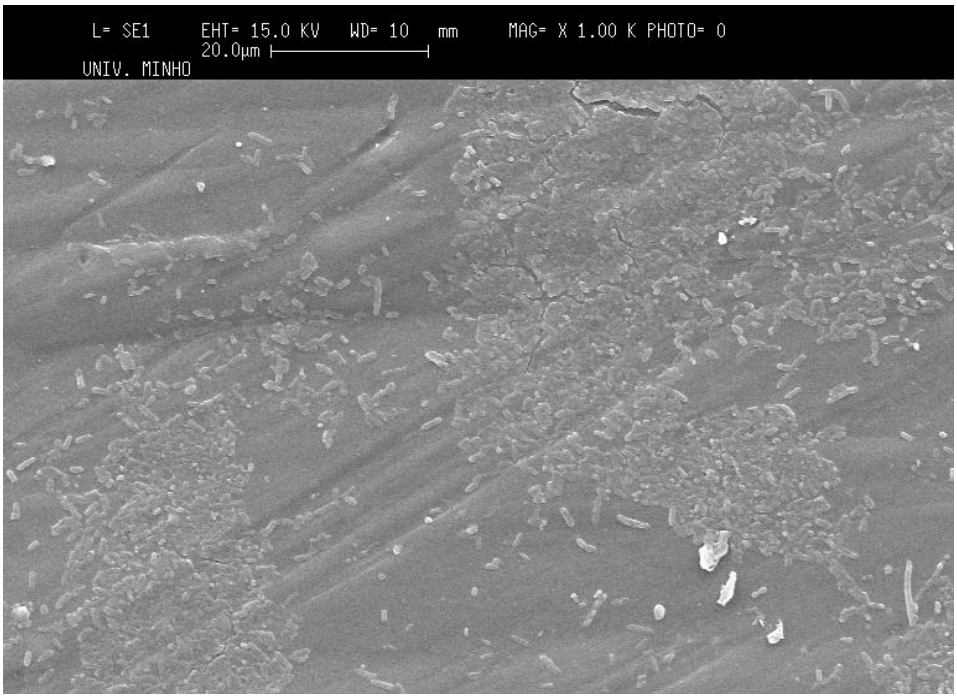


Name: P8WTPP1 (1000 X)

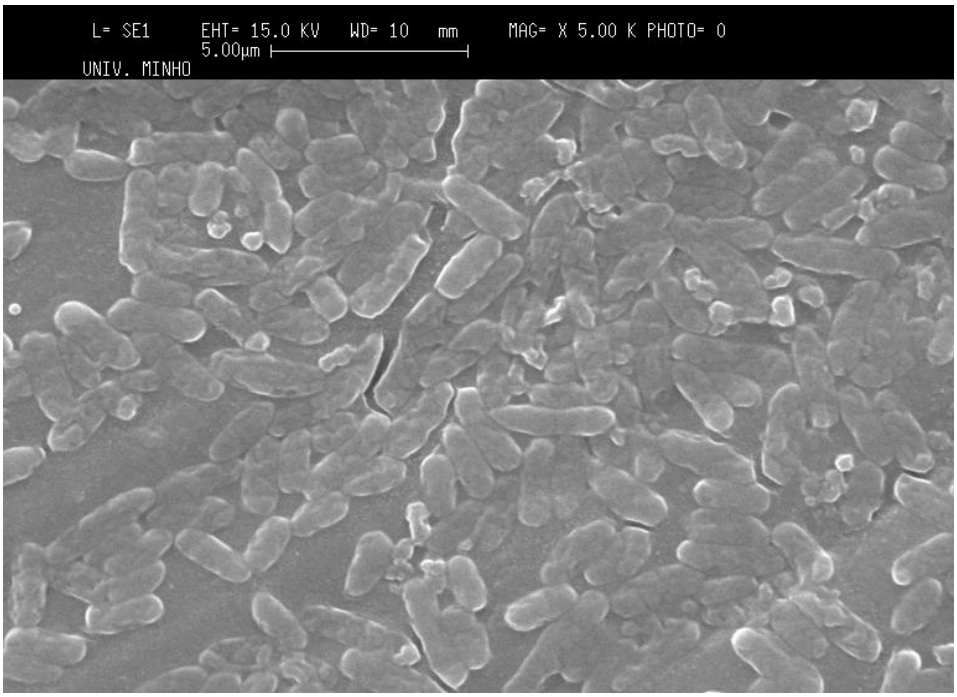


Name: P8WTPP2 (5000 X)

Figure 5.59: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 5 °C on polypropylene.



Name: P8WTSI1 (1000 X)



Name: P8WTSI2 (5000 X)

Figure 5.60: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 5 °C on silicon.

5.6.8 Adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel (48 h and 72 h stress @ 3 mM H_2O_2).

The conditions used in this study are listed in Table 5.9 and 5.10. For the images presented, the following designations are used. For example, P5WTSI 1/2 should read as,

P5 - Plate 5 (condition)

WT - Wild Type (strain), **B** - *bolA+/rpoS-*, **R** - *rpoS+/bolA-*

SI - Silicon (substrate), **ACO** – stainless style, **PP** – polypropylene

1/2 - magnification under 1000x (1) and 5000x (2).

Table 5.9: Condition used in this study with the designated names and remarks

Plate	Strain	Material	Name	Figure	Remarks
Plate 5 **48 h stress @ 3 mM H2O2 (24 h/37 °C + 24 h/H2O2)**	*WT*	Stainless Steel	P5WTACO 1/2	5.67	Less attachment
		Silicone	P5WTSI 1/2	5.68	Very well attached
		Polypropylene	-	NA	Nothing visible
	bolA+/rpoS-	Stainless Steel	P5BACO 1/2	5.61	Very well attached
		Silicone	P5BSI 1/2	5.63	Well attached
		Polypropylene	P5BPP 1/2	5.62	Thick biofilm
	rpoS+/bolA-	Stainless Steel	P5RACO 1/2	5.64	Less attachment
		Silicone	P5RSI 1/2	5.66	Very well attached
		Polypropylene	P5RPP 1/2	5.65	Less attachment

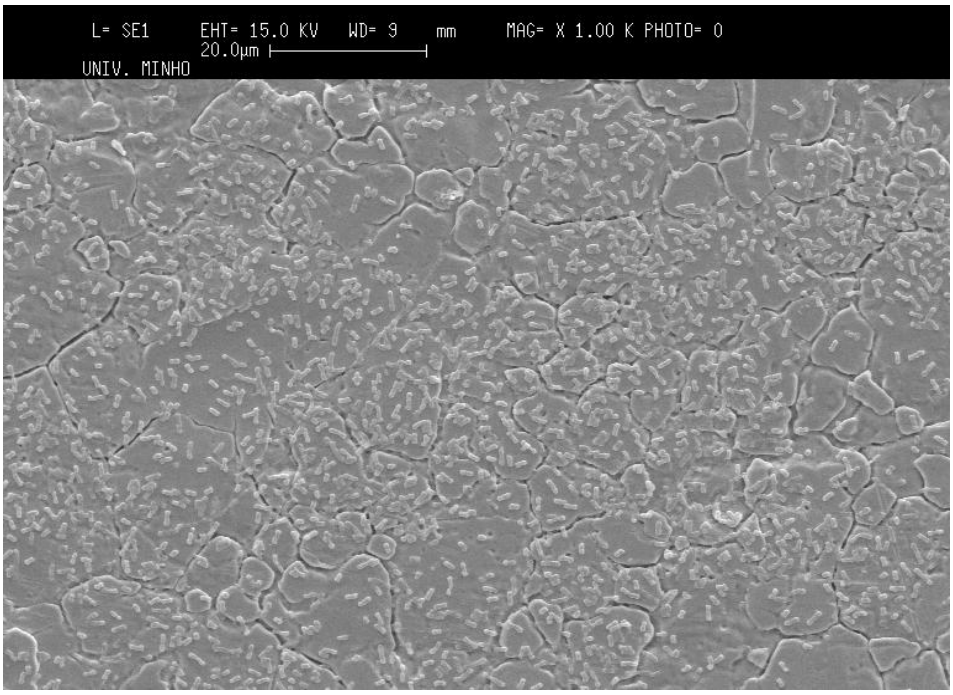
Table 5.10: Condition used in this study with the designated names and remarks

Plate	Strain	Material	Name	Figure	Remarks
Plate 9 **72 h stress @ 3 mM H2O2 (48 h/37 °C + 24 h/H2O2)**	*WT*	Stainless Steel	P9WTACO 1/2	5.73	Thick biofilm/round morphology
		Silicone	P9WTSI 1/2	5.75	Very less attachment
		Polypropylene	P9WTPP 1/2	5.74	Well attached/elongated cells
	bolA+/rpoS-	Stainless Steel	P9BACO 1/2	5.69	Less attachment
		Silicone	P9BSI 1/2	5.71	Well attached
		Polypropylene	P9BPP 1/2	5.70	Well attached/biofilm formation
	rpoS+/bolA-	Stainless Steel	-		
		Silicone	P9RSI 1/2	5.72	Less attachment
		Polypropylene	-		

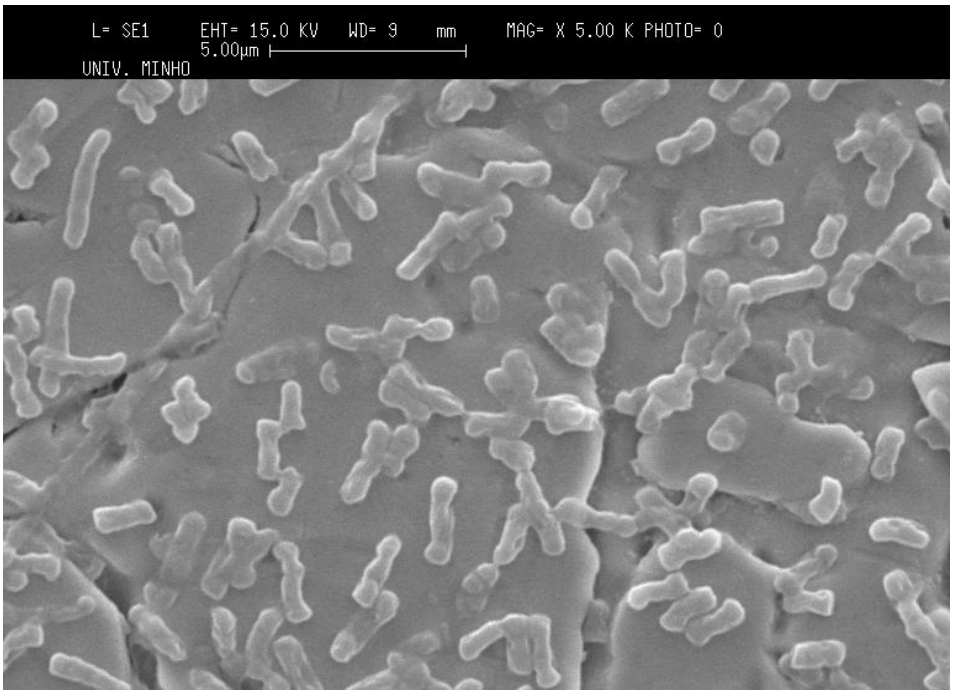
The results obtained under oxidative stress are shown here, i.e. in presence of 3 mM H_2O_2 after 48 and 72 hours. SEM shows that cells can attach to all the three surfaces investigated under H_2O_2 stress, but were unable to form biofilms until 72 hours had elapsed indicating that biofilm formation might be able to take place in later stages? Considerable morphological variation was seen under oxidative stress. In presence of *rpoS+/bolA-* large numbers of cells were seen attached to the surface of polypropylene after 48 hours (Fig. 5.64 and 5.75), whilst after 72 hours nothing was seen in presence of *rpoS+/bolA-* cells.

An interesting result was seen in presence of *bolA* after 72 hours (5.69-5.71). Cells were found to attach to the surface of polypropylene under oxidative stress, initiating the biofilm process, which in turn again shows the ability of *bolA* to respond under

stress conditions enabling the cells to attach to surfaces and form biofilms in absence of *rpoS*. On stainless steel surface, after 72 hours a high number of cells with a round morphology was seen (Fig. 5.73), while nothing was seen after 72 hours in the case of *bolA*-/*rpoS*+ containing cells on stainless steel. This confirms that mutation of the *bolA* gene make cells unable to adhere to the stainless steel and silicon surfaces.

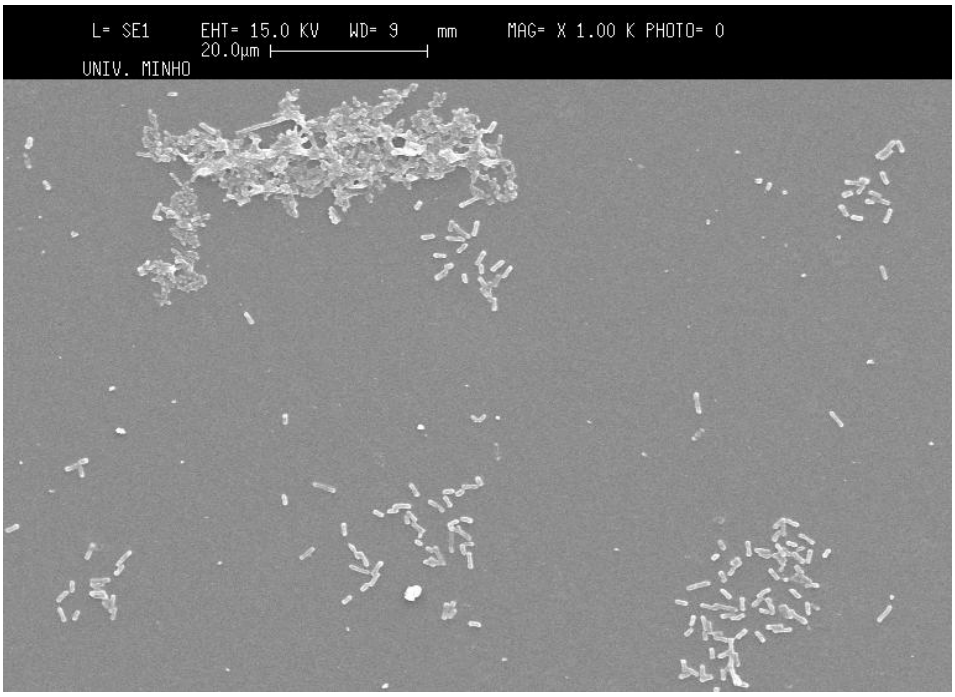


Name: P5BACO1 (1000 X)

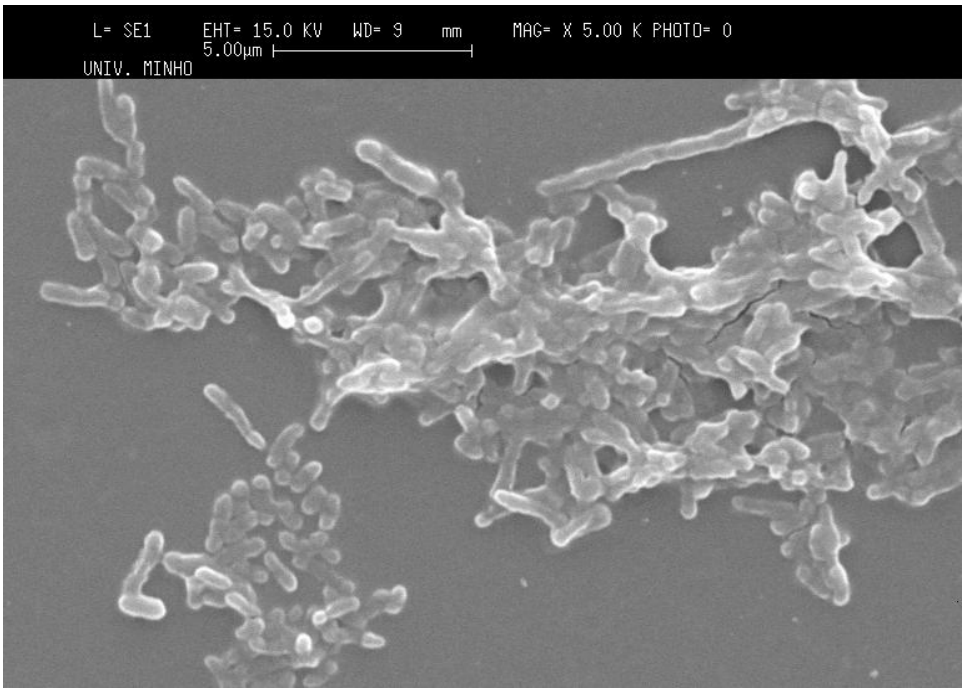


Name: P5BACO2 (5000 X)

Figure 5.61: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 3 mM H_2O_2 stress on stainless steel.

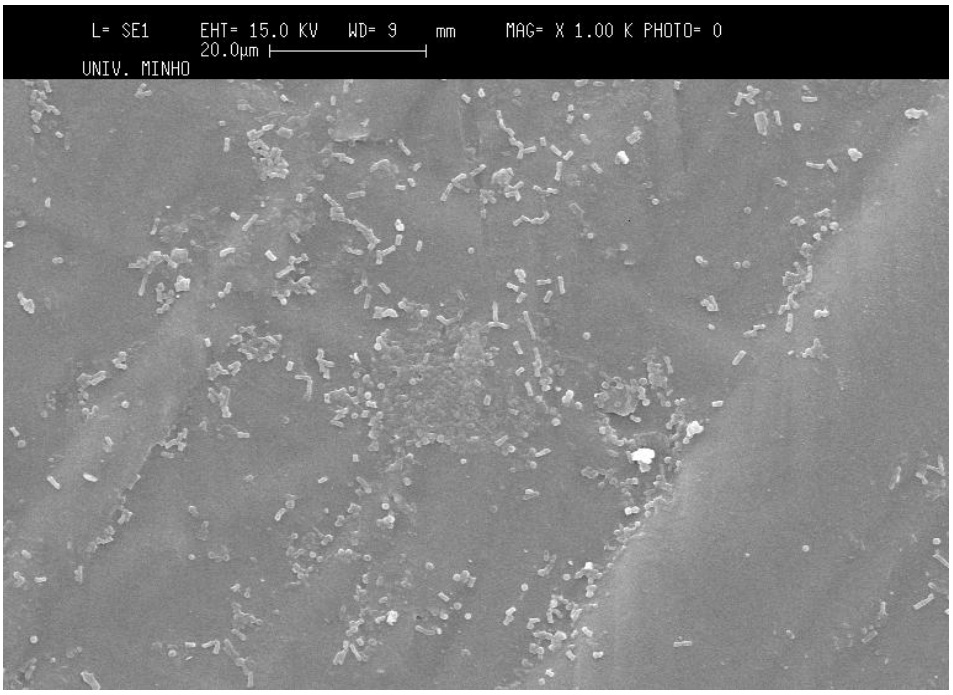


Name: P5BPP1 (1000 X)

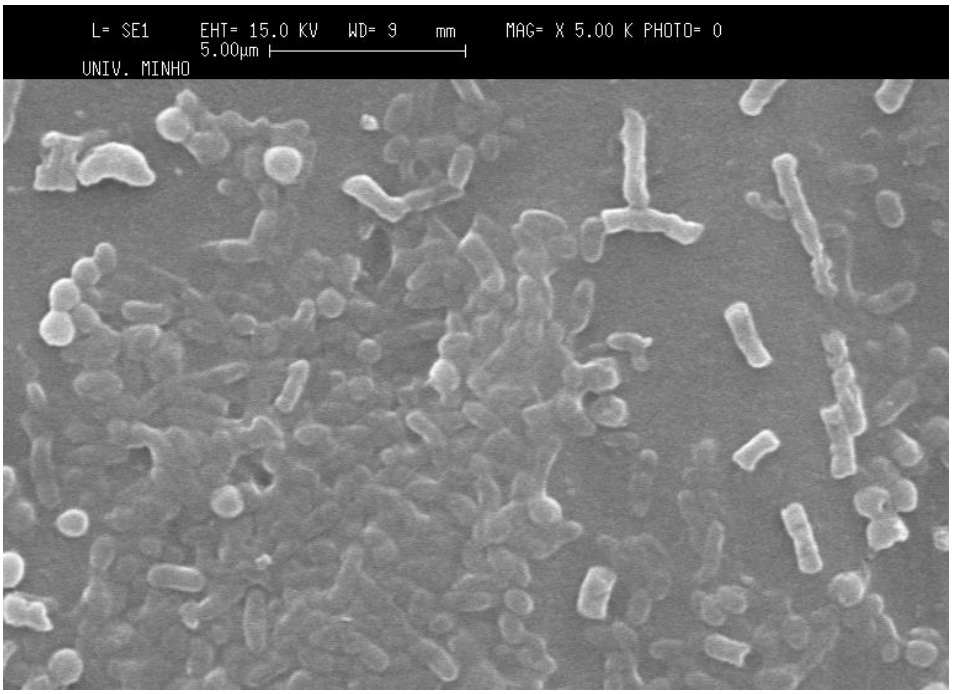


Name: P5BPP2 (5000 X)

Figure 5.62: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 3 mM H_2O_2 stress on polypropylene.

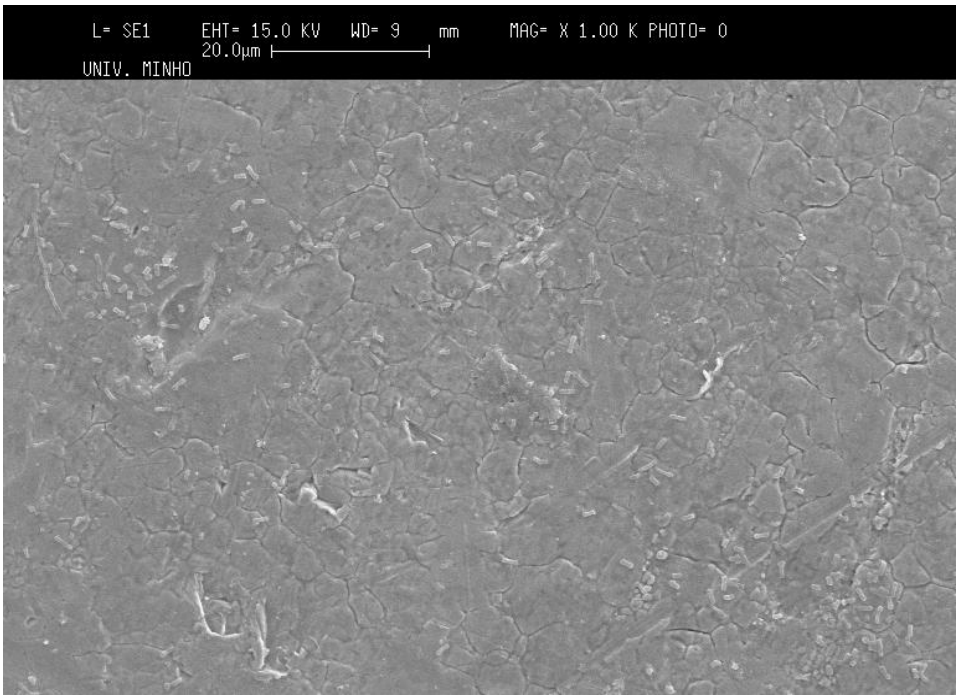


Name: P5BSI1 (1000 X)

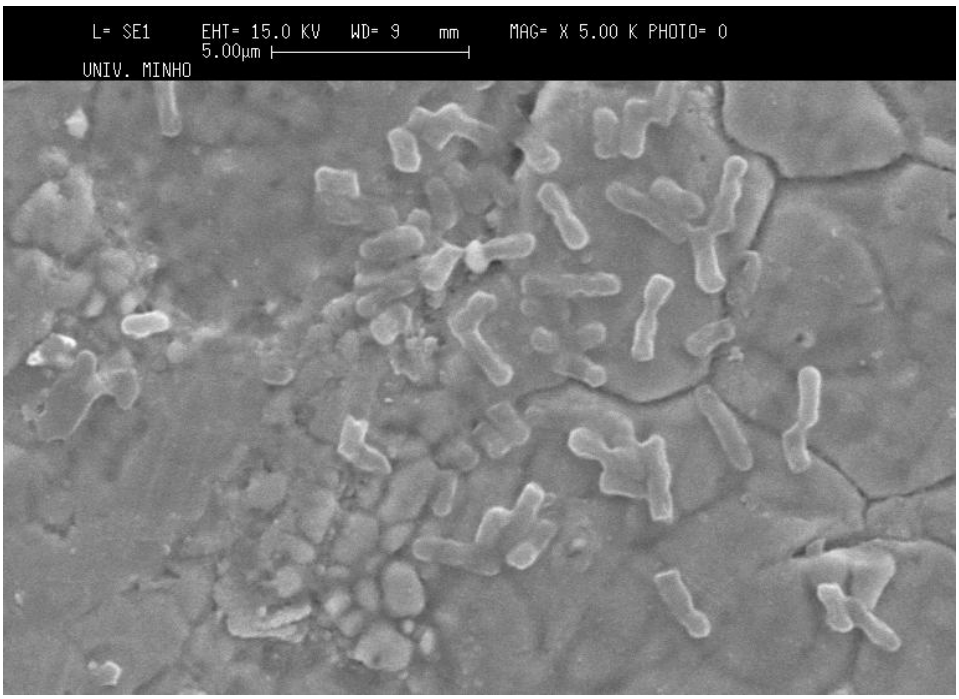


Name: P5BSI2 (5000 X)

Figure 5.63: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 48 hours at 3 mM H_2O_2 stress on silicon.

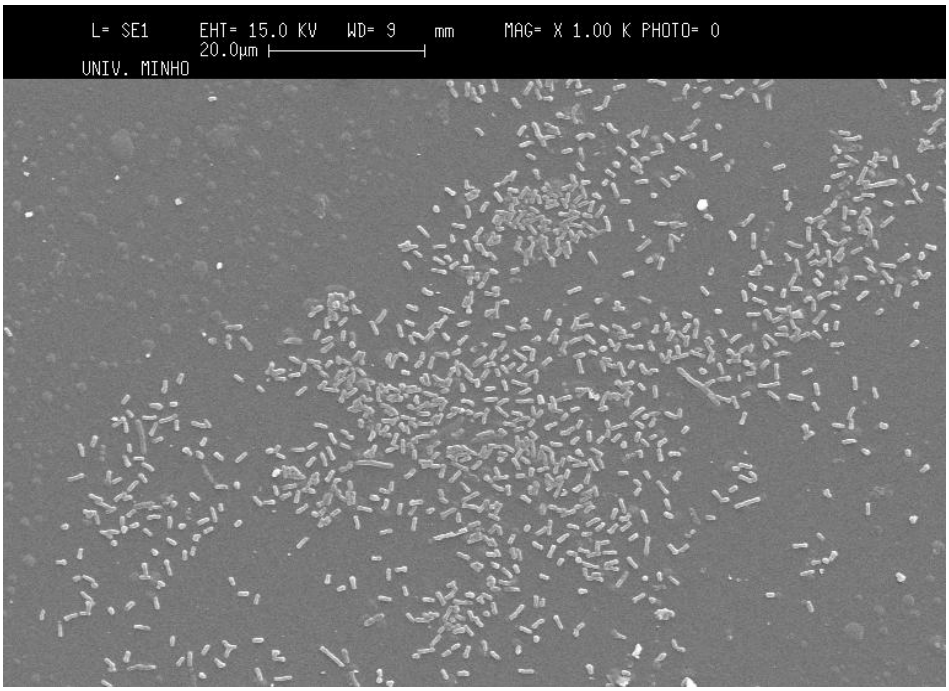


Name: P5RACO1 (1000 X)



Name: P5RACO2 (5000 X)

Figure 5.64: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 3 mM H_2O_2 stress on stainless steel.



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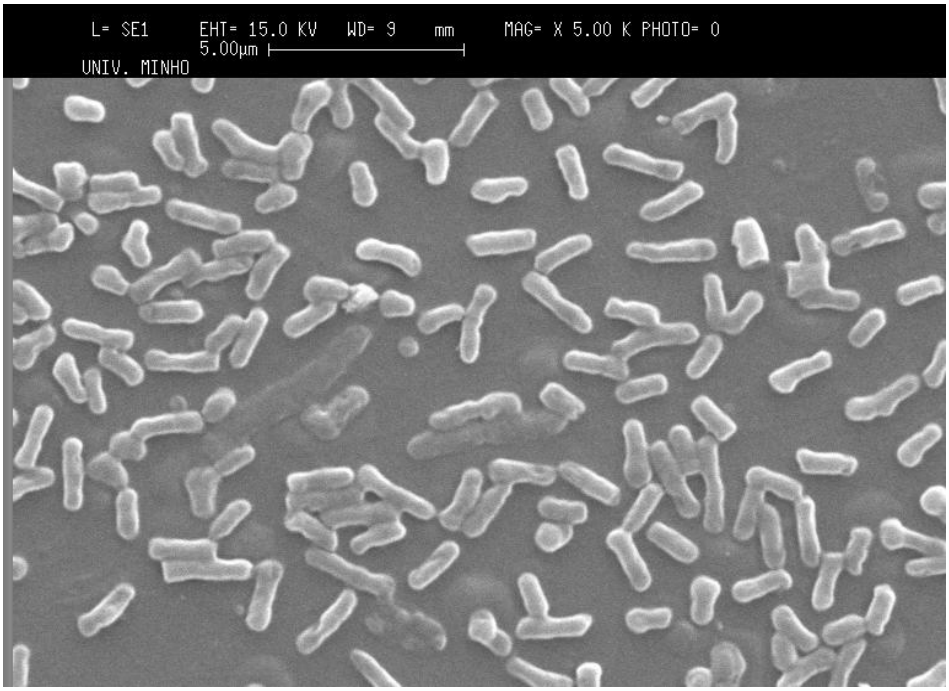
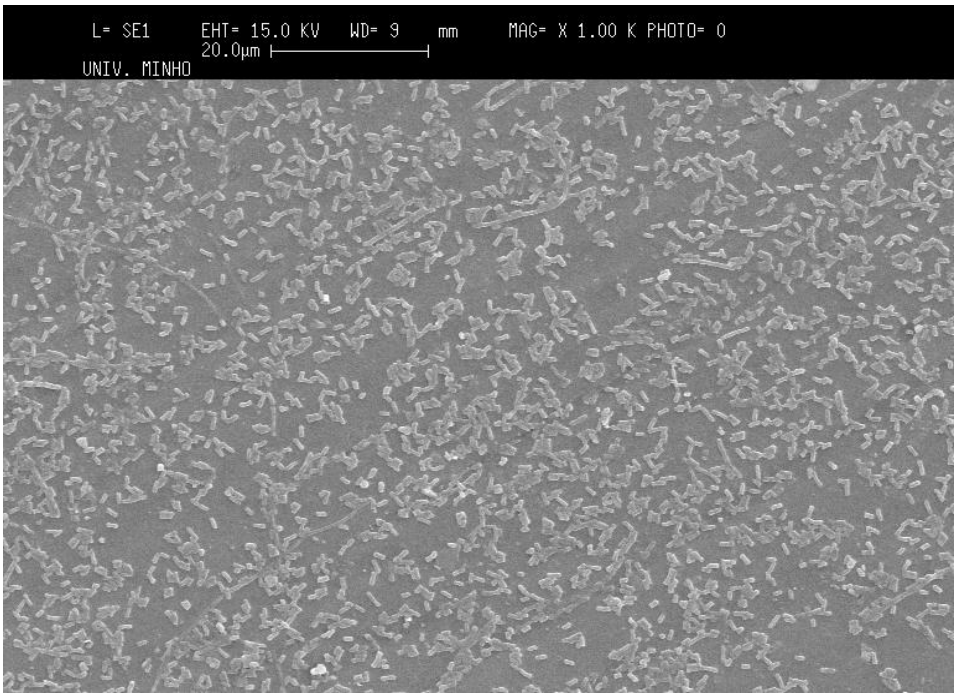


Name: P5RPP2 (5000 X)

Figure 5.65: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 3 mM H_2O_2 stress on polypropylene.



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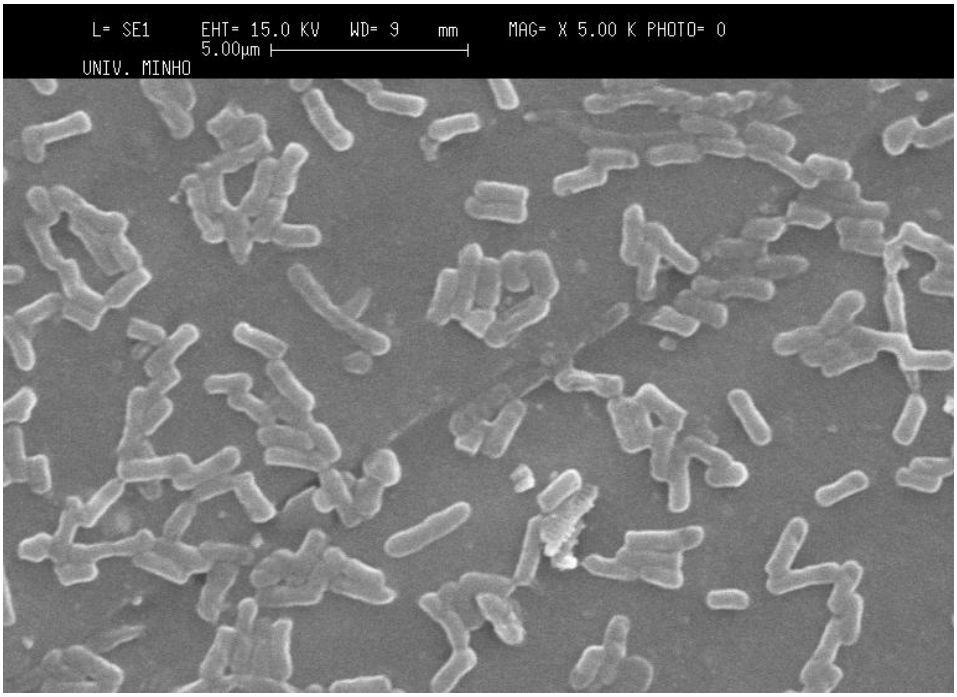
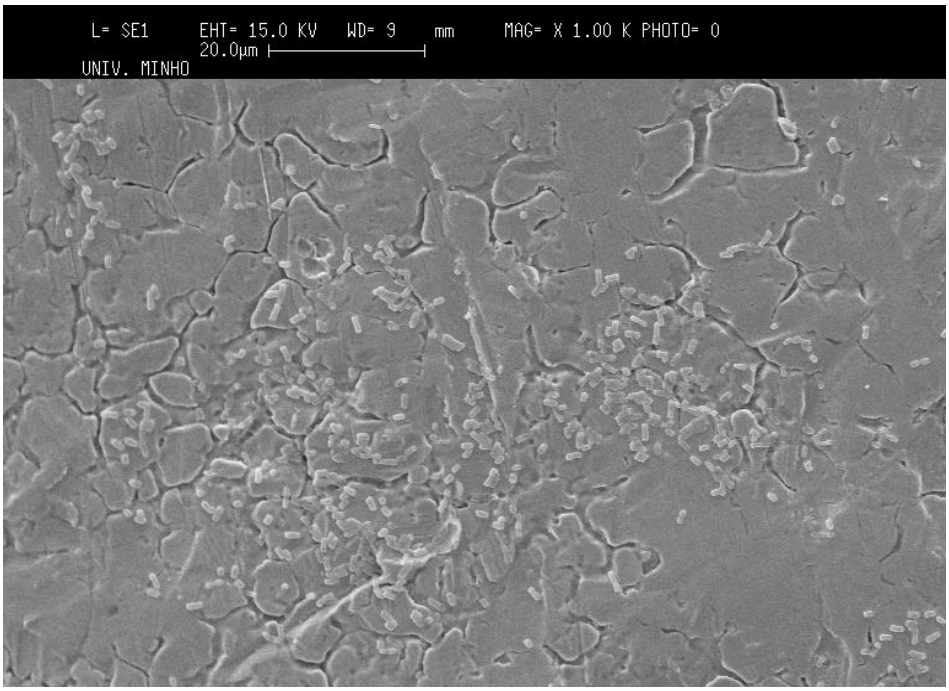
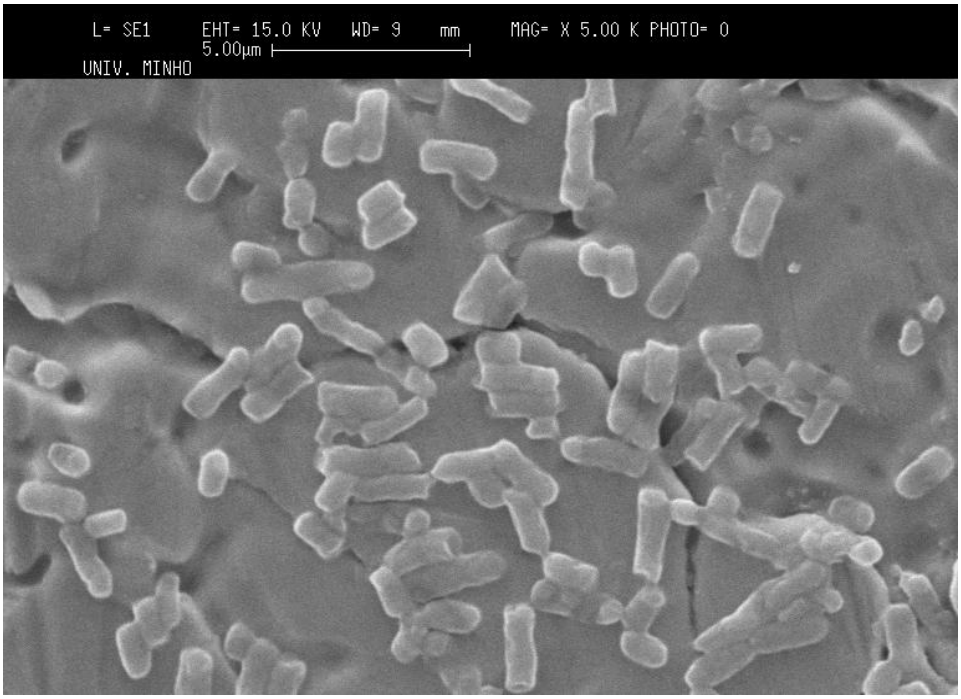


Name: P5RSI2 (5000 X)

Figure 5.66: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 48 hours at 3 mM H_2O_2 stress on silicon.

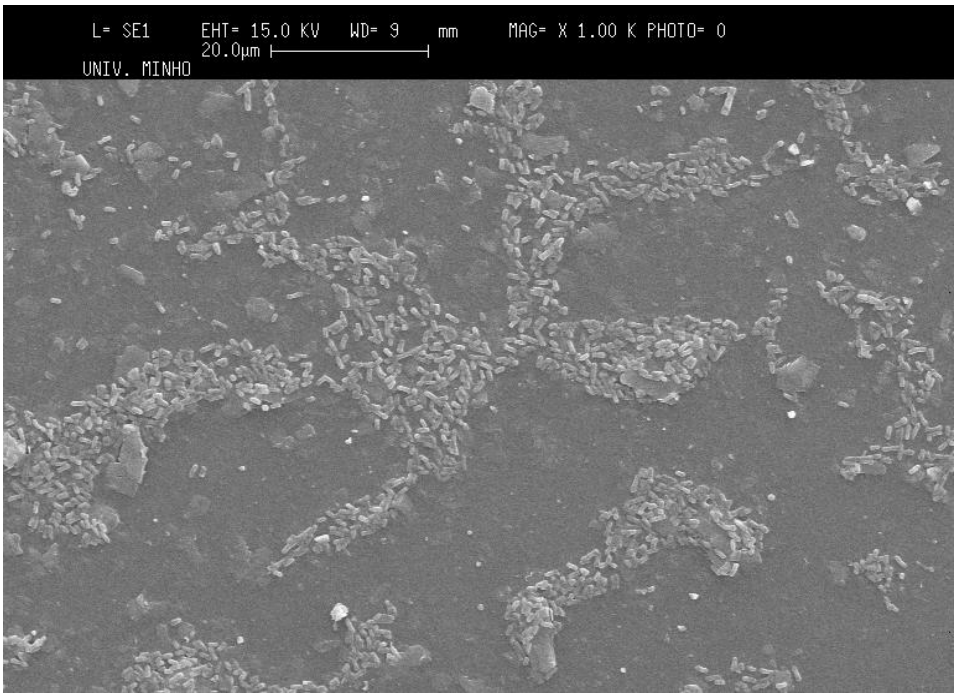


Name: P5WTACO1 (1000 X)

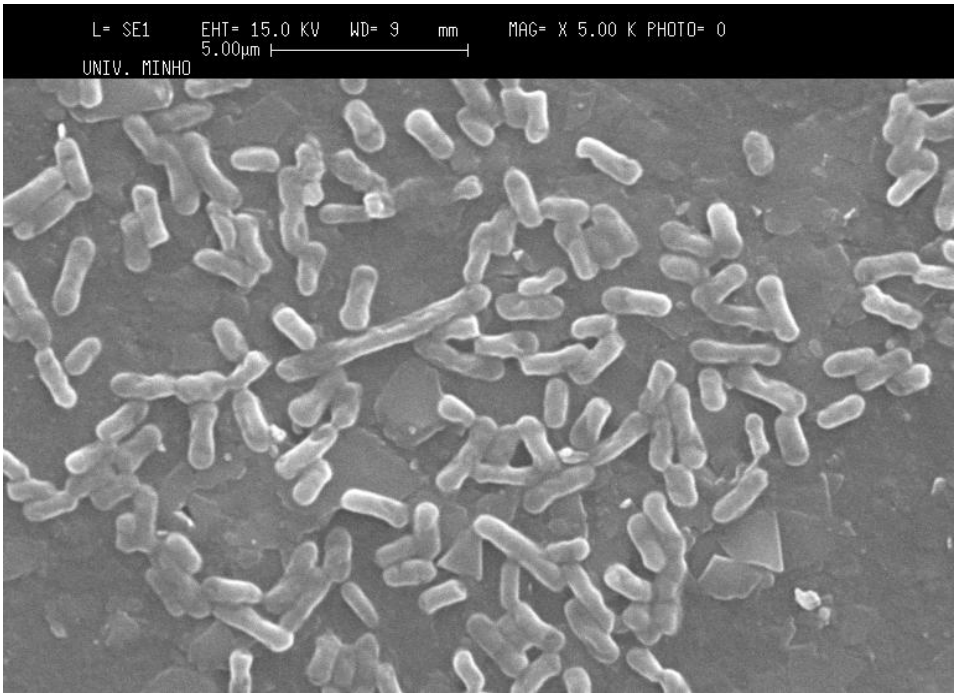


Name: P5WTACO2 (5000 X)

Figure 5.67: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 3 mM H_2O_2 stress on stainless steel.

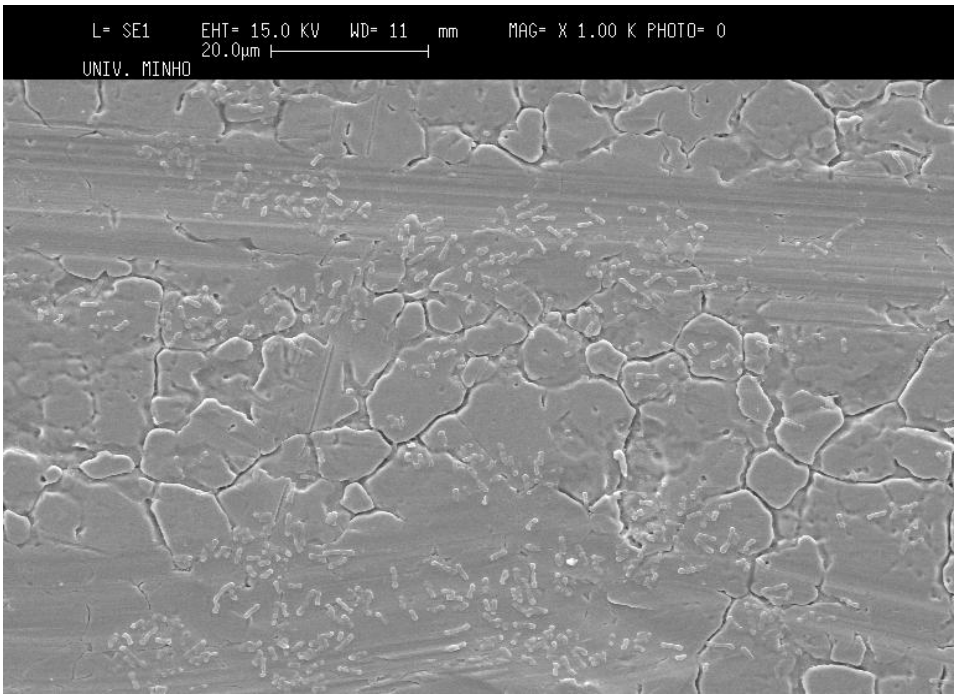


Name: P5WTSI1 (1000 X)

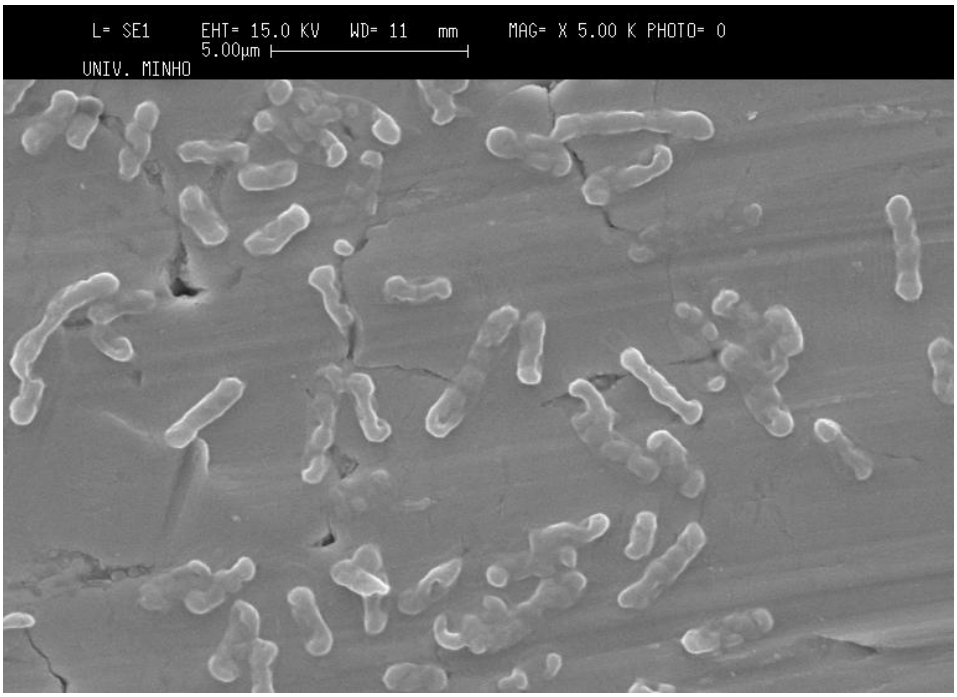


Name: P5WTSI2 (5000 X)

Figure 5.68: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 48 hours at 3 mM H_2O_2 stress on silicon.

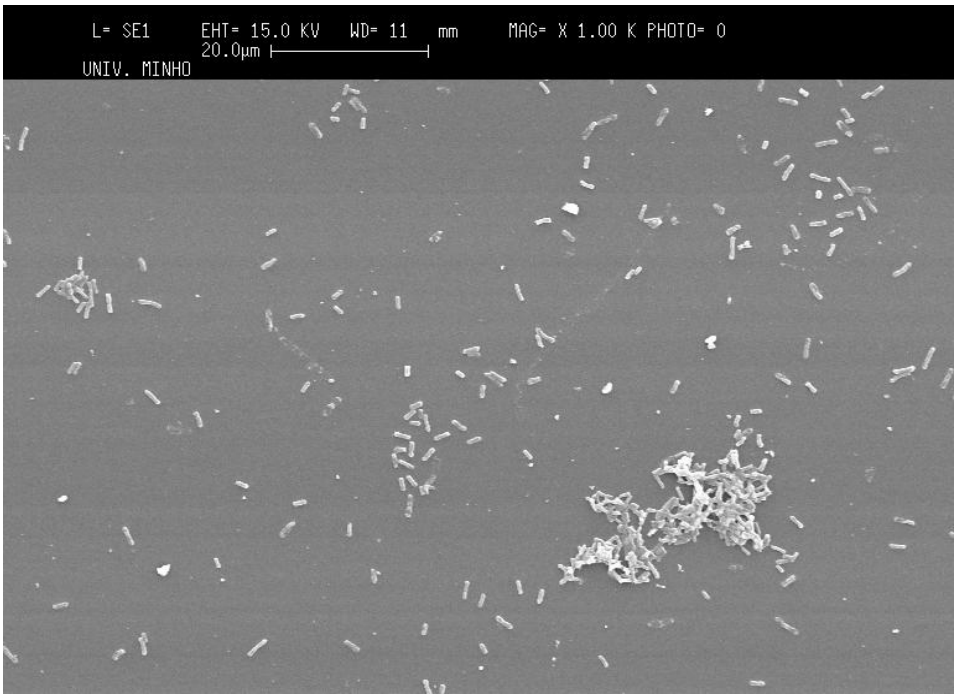


Name: P9BACO1 (1000 X)

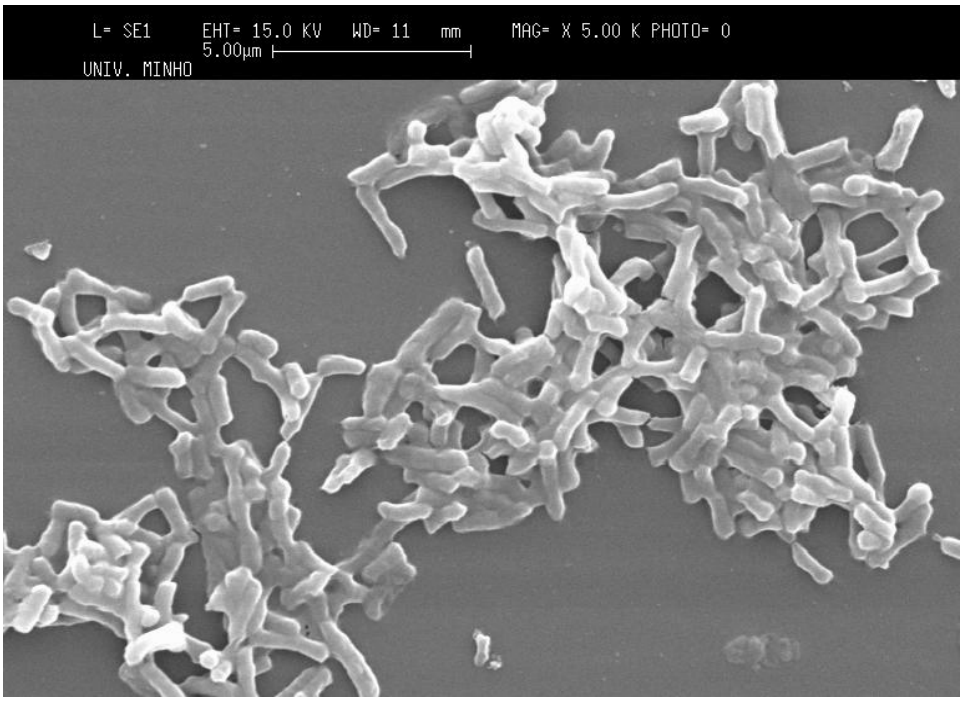


Name: P9BACO2 (5000 X)

Figure 5.69: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 3 mM H_2O_2 stress on stainless steel.

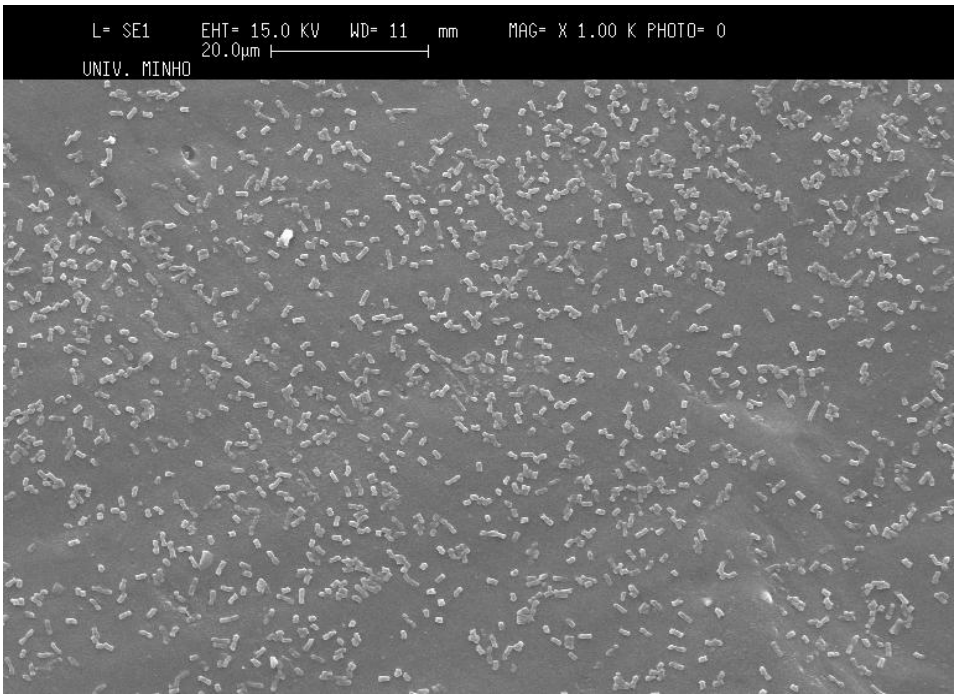


Name: P9BPP1 (1000 X)

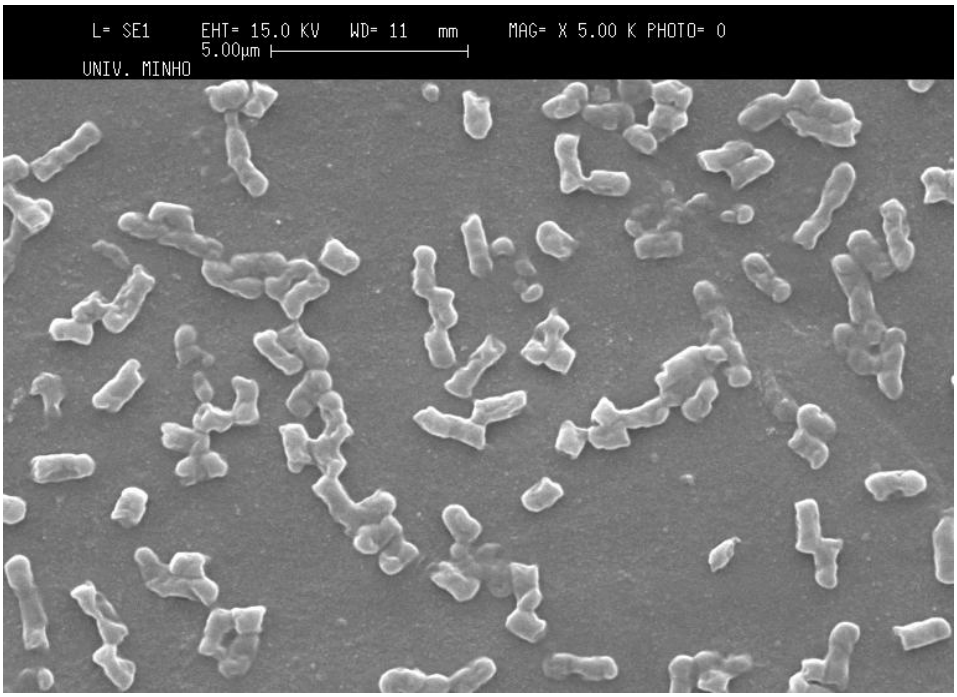


Name: P9BPP2 (5000 X)

Figure 5.70: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 3 mM H_2O_2 stress on polypropylene.

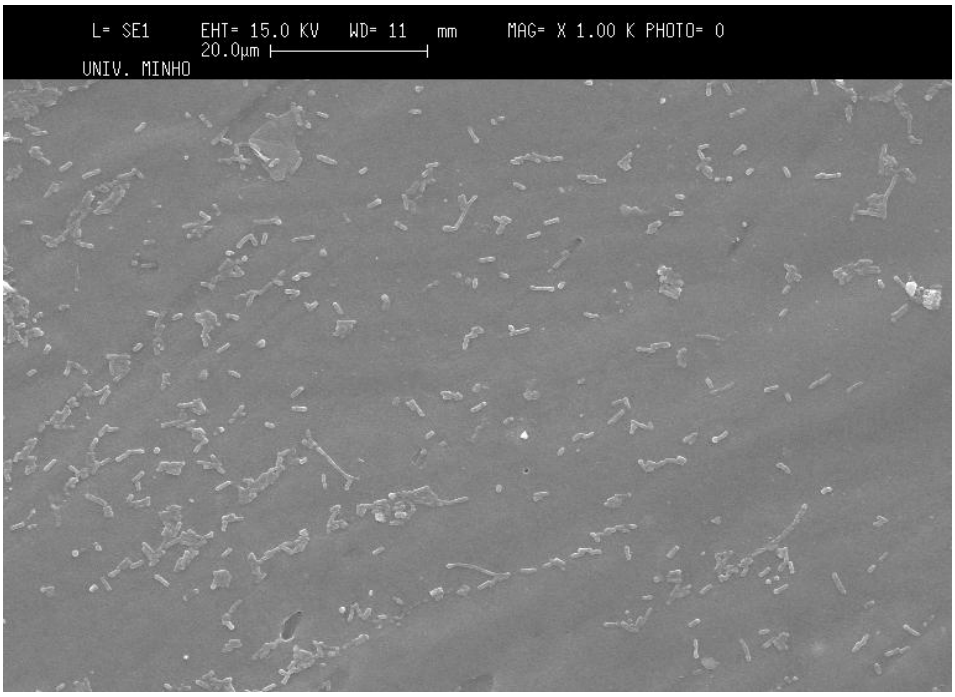


Name: P9BSI1 (1000 X)

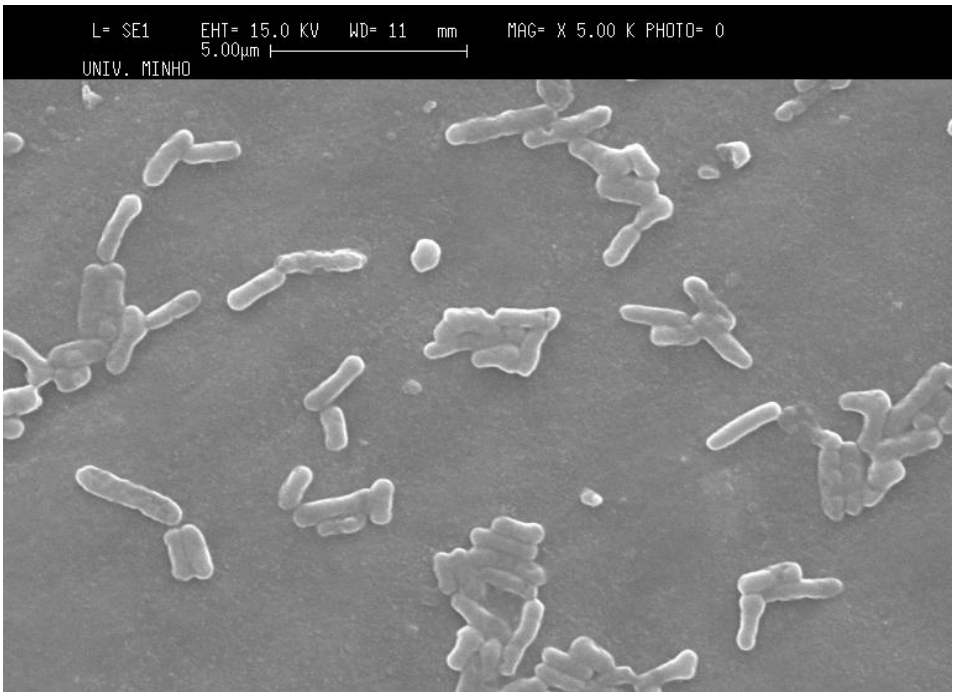


Name: P9BSI2 (5000 X)

Figure 5.71: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA+/rpoS-)* after 72 hours at 3 mM H_2O_2 stress on silicon.

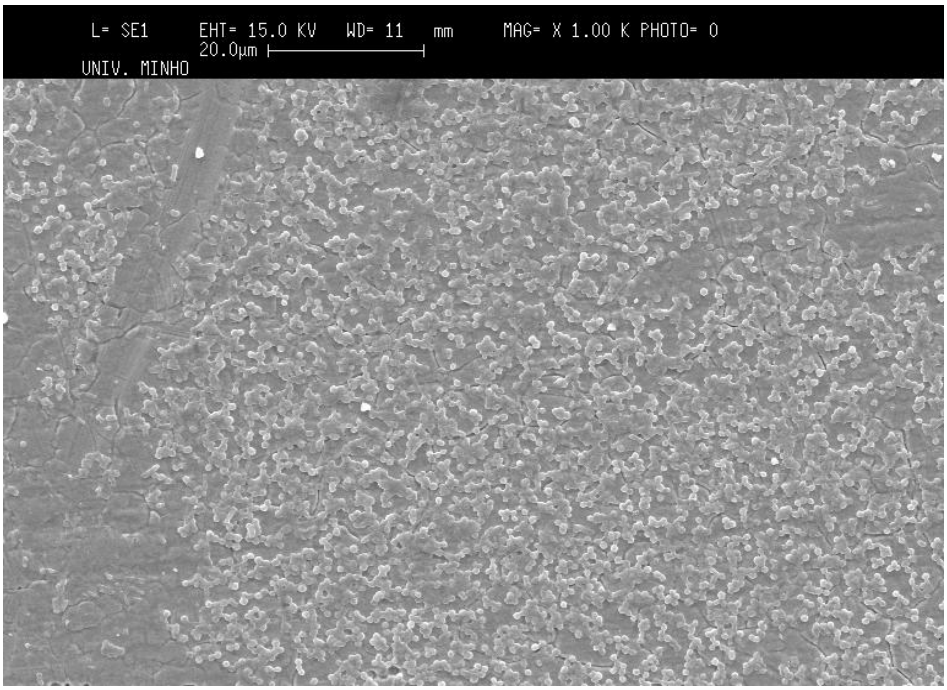


Name: P9RSI1 (1000 X)

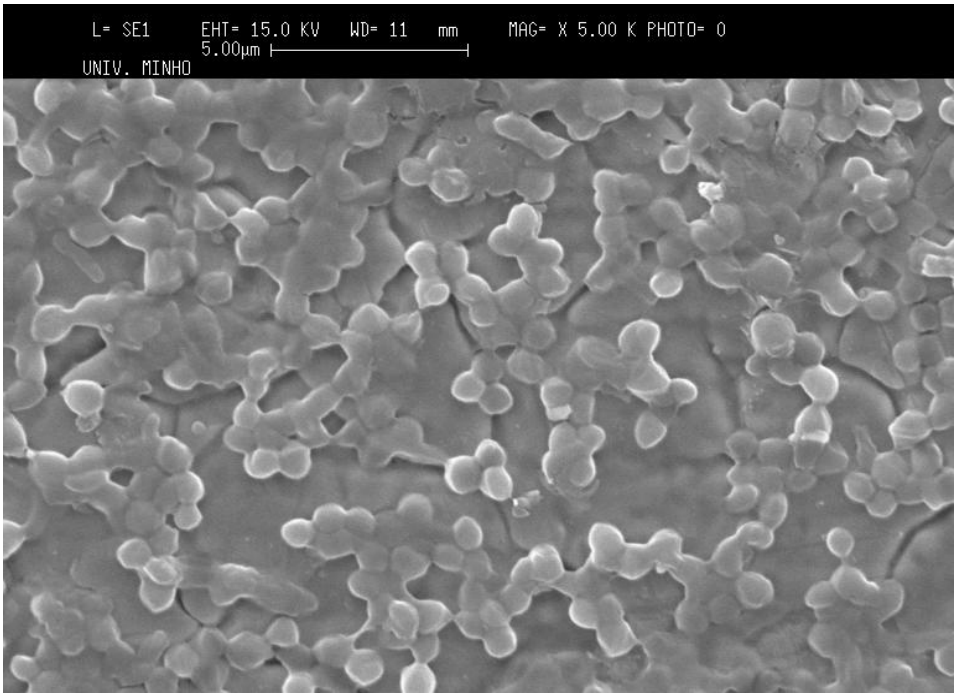


Name: P9RSI2 (5000 X)

Figure 5.72: Scanning electron micrograph of *E. coli* K-12 MG1655 *(bolA-/rpoS+)* after 72 hours at 3 mM H_2O_2 stress on silicon.

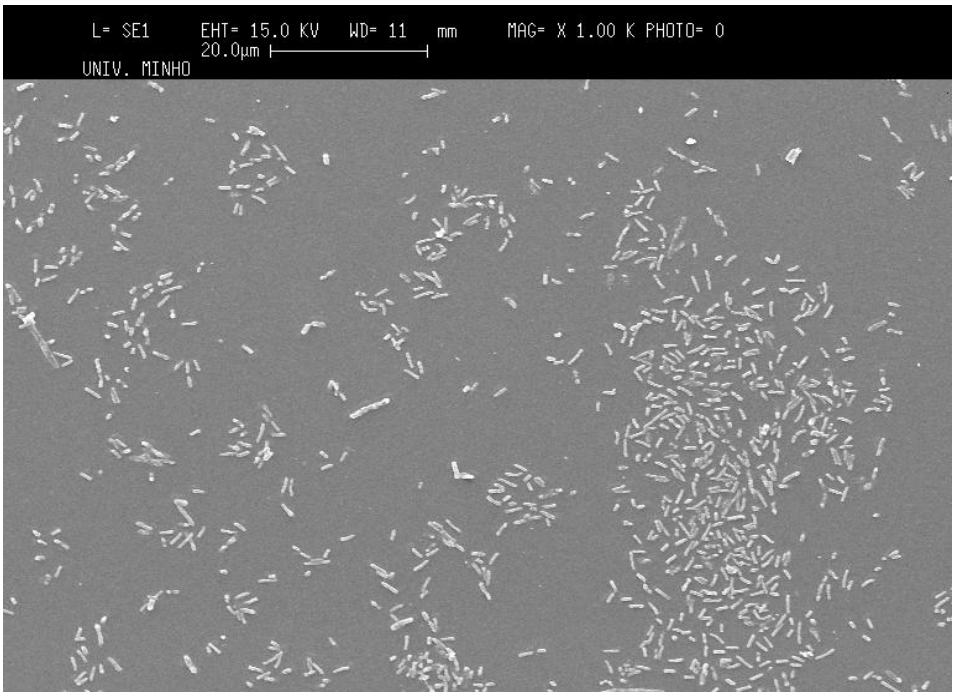


Name: P9WTACO1 (1000 X)

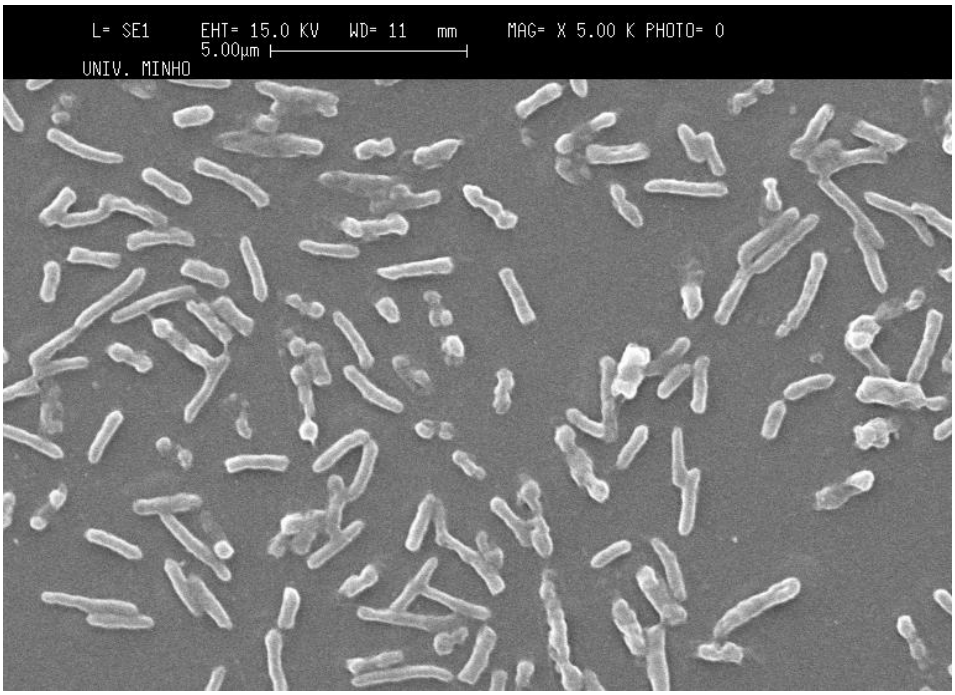


Name: P9WTACO2 (5000 X)

Figure 5.73: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 3 mM H_2O_2 stress on stainless steel.

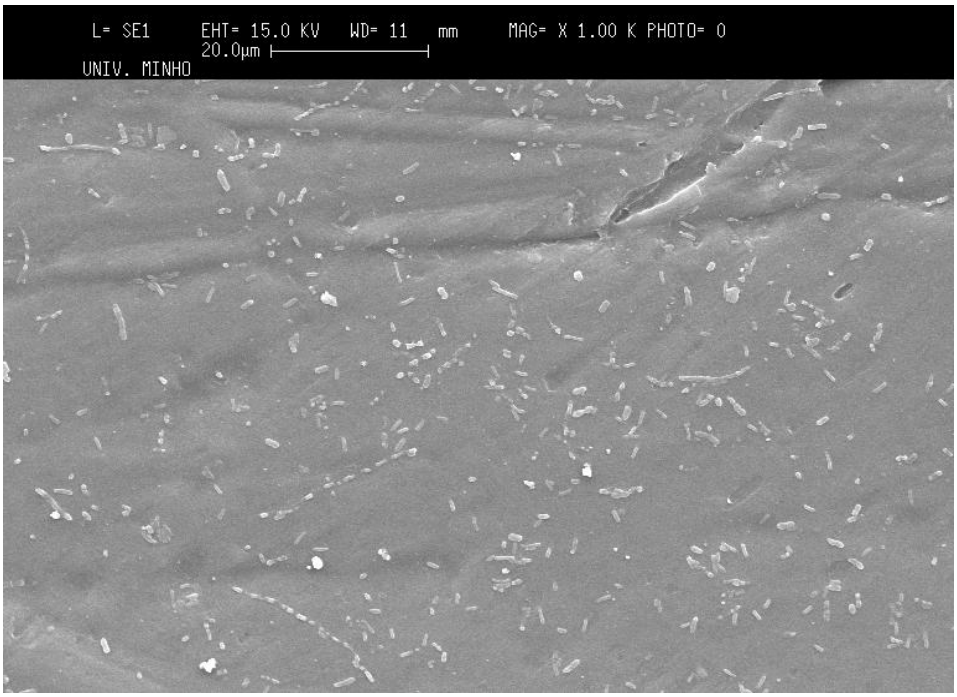


Name: P9WTPP1 (1000 X)

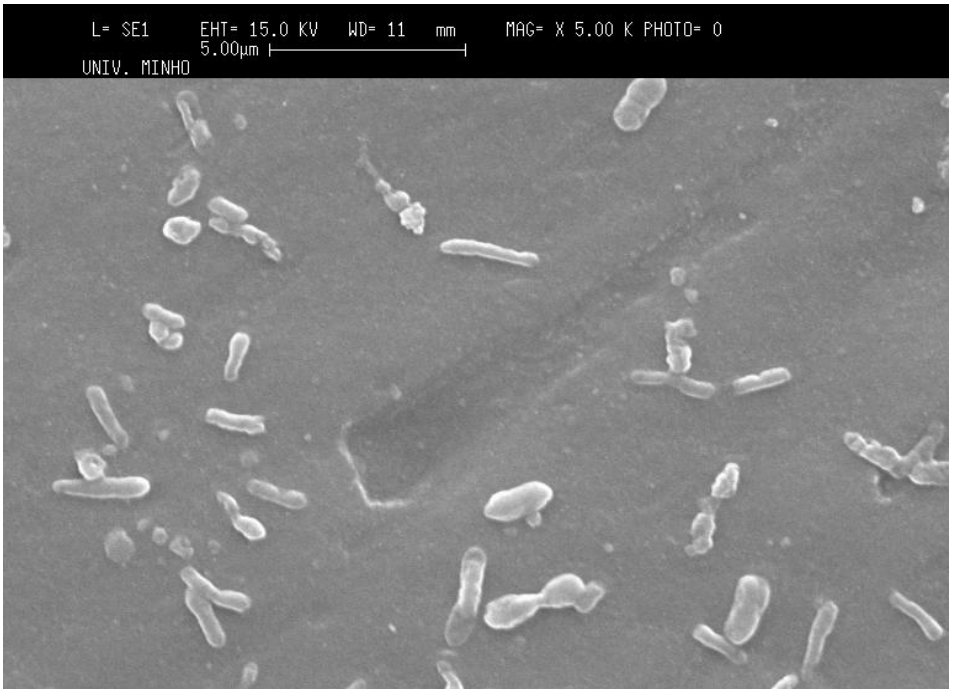


Name: P9WTPP2 (5000 X)

Figure 5.74: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 3 mM H_2O_2 stress on polypropylene.



Name: P9WTSI1 (1000 X)



Name: P9WTSI2 (5000 X)

Figure 5.75: Scanning electron micrograph of *E. coli* K-12 MG1655 (WT) after 72 hours at 3 mM H_2O_2 stress on silicon.

Scanning electronic microscopy allows the observation of bacteria/surface interaction and may be used as a semi-quantitative technique. It is almost impossible to quantify surface microorganisms since they may be clustered and cells may be arranged in overlapping layers. Generally, it was found that there were more cells attached on stainless steel to form biofilms, than on the silicon and polypropylene surfaces. This observation suggests the ability of the cells to adhere to stainless steel is greater than their ability to adhere silicon or polypropylene, indicating in our study that stainless steel is the optimal environment for most bacterial biofilms to attach and develop.

The extent of biofilm accretion on surfaces is controlled by the amount of nutrient available for cell replication and EPS production. A rich nutrient environment provides an ideal environment for bacteria to adhere, thus triggering biofilm formation through the secretion of EPS (Costerton 1995). Bacteria do not form biofilms where the nutrients are lacking. They will leave the environment and revert back to the free-swimming life style (Williams et al. 1995). Similarly, *E. coli* cells reach the highest population density when the nutrients in the environment are optimal. During the 3rd day, the reduced nutrient availability, diminished oxygen concentrations due to the crowded environment, and possible release of potentially damaging metabolic by-products, probably cause the drop in cell density. Bacteria need to re-locate from a biofilm when the nutrient supply is exhausted for a more favourable environment. At this time, polysaccharide lyase is secreted to facilitate the dispersal of cells, in order to reduce the population of cells in a biofilm (Allison et al. 1998).

SEM shows that, *E. coli* was mostly found on the surface of the samples under various stress environments. In many cases after 48 hours, the cell density increased and biofilm formation was seen, especially in cold shock condition. Again, the adhesion by *E. coli* cells to a surface is substrate specific. Results in this study revealed that *bolA* and *rpoS* play an important role in cell attachment and biofilm formation under various stress-induced conditions.

The biofilm bacteria reached a stationary growth phase after 24 to 48 hours. The development of a biofilm is caused by the formation of EPS to facilitate the attachment of bacterial cells to the surface. In addition to the facilitation of initial attachment of bacteria to the surface and the formation of robust biofilm architecture, EPS protects biofilms from exogenous effect of the environment (O'Toole et al. 2000). Indeed, the secretion of EPS in *E. coli* biofilms is also captured by SEM. EPS became crowded on day 2 during biofilm maturation, indicating the rigidity of a biofilm.

The scanning electron microscopic evidence provided by this study shows that attachment of *E. coli* on various substrates in various stress environments, i.e. heat, cold and oxidative stress, was varied and in many cases morphologically different. The differences were presumably related to the different selective conditions in these environments. In this study two of the possible important genes, *rpoS* and *bolA* were studied. The *rpoS* gene has been known as the alternative sigma (σ) factor, which controls the expression of a large number of genes, which are involved in responses to a varied number of stresses, as well as being transitional to the stationary phase from the exponential form of growth. Morphogene *bolA* response to environmental stress results in a round morphology of the *E. coli* cells, but nothing is known as yet,

about its involvement in the attachment of *E .coli* cells to various substrates and grows as a biofilms. Morphogene *bolA* was first reported to be involved in adaptation to the stationary growth phase by Santos et al. in 1999. However, its function is still not fully understood. Its expression might be induced by different forms of stress that result in the high-level expression of *bolA* mRNA which can lead to biofilm formation. This research demonstrates the capability of *rpoS* and *bolA* genes in cells of *E. coli* to adhere to various substrates under various stress-induced environments and grow as biofilms. SEM was found to be a powerful tool in successfully investigating the cell density and interaction of biofilms on various substrates in presence and absence of *rpoS* and *bolA* genes. Attachment pattern of *E. coli* on various substrates can be considered in various ways and might be used productively for industrial, environmental and medical purposes.

5.7 Key Findings

1. This study suggests that *E. coli* has better adhesion to the surface but attachment patterns vary under different stress conditions.

2. Most of the SEM images show an increase in cell density of *E. coli* during the first two days, followed by a decline in the population on the last day, especially under oxidative stress environment.

3. Stainless steel surface was found to be the appropriate surface for the cells to attach, multiply and grow as biofilms.

4. Polypropylene surface was found to be the inappropriate surface for the cells for attachment in any condition.

5. Importance of polypropylene surface, under cold shock conditions was seen. It was found that cells can only attach and grow as biofilms on polypropylene surface.

Chapter 6

General discussion and future work

This chapter presents the general discussion of this thesis and identifies future research to understand more fully the molecular mechanisms of biofilm formation.

The main purpose of this project was to investigate the importance and involvement of *rpoS* and *bolA* genes in biofilm formation and respiratory activity in response to general stress, using *E. coli* K-12 MG1655 as a model organism. In addition, possible differences in biofilm formation/adherence patterns on different substrates under various stress-induced environments, using a scanning electron microscope (SEM) as an objective and non-destructive imaging tool for the visualisation of the biofilms, were investigated.

6.1 Overview

Significant advances have been made in imaging studies of biofilms in the wider scientific community, however, the research into molecular mechanisms of biofilm formation, their exact role and importance in stress conditions is on the increase. A large number of protein molecules play an important role in the complex task of biofilm formation under stressed conditions in *E. coli*. The main function of the stress-sensing pathway is to trigger the transcription of stress-response genes. Various genes have been found to be essential in this process.

Many strains of *E. coli* K-12 MG1655 are known to be biofilm producers. In natural environments, the biofilm mode of life is considered to be the predominant mode of growth for bacteria, because the film and its related factors confer the benefit of potential protection for the cells against environmental stresses such as antibiotics, UV, chemicals, dessication, shear force, immunological attacks and other environmental variables(Hall-Stoodley et al. 2004;Sutherland 2001). Biofilm formation is a process which is believed to be mediated by density dependent cell-to-cell communication referred to as

quorum sensing (Spoering and Gilmore 2006). The evolution of the biofilm theory has quickly grown and been applied to many causes of persistent diseases such as cystic fibrosis, catheter induced urinary tract infections, chronic wound infections, burn wound infections, oral cavity formation, implant devices, and otitis media, among many others. Many studies appear to produce strong data suggesting biofilm formation as a potential leading problem in many industrial environments, especially the food processing industry (Harvey et al. 2007).

This study demonstrates the role of *rpoS* and *bolA* genes, their expression in various stress induced environments and resultant biofilm formation. Real-time RT-PCR was performed to analyze the mRNA levels of *rpoS* and *bolA* genes in response to different stress conditions, both in planktonic and sessile cells. Studies of planktonic cells provide an incomplete picture of induced stress conditions when used as models for biofilm formation. The behaviour of bacterial cells when facing lethal environments was significantly different when the cells were in suspension or when they were embedded in a biofilm. This study investigates these aspects.

During this work a number of interesting questions were addressed. The role of *rpoS* and *bolA* genes in biofilm formation under stress conditions was studied, as well as the attachment pattern of cells, with and without *rpoS* and *bolA* genes in various stress conditions on different surfaces. In addition to the estimation of mRNA levels of *rpoS* and *bolA* genes in planktonic and biofilm cells, another novel aspect of the project was the measurement of the respiratory activity of *E. coli* K-12 MG1655 mutant and wild type strains under various stress-induced conditions was measured. The environment changes appeared to influence biofilm structure

and activity; however this can affect the respiratory activity of *E. coli* under stress conditions.

6.1.1 Key role of *rpoS* and *bolA* genes in adaptation to unfavourable environment

The survival of a bacterium depends on its ability to adapt to intense, rapidly changing detrimental environmental conditions. Common environmental stresses encountered by bacteria are extreme temperature, low and high pH, oxidative stress and nutrient depletion. Growth under these non-optimal conditions requires the activation of stress responses. These consist of programmed cascades of cellular events that modulate protein synthesis and activity. The result of a stress response is stress resistance, elimination of the stress agent and/or repair of cell injury (Heinzel 1998; Vorob'eva 2004).

A stress response is triggered when initial sensing of a change in environmental conditions is transduced into a change in gene expression and/or activity. In numerous cases, an effective stress response requires amodification of the cellular transcriptional machinery. σ^S/*rpoS*/σ^{38} probably acts as a master regulator of the general stress response of *E. coli*. It is structurally similar to the primary sigma factor, σ^{70}. However, its expression and activity are regulated by a complex network of proteins and small RNAs. The result is a low level of σ^S during exponential growth and rapid accumulation in response to an array of environmental insults, including heat or cold shock, pH downshift or nutrient starvation. Induction of σ^S provides a kind of 'preventative medicine' for the cell in that activation of σ^S dependent genes results in broad stress resistance, allowing a cell to avoid damage from a number of potential stressors. It is not

surprising, then, that σ^S is directly or indirectly involved in regulating up to 10% of the *E. coli* genome (Becker et al. 1999; Jishage et al. 1996). Therefore, the main aim of the current project was to understand the possible role of σ^S and its dependent gene *bolA* in biofilm formation as a stress response under various stress environments. In poor growth conditions, *bolA* was considered to be important for normal cell morphology duringexponential growth, stationary phase and in response to sudden carbon starvation (Lange and Hengge-Aronis 1991). Under stationary phase of growth, *bolA* is shown to promote round cell morphology (Vieira et al. 2004a). This study showed that *bolA* is not only confined to stationary phase response, but its expression is also induced under various stress conditions and it plays a major role in biofilm formation as a result of stress.

6.2 Analysis of *rpoS* and *bolA* Genes Expression Patterns under Heat Shock (Absolute Quantification)

The change in the level of expression of the *rpoS* and *bolA* genes during the heat shock conditions wasan important aspect of the project. Heat shock response in *E. coli* is one of the safest methods to protect the cells from heat-induced damage by synthesizing specific proteins called heat shock proteins (HSPs), which are mediated by alternative sigma factor σ^{32} and is controlled by *rpoH*. It has previously been observed that σ^{32} level in *E. coli* remains low at 30 ºC but rapidly increases when the temperature shifts from 30 to 42 ºC and HSPs are rapidly induced 10–15 fold within 5 min of the temperature elevation. However, there was lack of data available on another alternative sigma factor called σ^S, which is mediated by *rpoS* and is considered to be the general stress

sigma factor. It is strongly induced when a cell is in stationary phase exposed to various stress conditions. The effect of heat shock is to induce the expression of σ^S as in σ^{32} within a short span of time. Temperature elevation has been investigated in this study. Similarly, *bolA* expression might be induced under heat shock environments which may lead to biofilm formation, but there was no experimental data available. Therefore, it was important to study the possible role of these two genes in a heat shock environment and to understand the mechanism of biofilm formation as a response to this stress. This study demonstrates that in response to heat stress (increase from 37 °C to 46 °C), the *rpoS* and *bolA* genes are over expressed when cells are grown in a complex medium before reaching stationary phase (Fig. 3.14-chapter 3 and 3.15-chapter 3). Differences in the expression of *rpoS* were seen, during a shift from 37 ºC to 46 ºC. A major challenge for all organisms is to maintain an appropriate intracellular environment to overcome environmental stress. This requires a vigorous and receptive stress response that is capable of responding suddenly to small changes in the environment. Until now it was believed that σ^{32}, coded by *rpoH* was responsible for the response under heat shock environment.

Therefore, in this work σ^S, encoded by *rpoS* was studied to explore the importance of other sigma factors in heat shock response of *E. coli* which may lead to biofilm formation. As *bolA* is known to be induced in the stationary phase and it is under control of *rpoS*, its expression was also measured to substantiate its involvement in heat shock response which in turn leads to biofilm formation. The comparison between wild type, *rpoS* and *bolA* mutant strains, showed that both genes contributed to the ability to respond and adapt to heat shock conditions. A difference in the induction of the *rpoS* gene has

been observed, as *rpoS* encodes the σ^S subunit of RNA polymerase and is responsible for induction of the stationary-phase proteins in general stress.

6.3 Analysis of *rpoS* and *bolA* Genes Expression Pattern under Cold Shock (Absolute Quantification)

Bacteria have developed mechanisms that permit growth at low temperatures. It has been observed that several genes with increased expression at 23 ºC are involved in biofilm development. For example, *bolA* has been shown as one of the important genes in biofilm formation in *E. coli* and itstranscription is induced in response to a variety of stresses. Low temperature shift from 37 to 23 ºC causes an increased expression of *rpoS* and *bolA* during the exponential phase. This project has shown that there was a sudden variation in expression of *rpoS* at 20 ºC and 5 ºC in planktonic cells (Fig. 3.17-chapter 3). Rapid induction in the expression level of *rpoS* was seen; as cells were shifted from 37 to 5 ºC. Sensing a sudden change in the growth temperature, *E. coli* start producing cold shock proteins to adapt to a given temperature. In a heat shock response, σ^{32} plays a major role in the induction of heat shock proteins. However, no such sigma factor has been identified in cold shock response. In order to investigate this, the importance of *rpoS* was studied under cold shock response and discussed.

Interesting results were seen when *rpoS* and *bolA* expression was measured under cold shock conditions. It was found that the presence of *rpoS* is an important factor in the response of *bolA* under the cold shock environment and that it cannot respond to the cold shock condition in absence of *rpoS*. In presence of *rpoS*, a sudden change in the expression of *bolA* was measured, in

the biofilm mode of growth, as a 3 to 3.5 fold increase after shifting the cells from 37 to 20 ºC and 5 ºC. The result suggests that the expression of *bolA* is under the transcriptional control of σ^S (encoded by *rpoS*). It was demonstrated that temperature has a spectacular effect on gene expression, which signifies that adaptation to low temperature requires a co-ordinated, multifunctional response.

6.4 Analysis of *rpoS* and *bolA* Genes Expression Pattern under Acid Stress (Absolute Quantification)

Acid resistance is another important property of *E. coli*, as it enables the organism to survive under conditions of lethal acid stress. *E. coli* has both exponential phase and stationary phase acid resistance pathways. At least three stationary phase acid resistance pathways are known (Garren et al. 1998). In this study results of pH shock appeared more robust. The response of *rpoS* and *bolA* within 15 minutes of stress at various pH was sudden in the biofilm phase compared with the planktonic form of growth (Fig. 3.20 and 3.21-chapter 3). Morphogene *bolA* is recognized for its expression in the stationary phase of growth, however, its expression in the biofilm mode at exponential level, in absence of the *rpoS* gene has not been reported previously (Vieira et al. 2004b). This indicates that it might play a role in biofilm formation under pH stress conditions because of its sudden response to different pH with a 10-15 fold increase in the expression of *bolA* gene under biofilm phase. This study also showed that acidic and alkaline conditions have a remarkable effect on expression of the *rpoS* and *bolA* genes, which indicates adaptation of *E. coli* to different pH values and may lead to biofilm formation as response to

environmental stress. The importance of *rpoS* and *bolA* genes and their involvement in biofilm production has been shown for the first time in this study, as the data on acid tolerance response (ATR) has only been available for a number of food-borne bacteria.

6.5 Analysis of *rpoS* and *bolA* Genes Expression Pattern under Oxidative Stress (Absolute Quantification)

Cross resistance mechanisms and resistance to hydrogen peroxide has been previously studied which report that the death of *E. coli* when exposed to high doses of H_2O_2, was mainly due to DNA damage. While some strains of *E. coli,* when exposed to low doses of H_2O_2, develop resistance to many other environmental stresses including heat, UV and ethanol (Asad et al. 1998; Rosner and Storz 1997). However, the role of the oxidative stress response which may lead to biofilm development is not yet defined, especially with regards to *rpoS* and *bolA* genes. A response to oxidative stress might lead to biofilm mode of growth and serve as a defence mechanism. This observation might provide some insights into the nature of H_2O_2-induced gene expression. Nevertheless, it is important to realizethat the oxidative stress produced by H_2O_2 results in the induction of a diverse set of physiological responses, which include some paradoxical effects. In this context, the mostunexpected phenomenon investigated so far, is the involvement of *rpoS* and *bolA* genes in oxidative stress and their involvement in biofilm formation under sudden shock. However, there was no data available on the expression analysis of *rpoS* and *bolA* gene in biofilm mode under various hydrogen peroxide concentrations.

It is evident that *rpoS* and *bolA* respond suddenly after exposure to oxidative stress conditions. Hydrogen peroxide probably has a lethal effect on *E. coli* at high concentrations. In this study low levels of hydrogen peroxide concentration were used to determine the efficient response of *rpoS* and *bolA* gene under oxidative stress. The results showed affirmative response of *rpoS* and *bolA* gene towards oxidative stress.

6.6 Analysis of *rpoS* and *bolA* Genes Expression Pattern (Relative Quantification)

Reverse transcription quantitative PCR (RT-qPCR) distinguishes itself from other methods available for gene expression in terms of accuracy, sensitivity, and fast results. Because of this, the technology has established itself as the gold standard for medium throughput gene expression analysis (Derveaux et al. 2010). In this study, both of the quantification methods (Absolute and Relative Quantification) have been used to produce reliable data. Accurate quantification by real-time RT-PCR relies on normalisation of the measured gene expression data. Reference genes that have been used until now are "housekeeping genes", which are involved in basic cellular processes, and they were supposed to have a uniform level of expression across different treatments (Remans et al. 2008). 16S ribosomal RNA has been used throughout this study as a reference gene. Many publications exist that describe the identification of multiple reference genes but similar reports are extremely rare in biofilm research. The mRNA expressions of the two genes examined had wide ranges, suggesting that quantitative Real-time RT-PCR is a highly sensitive method, with demonstrable ability to detect mRNA expression differences from less than 0.01-fold (Hu et

al. 2006). Despite the technical differences between the two approaches to study gene expression, the results from the methods are very similar.

A noticeable difference in gene expression of *rpoS* and *bolA* gene under various stress-induced environments in both the planktonic and biofilm phases was seen. In this study, the data are presented as the *N*-fold change in target gene expression under various stress-induced environments normalized to the internal control gene (16S rRNA). Results showed the *N*-fold change in the expression of both *rpoS* (Fig. 3.25-chapter 3) and *bolA* (Fig. 3.26-chapter 3) genes under heat shock temperatures (42 and 46 °C), cold shock temperatures (5 and 20 °C), pH stress levels (pH 5, 6, 8, and 9), and different concentrations of H_2O_2 (3, 4, and 5 mM). An interesting result was noticed in case of *bolA* where a 5-6 fold change in expression was seen under oxidative stress by planktonic cells; however, no sudden change in expression was noticed by *rpoS* under oxidative shock. The data indicate that gene expression within biofilm is different from that observed in standard planktonic cultures. pH change induces the expression of *rpoS* and *bolA* genes up to 5.5 fold in case of biofilms, which shows the importance of these two genes under pH variation. It hypothesizes that cells in biofilms were under stress and required the expression of *rpoS* and *bolA* as a sudden response to environmental change. These results are nearly the same as those obtained for absolute quantification. Only Ct value was generated using absolute quantification in order to obtain relative quantification.

6.7 Respiratory Activity of *E. coli* K-12 MG1655 under Various Stress-Induced Conditions

The complex architecture of the biofilm provides an opportunity for metabolic co-operation within this well-organised system. Consequently, it is important to understand the effect of stress on the metabolic activity of *E. coli* cells. The effects of a variety of stress-induced shocks including oxidative shock, high and low temperatures on respiration of *E. coli* in presence and absence of *rpoS* and *bolA* genes was investigated during this work (Chapter 4).

The respiratory activities of samples are evaluated by measuring oxygen uptake rates using a biological oxygen monitor (BOM). The results of this study have shown marked changes in the respiratory activity of *E. coli*. The data shows that, overall metabolic activity, i.e. the oxidation of glucose occurred at faster rate in presence of *bolA* and *rpoS* genes after the cells were shifted from optimal growth conditions to various stress-induced conditions. Study of metabolic specialization helps to explain the remarkable metabolic efficiency of cells and their resistance to external stress conditions and this study showed the response of *E. coli* to changes in their immediate environment. Bacterial respiratory activity has been used to measure the efficacy of antimicrobial agents (Simoes et al. 2003), but this technique has been used here for the first time to investigate the involvement of genes in respiration and metabolic activity of the cells under various environmental stress conditions. It is considered that respiratory activity, using oxygen consumption rate, represents a fast, consistent and easy methodology that can be used to evaluate respiratory activity of bacteria under stress. Overall, this study has lead to a better

understanding of the behaviour of cells in response to the external environment and the stress conditions under which the biofilms are formed, especially the importance of *bolA* gene than *rpoS* in respiratory activity of *E. coli*, under heat shock conditions (Fig. 4.3), where consumption of oxygen and oxidisation of glucose is recorded at a faster rate.

6.8 Scanning Electron Microscopic Analysis of *E. coli* Adherence Pattern on Various Substrates and Formation of Biofilms

Biofilms are complex microbial communities that grow at interfaces, often on various surfaces. They are highly robust and exhibit significant phenotypic changes(Costerton et al. 1987; Costerton 1995). Bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming (planktonic) state. Biofilm formation initiates with the attachment of cells on to surface, Once initial contact with a surface is accomplished, microbes develop different types of behaviour (Costerton et al. 1999;Poortinga et al. 2001). This study was undertaken to identify the surface where cells were unable to attach and grow into biofilms .and to study the role of *bolA* and *rpoS* genes in the biofilm forming process under various stress-induced conditions.

The major factors regulating attachment of bacteria onto, or detachment from surfaces are nutrient availability, the electrochemical properties of the surface, and liquid flow (Watnick and Kolter 1999). When nutrients are non-limiting in the liquid phase bacteria do not attach to surfaces. Stress situations such as a depletion of nutrients makes sessile growth more favourable inflowing liquids. Any stress factor at sub lethal level can initiate biofilm formation. For this

reason this study investigates the importance of *rpoS* and *bolA* genes in the attachment of *E. coli* on stainless steel, polypropylene and silicon under various stress conditions. Scanning electron microscopy has been used to provide detailed, scaled images on dehydrated biological specimens. Chemical fixation and dehydration under a high vacuum are needed to allow imaging and to preserve the cellular structures.

Although the result for patterns of attachment obtained for the surfaces and stress conditions vary in this study. Overall, they show that stainless steel is the most favourable surface for the attachment of *E. coli* under any of the stress environments studied. The importance of *bolA* was seen in many cases, where cells couldn't attach to the surface in its absence. Morphological changes in cells were also seen in presence of *bolA* after 48 hours and 72 hours of growth (Fig. 5.21 and 5.35-chapter 5). Increase in cell density and thickness of biofilms was seen after 48 hours of growth on silicon and stainless steel surfaces in presence of *bolA*. Polypropylene surfaces were not found to be the best for the cells to attach and grow as biofilms, except under cold shock conditions. Under low temperature, cells failed to produce thick biofilms on stainless steel and silicon surfaces, while polypropylene was found to produce the best biofilms under cold shock environment. After two days, SEM images show an increase in cell density of *E. coli*, followed by a decline in the population, especially under oxidative stress environment. SEM indicates that *E. coli* are mostly found on the surface under various stress environments and suggests that *E. coli* has a better adhesion to the surface but the attachment patterns vary with time to stress conditions.

6.9 Perspectives for Further Research

The consequence of biofilms is not a well understood phenomenon because of a lack of research using model systems that closely simulate the environmental system and lack direct observation of biofilms in their environment. Most investigations involving biofilms have been performed using *in vitro* laboratory models. However, all these *in vitro* models of biofilms fail to identify the contribution of important individual parameters. One of the important aspects in the study of multifaceted biological systems seems to be the development of accurate and realistic models of natural communities in the laboratory. With this in mind the identification of bacteria forming natural biofilms seems to have greatest potential. Subsequently, the assessment of the potential of the bacteria for biofilm formation of single and mixed species and the evaluation of the interspecies interactions will possibly provide new information in order to understand the phenomenon behind biofilm recalcitrance and will provide new mechanisms for biofilm self-regulation. So, the intense scrutiny of these interactions provides one of the future challenges in biofilm research/control.

The environmental changes appear to influence the biofilm structure and activity, where this complex biofilm architecture obviously provides an opportunity for metabolic cooperation and niches are formed within this spatial well-organized system. Consequently, an understanding of the structure-function relationships in microbial biofilms seems to be fundamental to interpret and predict biofilm impacts on the habitat where they are developed.

Since the altered phenotype is believed to be responsible for the distinct properties of bacteria including the round morphology in cells of *E. coli* under

stress conditions, growing in biofilms, and since it has also been suggested that it is related to the enhanced resistance of biofilm cells to external environments, it follows that it will be important in explaining the adaptive mechanisms involved in the bacterial resistance to general as well as other forms of stress. Another important fact is to identify the essential proteins involved in the enhanced resistance of cells within biofilms to antimicrobial agents. The analysis of the microbial resistance to antimicrobial agents will be assessed in the future using proteomic techniques, by identifying the target proteins that are necessary to the biofilm state, *i.e.*, proteins that are common to the biofilm state among different adherent species and the inhibition of the synthesis of which would prevent biofilm formation or accelerate biofilm eradication.

The current work indicates that further investigation into the behaviour of the *rpoS* and other stationary phase induced genes like morphogene *bolA* is required. The next logical step in this research would be to study the difference in expression of *rpoS* and *bolA* at various time intervals in a combination of stress induced environments. Another possible step could be a practical study that could be conducted on the $rpoS^{+}/rpoS^{-}$ and $bolA^{+}/bolA^{-}$ strains to assess their effects on the biofilm architecture on various substrates/surfaces. This study has confirmed the relationship between *rpoS* and *bolA* activation at various stress levels, however, the study does not establish the role of *rpoS* and *bolA* under combination of stress induced environments at various time intervals. Future work should consider this by exploring the *rpoS* and *bolA* regulation in order to fully understand the physiological role of these two genes.

6.10 Conclusion

Research into microbial biofilms is ongoing with special emphasis on the identification of the genes which are specifically expressed under biofilm conditions in stress-induced environments. In summary, biofilm formation is a mechanism by which bacteria protect themselves against environmental stresses. It involves different forces which stimulate bacteria to change from a planktonic phase to a biofilm phase which in turn is beneficial to the bacteria. The stress response of *E. coli* is complex and its adaptation to the stress environments needs to be addressed in more detail. More research is required on the combined and individual roles of these two genes in biofilm formation. The key to success will be, to understand what makes biofilm bacteria so different from planktonic bacteria.

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Appendix 1

Microbiological Media and Reagents

1. Luria-Bertani Agar

10 g Tryptone

5 g Yeast extract

5 g sodium chloride and

10 g agar

1 litre distilled water

Adjust pH to 7.4 with 1 N NaOH

Autoclave; aliquot using aseptic technique

2. MOPS buffer (10x)- 3- [N- morpholino]propanesulfonic acid

200 mM MOPS, pH 7.0

50 mM sodium acetate

10 mM Na_2-EDTA, pH 8.0

3. NaOH (10N)

Add 40 g NaOH pellets to 60 ml water. Increase volume to 100 ml.

4. TAE (50x) - Tris-Acetate-EDTA

Per litre: 242 g Tris base

100 ml 0.5 M Na_2-EDTA, pH 8.0

57.1 ml glacial acetic acid

Autoclave; working concentration is 1x TAE.

5. TE buffer

10 mM Tris, pH 7.5

1 mM EDTA

Autoclave; store at room temperature

Adjust pH to 8.0 for DNA storage.

6. **DEPC Water (Diethyl pyrocarbonate- treated water)**

   Add DEPC to laboratory grade (deionised/distilled) water to a final concentration of 0.05 to 0.1% (v/v). Mix thoroughly or stir rapidly on a magnetic stirrer for 30 min or more. Autoclave to destroy DEPC.

7. **EDTA-Na_2 (500 mM)- Ethylene-di-amine tetra acetic acid**

   Adjust to final pH of 8.0 (will not dissolve below pH 7.5) with 10 N NaOH or NaOH pellets. Autoclave; store at room temperature.

Preparation of 1 x TE buffer: From a 100 x stock solution, 1 ml of 1 x TE was prepared by pipetting 10 µl of 100 x TE with 990 µl of distilled water.

Preparation of 1 x TAE buffer: For the preparation of 1 L of 1 x TAE buffer from 50 x TAE, 20 ml of 50 x TAE were added to 980 ml of distilled water.

Preparation of Agarose Gel (AGE): For the preparation of 25 ml of 1% agarose gel, 0.25 g of agarose were added to 25 ml of 1 x TAE buffer and boiled until all solid agarose dissolved. Solution was then allowed to cool slightly, poured in the casting tray and allowed to settle.

Appendix 2

List of *rpoS*-regulated genes in Luria-Bertani media with their functions

***RpoS*-dependent Genes**	**Functions**	**Major regulators**
aidB	Isovaleryl CoA dehydrogenase	*RpoS, Ada, Lrp*
bfr	Bacterioferritin	*RhyB*
cbpA	Co-chaperone of DnaK	*RpoS*
csgF	Curli production assembly	*CRP, RpoS, CsgD, CpxR OmpR, RstA*
dnaKJ	Chaperone Hsp70	*RpoH*
dppAC	Dipeptide transporter	*Fnr, IHF*
dps	Stationary phase DNA protein	*OxyR, IHF, RpoS*
fbaB	Fructose-bisphosphate aldolase class I	*RpoS*
fhuF	Ferric reductase	*Fur*
fic	Stationary-phase protein, folate biosynthesis	*RpoS*
fimAC	Type I fimbriae	*IHF, Lrp, H-NS*
flgAMN	Flagellar biosynthesis	*FlhDC*
flgBCDEFGHIJ	Flagellar biosynthesis	*FlhDC*
fliAZ	Flagellar biosynthesis	*FlhDC, ArcA, H-NS*

fliD	Flagellar biosynthesis	*FlhDC*
fliLMN	Flagellar biosynthesis	*FlhDC*
flxA	Flagellar motility	*FliA*
gadAX	Glutamate dependent acid resistance	*RpoS, Fis, GadEXW CRPHNSTorR, Fnr*
gadBC	Glutamate dependent acid resistance	*GadX, GadE, GadW, Fis, CRP, RpoS*
gadE	Glutamate dependent acid resistance	*RpoS, GadEWX, CRPEvgA*
gadXW	Glutamate dependent acid resistance	*RpoS, GadEW, RutR, Fnr, H-NS*
hchA	Hsp31 molecular chaperone	*H-NS*
hdeAB	Acid-resistance protein	*RpoS, H-NS, GadEX, Lrp TorR, MarA*
hdeD	Acid-resistance membrane protein	*GadEX, H-NS*
hyaAB	Hydrogenase 1	*RpoH*
mopA	Heat shock chaperone GroEL Hsp60	*CpxR, FliA*
motAB-cheAW	Chemotaxis	*RpoS*

msyB	Suppresses heat sensitivity of secY mutants	*CRP, OmpR, IHF*
nmpC	Outer membrane porin protein	*RpoH, Fur, NrdR*
nrdHI	Ribonucleotide reduction	*CpxR, OmpR, IHF, CRP, RstA, Lrp, EnvY, Fur*
ompF	Outer membrane protein F precursor	*H-NS, Lrp, NhaR, RcsB, RpoS*
osmC	Stress-inducible membrane protein	*RpoS, Fis, CRP, IHF, Lrp*
osmY	Stress-inducible periplasmic protein	*RpoS*
otsB	Trehalose-phosphatase	
rpsV	30S ribosomal protein S22	*ArcA, CRP, Fur, Fnr*
sdhCDAB	Succinate dehydrogenase	*MarA, GadXW, RpoS*
slp	Resistance to metabolic end products	*OxyR, IscR, IHF, Fur*
sufABDS	Iron–sulfur cluster assembly	*RpoS*
talA	Transaldolase A	*Fnr, FliA*
tar-tap	Chemotaxis	*RpoS*

tktB	Transketolase 2	*CpxR*, *RpoF*
tsr	Chemotaxis	*RpoS*
wrbA	NAD(P)H:quinone oxidoreductase	*GadX*
ybaS	Glutaminase	*RpoS*
yeaG	Stationary-phase-induced protein kinase	

Appendix 3

Location and Gene Card for *rpoS* and *bolA*

Appendix 3.1

Location and Gene Card for *rpoS*

GI Number	16130648
Gene Position	2864581-2865573
Gene Name	*rpoS*
GC Content [%]	52.06
Preceding Gene	*ygbN*
Following Gene	*nlpD*

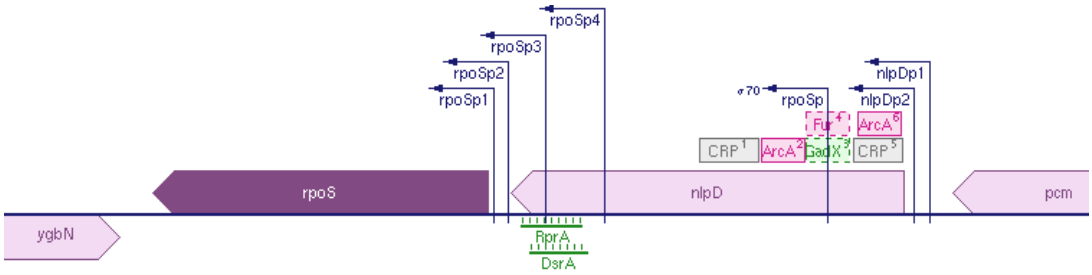

Figure A3.1: Map position of *rpoS* with its neighbouring genes

Upstream >100 bases

AATCCGTAAACCCGCTGCGTTATTTGCCGCAGCGATAAATCGGCGGAA
CCAGGCTTTTGCTTGAATGTTCCGTCAAGGGATCACGGGTAGGAGCCA
CCTT

Gene Sequence >993 bases

ATGAGTCAGAATACGCTGAAAGTTCATGATTTAAATGAAGATGCGGA

ATTTGATGAGAACGGAGTTGAGGTTTTTGACGAAAAGGCCTTAGTAGA

ACAGGAACCCAGTGATAACGATTTGGCCGAAGAGGAACTGTTATCGC

AGGGAGCCACACAGCGTGTGTTGGACGCGACTCAGCTTTACCTTGGTG

AGATTGGTTATTCACCACTGTTAACGGCCGAAGAAGAAGTTTATTTTG

CGCGTCGCGCACTGCGTGGAGATGTCGCCTCTCGCCGCCGGATGATCG

AGAGTAACTTGCGTCTGGTGGTAAAAATTGCCCGCCGTTATGGCAATC

GTGGTCTGGCGTTGCTGGACCTTATCGAAGAGGGCAACCTGGGGCTGA

TCCGCGCGGTAGAGAAGTTTGACCCGGAACGTGGTTTCCGCTTCTCAA

CATACGCAACCTGGTGGATTCGCCAGACGATTGAACGGGCGATTATGA

ACCAAACCCGTACTATTCGTTTGCCGATTCACATCGTAAAGGAGCTGA

ACGTTTACCTGCGAACCGCACGTGAGTTGTCCCATAAGCTGGACCATG

AACCAAGTGCGGAAGAGATCGCAGAGCAACTGGATAAGCCAGTTGAT

GACGTCAGCCGTATGCTTCGTCTTAACGAGCGCATTACCTCGGTAGAC

ACCCCGCTGGGTGGTGATTCCGAAAAAGCGTTGCTGGACATCCTGGCC

GATGAAAAAGAGAACGGTCCGGAAGATACCACGCAAGATGACGATAT

GAAGCAGAGCATCGTCAAATGGCTGTTCGAGCTGAACGCCAAACAGC

GTGAAGTGCTGGCACGTCGATTCGGTTTGCTGGGGTACGAAGCGGCAA

CACTGGAAGATGTAGGTCGTGAAATTGGCCTCACCCGTGAACGTGTTC

GCCAGATTCAGGTTGAAGGCCTGCGCCGTTTGCGCGAAATCCTGCAAA

CGCAGGGGCTGAATATCGAAGCGCTGTTCCGCGAGTAA

Appendix 3.2

Location and Gene Card for *bolA*

GI Number	49176023
Gene Position	453663-454013
Gene Name	*bolA*
GC Content [%]	48.15
Preceding Gene	*yajG*
Following Gene	*tig*

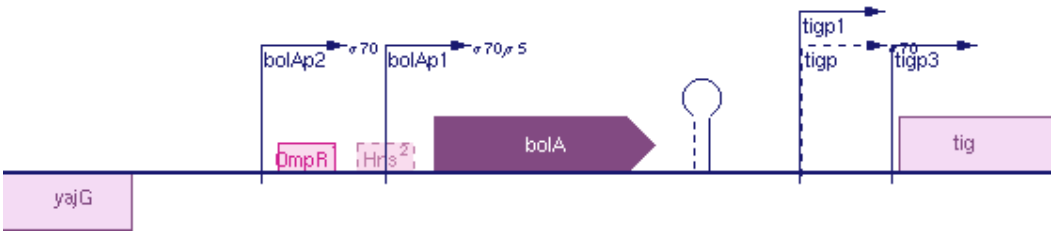

Figure A3.2: Map position of *bolA* with its neighbouring genes

Upstream >100 bases

TAAACTTCATACGCTTGACGGAAAAACCAGGACGAAACCTAAATATTT

GTTGTTAAGCTGCAATGGAAACGGTAAAAGCGGCTAGTATTTAAAGG

GATGG

Gene Sequence >351 bases

ATGACATCTCAGCGTTGTCGGAGGAGATATTTCATGATGATACGTGAG

CGGATAGAAGAAAAATTAAGGGCGGCGTTCCAACCCGTATTCCTCGA

AGTAGTGGATGAAAGCTATCGTCACAATGTCCCAGCCGGCTCTGAAAG

CCATTTTAAAGTTGTGCTGGTCAGCGATCGTTTTACGGGTGAACGTTTT

CTGAATCGTCATCGAATGATTTACAGTACTTTAGCGGAGGAACTCTCT

ACTACCGTTCATGCGCTGGCTCTGCATACTTACACTATTAAGGAGTGG

GAAGGGTTGCAGGACACCGTCTTTGCCTCTCCTCCCTGTCGTGGAGCA

GGAAGCATCGCGTAA

Appendix 4

Publications arising from this work

Mol Cell Biochem (2010) 342:207–213
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Contribution of *rpoS* and *bolA* genes in biofilm formation in *Escherichia coli* K-12 MG1655

Mohd Adnan · Glyn Morton · Jaipaul Singh · Sibte Hadi

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Abstract Flexibility of gene expression in bacteria permits its survival in varied environments. The genetic adaptation of bacteria through systematized gene expression is not only important, but also clinically relevant in their ability to grow biofilms in stress environments. Stress responses enable their survival under more severe conditions, enhanced resistance and/or virulence. In *Escherichia coli* (*E. coli*), two of the possible important genes for biofilm growth are *rpoS* and *bolA* gene. *RpoS* is also called as a master regulator of general stress response. Even though many studies have revealed the importance of *rpoS* in planktonic cells, little is known about the functions of *rpoS* in biofilms. In contrast, *bolA* which is a morphogene in *E. coli* is overexpressed under stressed environments resulting in round morphology. The hypothesis is that *bolA* could be implicated in biofilm development. This study reviewed the literature with the aim of understanding the stress tolerance response of *E. coli* in relation with *rpoS* and *bolA* genes in different environmental conditions including heat shock, cold shock, and stress in response to oxidation, acidic condition and in presence of cadmium. Knowledge of the genetic regulation of biofilm formation may lead to the understanding of the factors that drive the bacteria to switch to the biofilm mode of growth.

Keywords *E. coli* · Biofilm · Stress environment · *rpoS* · *bolA*

M. Adnan (✉) · G. Morton · J. Singh · S. Hadi
School of Forensic and Investigative Sciences, University of Central Lancashire, Maudland Building, Preston PR1 2HE, Lancashire, England, UK
e-mail: madnan@uclan.ac.uk

Introduction

Microorganisms were initially characterized as freely suspended cells and described on the basis of their morphology and growth characteristics [1]. Scientists have recently realized that more than 99% of all bacteria exist as biofilms which are defined as a collection of microorganisms that are irreversibly attached to a surface and enclosed in an extracellular matrix allowing growth and survival in sessile environment [1]. This is due to the fact the biofilm organisms can transcribe genes, whereas planktonic organisms cannot [2]. Current research is focused in understanding the genomics of biofilm formation through gene expression profiling which is slowly becoming available in the literature.

Significance of biofilms

Biofilms are omnipresent; almost every material that comes into contact with naturally occurring fluids is susceptible to their form of bacterial colonization. Environmental microbiologists have long known that composite bacterial communities are responsible for driving the biogeochemical cycle that maintains the biosphere [3]. Industrial pipelines, nuclear power stations, air conditions systems, water distribution systems and the hospital are all vulnerable to colonization by microorganisms mounting in biofilms [4]. The majority of them persist attached to surface within an arrangement and not as free-floating organisms [5]. Biofilms encompass around single or multiple microbial species and can form on an array of surfaces. Although mixed-species biofilms prevail in most environments, single species biofilms exist in a variety of infections and on the surface of medical implants [6].

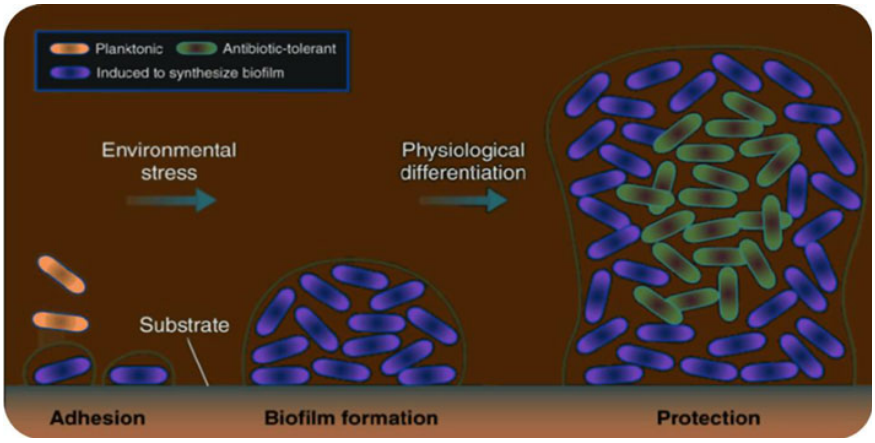

Fig. 1 Environmental stress induces the expression of *bolA* and *rpoS*, which results in physiological differentiation and biofilm formation [40]

Bacteria seem to instigate biofilm development in response to specific environmental cues (Fig. 1). Although these surroundings vary widely, the Gram-negative organisms with some exclusion undergo a shift from free living planktonic cells to sessile form of growth in response to a nutrient-rich medium. These biofilm continues to extend as long as fresh nutrients are provided, but when they are nutrient deprived, they detach from the surface and return to planktonic mode of growth [7]. This starvation response allows the cells to search for a fresh source of nutrients, and this adaptation when nutrient become inadequate is well studied. It is noteworthy that most microorganisms seem able to make the transition to life on a surface, irrespective of their physiological capabilities [5].

Formation of three dimensional (3D) structures inherent within biofilms is a dynamic process and involves a coordinated series of molecular events that includes mechanisms for adhesion, aggregation and community expansion [8]. The developmental steps in biofilm formation include

1. Initial attachment to a surface;
2. Formation of microcolonies;
3. Maturation of microcolonies to mature biofilms;
4. Detachment and return to the planktonic growth mode

Conversion of bacterial cells from planktonic to biofilm form involves a highly complex regulatory process which affects the expression of diverse group of genes. For example, changes in the expression levels of about 38% of *E. coli* genes were observed during the transition from planktonic to biofilm form [9]. Gene expression in *E. coli* biofilms show change in more than 600 genes which comprise around 9% of the whole genome being activated and 4.5% repressed in the biofilm cells [10].

Under normal conditions, microorganisms have their own signal transduction systems to sense the environmental stresses and to control their coordinated expression of genes which are involved in their cellular defence mechanisms [11]. A common regulatory mechanism, which involves sigma factors like σ^{70}, is responsible for transcription from various gene promoters under non-stress conditions. However, under stress conditions alternative σ factors can be induced, and these sigma factors are small proteins that bind to the RNA polymerase.

Under stress conditions, various sigma factors work differently, resulting in the expression of specialty regulons (Table 1), a system in which either two or more structural genes are subject to coordinated function by a common regulator molecule in response to various stresses [10]. By this way, gene expression is altered by different sigma factors. Specifically in *E. coli* and other enteric bacteria, σ^{s} (*rpoS*) is known to be the master regulator of the general stress response [10, 12].

Under normal conditions, the level of *rpoS* in the cell is low because the mRNA of *rpoS* forms a stable secondary structure that results in poor translation under normal conditions. Sigma factors (e.g. *rpoS* Protein) also get degraded continuously in normal conditions by the ClpXP protease in *E. coli* (Fig. 2). As a result, non-stressed cells contain low level of σ^{S} than σ^{70} [13, 14]. Furthermore, the expression of *rpoS* is induced while the cells enter into either stationary phase or any other general stress condition like heat shock, cold, acid, cadmium etc.

Morphogene *BolA*

Stress response genes are induced whenever a cell needs to adapt and survive under unfavourable growth conditions—morphogene *bolA* in *E. coli* is one of the examples of those genes [15]. It was foremost described to be implicated in adaptation to stationary form of growth [16, 17]. However, its function is still not completely understood and is not

Table 1 *E. coli* sigma factors with their genes and functions are listed below [39]

Sigma factors	Gene	Functions
σ^{70}	*rpoD*	Housekeeping functions
$\sigma^{54}(\sigma^{N})$	*glnF*, *nrtA*, *rpoN*	Nitrogen-regulated genes
σ^{32}	*htpT*, *rpoH*	Heat-shock genes
$\sigma^{24}(\sigma^{E})$	*rpoE*	Heat-shock genes
σ^{28}	*flbB+flaI*, *rpoF*	Flagella synthesis/chemotaxis
$\sigma^{38}(\sigma^{S})$	*rpoS*, *katF*	Starvation/general stress response

only confined to stationary phase, but its expression might be also induced by different forms of stresses such as heat shock, acidic stress, cold shock etc., which results in high level of expression of *bolA* mRNA and may lead to the formation of biofilms [16, 17]. It also has a major effect on the bacterial envelope and, therefore, is probably involved in cellular protection under adverse growth conditions [18]. *BolA* gene was also shown to regulate the transcript levels of D,D-carboxypeptidases PBP5 (encoded by dacA gene), PBP6 (dacC), and β-lactamase AmpC (ampC), all of which are involved in murein metabolism [17, 18].

In order to survive within stressed environments, over-expression of *bolA* leads to the round morphology to render the cell shorter and rounder, causing a decrease in surface to volume ratio and a reduction in the surface area exposed to the damaging or unfavourable environments, which may further leads in the development of biofilms (Fig. 3). *BolA* seems to be drawn in switching between cell elongation and septation systems during the cell division cycle. Normally, the expression of *bolA* is growth rate regulated, and induced during the transition into stationary phase from exponential phase [15].

Expression of *bolA* is governed by two promoters, P1 and P2. Main promoter P1 is proximal to the structural gene, and is a gearbox promoter under the control of σ^{S}. P2 is located further upstream from the structural gene, is under the control of σ^{D} and transcribes *bolA* constitutively. The high level of expression of *bolA* mRNA is mainly due to the specific transcription of the *bolA*1p promoter by the σ^{s} factor. Increased expression and morphological changes due to sudden carbon starvation and osmotic shock still occurs when σ^{S} is not present, which shows that expression of *bolA* is not confined to stationary phase, but it can also play an important role in general stress response [15, 17, 18]. This minireview is related to *E. coli* genetic responses to different types of stress conditions with particular reference to *rpoS* and *bolA* genes.

Biofilm bacteria can be 1,000-fold more resistant to antibiotic treatment than planktonic bacteria, but the mechanism by which the biofilm bacteria attain this

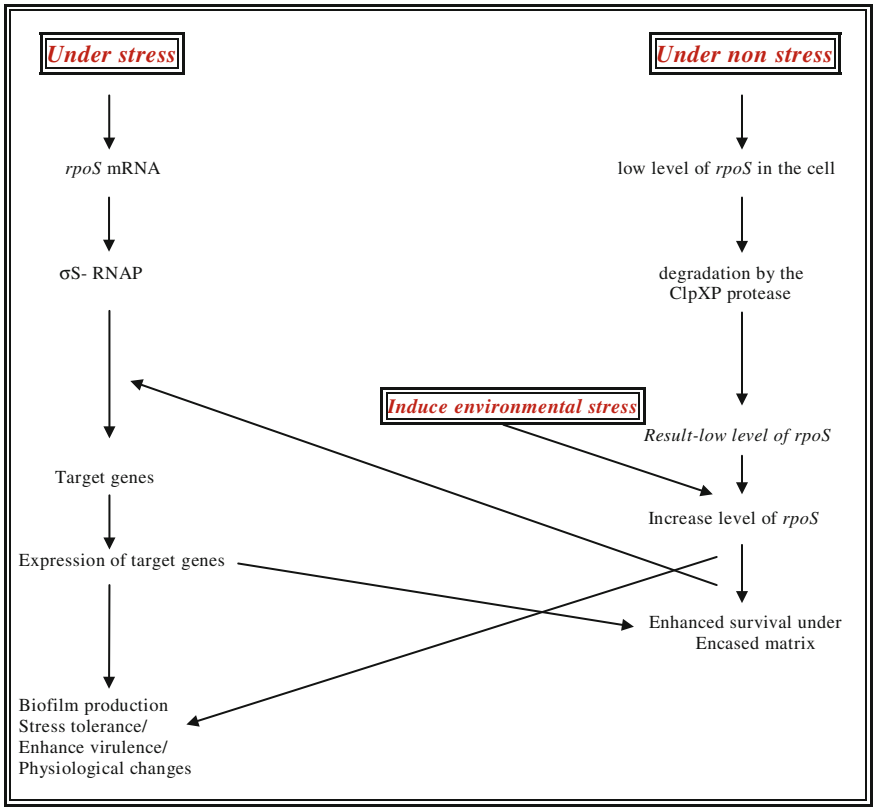

Fig. 2 Schematic model of stress and non-stress responses (*rpoS* response) of *E. coli* under different environmental conditions. Note that, in stress and non-stress conditions, induction of external environmental parameters results in expression of target genes, which lead to biofilm production, enhance virulence and other physiological changes

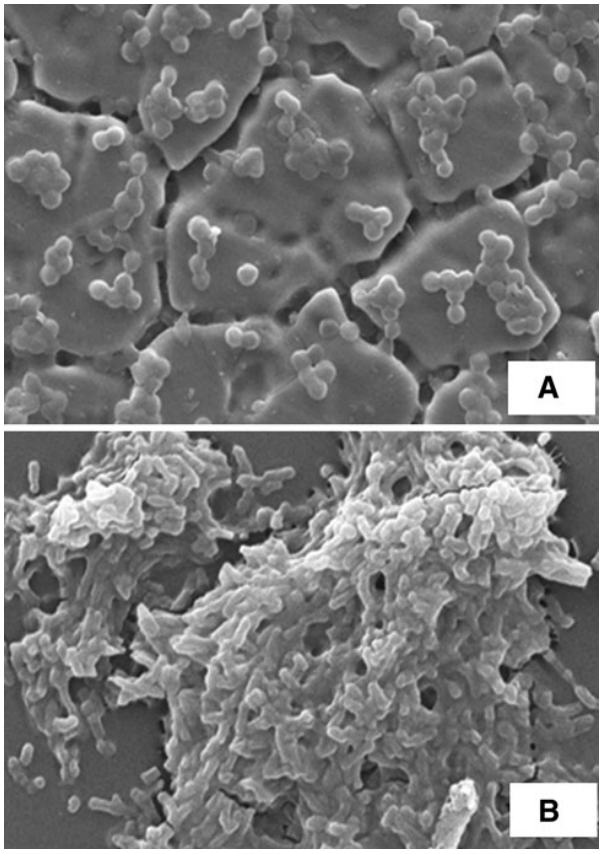

Fig. 3 Morphological changes (round morphology) in *E. coli* at stationary phase of growth in the presence of *bolA* (**a**). Biofilm formation by *E. coli* K-12 MG1655 after 72 h on stainless steel coupon in nutrient deprivation state (**b**)

resistance is still a matter of speculation. There are multiple mechanisms by which microorganisms develop resistance (Fig. 4). These include:

1. Phenotypic changes in bacteria resulting in resistance occurring within the biofilm environment.
2. Inactivation of the antibiotics by extracellular polymers or modifying enzymes and
3. Nutrient limitation resulting in slowed growth rate etc.

Heat shock

Heat shock response in *E. coli* is one of the safest methods to protect themselves from heat-induced damage by synthesizing specific proteins called heat shock proteins (HSPs) which are mediated by alternative sigma factor σ^{32} [19]. It has previously been observed that σ^{32} level in *E. coli* remains low at 30°C but rapidly increases when the temperature shifts from 30 to 42°C and HSPs are rapidly induced 10–15 folds within 5 min of the temperature elevation. In comparison to σ^{32} in *E. coli*, σ^{24} (σ^{E}) regulons

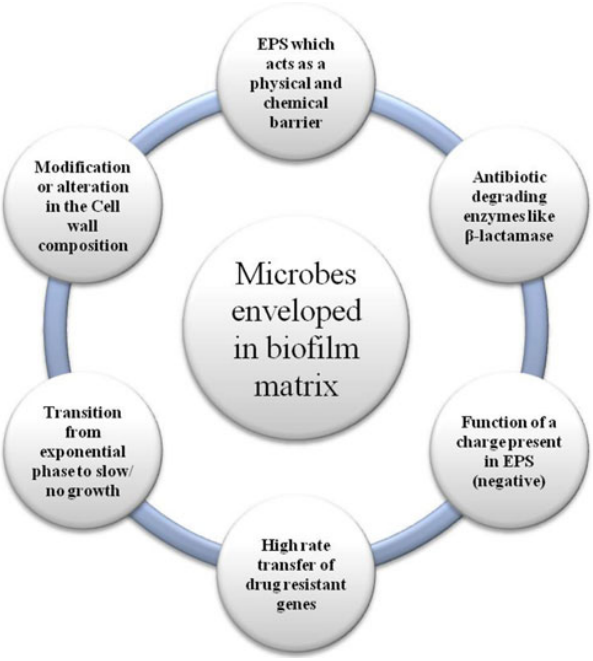

Fig. 4 Diagrammatic representation showing the different ways in which biofilm bacteria develop antimicrobial resistance

also play a key role in protecting cells against severe damage. σ^{32} regulon is confined with cytoplasmic protein damage while σ^{E} protects cells against extracytoplasmic (periplasmic) stress. Under periplasmic stress, synthesis of most proteins stops due to inactivation of σ^{70}, and synthesis of HSPss increased [20]. Another heat shock regulon in *E. coli* is controlled by σ^{54} (σ^{N}) which is also an alternative sigma factor that requires an activator for transcriptional activation [21]. The σ^{S} or *rpoS* subunit is considered the general stress sigma factor. It is strongly induced when a cell is in stationary phase or in exponential phase exposed to various stress conditions, including: ultra-violet radiation, hyperosmolarity, pH downshift, and non-optimal high or low temperature regimes [22]. The σ^{S} increase is often accompanied by a reduction or cessation of growth and provides cells with the ability to survive the actual stress as well as additional stresses not yet encountered, ('cross-protection') [23]. The dual nature of the σ^{S} stress response is opposite to other specific stress responses, which are triggered by a single stress signal and result in the induction of proteins that allow cells to overcome only a specific stress.

The *rpoS* gene occurs in the γ branch of the proteobacteria, a group of Gram-negative bacteria that includes many human pathogen species and also some beneficial organisms. With minor variations, the general function of σ^{S} in these bacteria appears to be similar to that in *E. coli* [24]. The synthesis and accumulation of σ^{S} are controlled

at multiple levels, including transcription, translation, proteolysis and activity. *BolA* expression might be induced under heat shock environments which may lead to biofilm formation, but there is no experimental data available. Although it is a complex process involving many steps, transcription initiation through promoter clearance and release from the RNA polymerase is the most important control point in determining whether or not most genes are expressed.

Cold shock

Temperature change serves as a good signal to regulate gene expression in *E. coli* and other bacteria. *E. coli* are likely to encounter shifts to lower temperature either for short-term or long-term gain during their life cycle [25]. It is particularly important to understand how *E. coli* are able to adapt to low temperatures in all industrial settings where prevention of bacterial contamination is utmost important. Low temperature shift from 37 to 23°C causes an increased expression of *rpoS* and *bolA* during the exponential phase [25]. As *bolA* is considered to be an *rpoS*-dependent gene [16, 17], it is expected to be induced at low temperature in exponential phase. Many bacteria synthesize increased amount of small proteins known as cold shock proteins (CSPs) when shifted to cold temperature. This is an area which has been extensively studied [26]. Generally, there are 15 different types of CSPs which have been induced in *E. coli* and they are all involved in essential functions like transcription, translation, protein synthesis, mRNA degradation and recombination in *E. coli* [27, 28]. However, specific sigma factor has not been specifically identified in this case of cold shock response unlike heat shock response [29].

It has been noticed that several genes with increased expression at 23°C are involved in biofilm development [25]. For example, *bolA* has been shown as one of the important genes in biofilm formation in *E. coli* and its transcription is induced in response to a variety of stresses. One study revealed that low temperature increases the expression of *bolA* 3.5 folds [25]. These data suggest the importance of low temperature which increases the expression of important biofilm genes.

Oxidative stress (exposure to hydrogen peroxide)

Oxidative stress is caused by increased levels of superoxide anion and H_2O_2 due to oxidative bursts. Oxidative stress in *E. coli* can significantly influence the virulence factors of the bacterium which in turn can determine the pathogenesis of *E. coli* infections. Previous studies have demonstrated that *E. coli* exposed to 1–3 mM H_2O_2 resulting in death of the bacteria, and this was due mainly to DNA damage [30]. In the presence of specific repair enzymes, glycosylases, AP lyases and AP endonucleases in wild-type *E. coli* strains, H_2O_2 exposure failed to induce cell death. This is not the only method by which a bacterium is able to repair its lesions. The recombinational repair is an important pathway in repairing H_2O_2-induced DNA lesions [30]. *E. coli* always show an adaptive response when exposed to oxidising agents suggesting that exposure to low levels of H_2O_2 allows bacteria to survive to subsequent exposures to increased toxic agents. Nine of the proteins induced by H_2O_2 treatment are expressed under the control of oxyR gene [31], including catalase and alkyl hydroperoxide reductase (Ahp) encoded by katG gene and ahpC and ahpF for Ahp [32]. It has been previously demonstrated that the *E. coli* cells which have been exposed to low doses of H_2O_2 develop resistance to heat, ethanol [33], ultra-violet (UV), formaldehyde and menadione and this referred to cross adaptive response process [30]. However, the role of oxidative stress response which may lead to biofilm development is not yet defined, especially in regards with *rpoS* and *bolA* genes. Can, a response to oxidative stress lead to biofilm mode of growth and serve as a defence mechanism? Question remains still unanswered.

Acid stress

In a variety of pathogenic and natural situations, bacteria encounter a variety of potentially lethal acid stress conditions. Acid stress is defined as the combined biological effect of H^+ ion (pH) and weak acids including butyrate, propionate and acetate which can lead to fermentation [34, 35]. This in turn results in an intracellular acidification that can damage the microbial biochemical processes [34]. Under severe acidic stress, e.g. (pH 3) proton leakage is faster, and cells tend to lose their ability to maintain their homeostasis. Organic acids in their uncharged protonated form enter the cells resulting in acidification which causes cell death [34]. In order to overcome this threat, microorganisms undergo a programmed molecular response leading to the synthesis of stress inducible proteins. These proteins in turn then repair the macromolecular damage caused by the stress. Three types of acid resistance (AR) systems have been identified in all types of *E. coli* when they are confined to stationary phase [36]. The first is a complex medium-dependent AR system which includes an oxidative system (AR1), while the other two are fermentative AR systems involving glutamate decarboxylase (AR2) and an arginine decarboxylase system (AR3) [34, 36, 37]. The oxidative system requires *rpoS* (σ^S) for the protection against organic acids, and it is induced by growth to stationary phase in Luria–Bertani broth. In contrast, the arginine and glutamate AR systems are partially dependent on alternative sigma factor σ^S [37]. Data

on acid tolerance response (ATR) are available for a number of foodborne bacteria or related to food microbiology, but none has so far explained their involvement in biofilm. In short, biofilm production in relation to acid tolerance is a complex biological phenomenon where different systems are involved, thus varies from organism, growth phase, medium, type of acid stress, and other environmental factors [34].

Cadmium (Cd^{2+}) stress

Cadmium is widely used in variety of industrial applications, and it is a possible source of environmental contamination. It is toxic with no known biological functions and is available in a variety of chemical forms [38]. Cadmium is a potent oxidative agent, and it can seriously damage the cells in various ways. These include inhibition of DNA replication and nucleolytic attack which result in single strands DNA break. Bacterial cells can readily take in the Cd^{2+} with the help of a Mg^{2+} uptake system. As in the case of other stress responses, *E. coli* can also repair the cadmium-mediated cellular damage and adjust its cell physiology to restrict the distribution of the toxic ion in the cell. This is done through the most common defence mechanism in which *E. coli* produce intracellular cadmium binding proteins. Cadmium can also activate global complex regulatory systems in which *E. coli* are able to synthesize a group of proteins called cadmium- induced proteins, CDPs which together are known as cadmium stress stimulon [38].

Conclusion

Research on microbial biofilms is scheduled on many fronts, with special emphasis on elucidation of the genes which are specifically expressed under biofilm mode in all the stress-induced environments. Biofilm formation is a programmed developmental process, and it is still difficult to know which transcription factors regulate genes under planktonic conditions and whether these are the same transcription factors which regulate genes under biofilm mode. The stress response of *E. coli* is complex, and its adaptation to the stress environments needs to be addressed in more detail. More research is required on the combined and individual roles of these two genes in biofilm formation. The key to success is to understand what makes biofilm bacteria so different from the planktonic bacteria. Biofilm formation is a mode of action by which the bacteria can protect themselves against the environment stress. This involves different forces which motivate bacteria to change from a planktonic phase to a biofilm phase, which in turn is beneficial to the bacteria.

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Analysis of *rpoS* and *bolA* gene expression under various stress-induced environments in planktonic and biofilm phase using $2^{-\Delta\Delta CT}$ method

Mohd Adnan · Glyn Morton · Sibte Hadi

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Abstract Genetic adaptation is one of the key features of *Escherichia coli* (*E. coli*) that ensure its survival in different hostile environments. *E. coli* seems to initiate biofilm development in response to specific environmental cues. A number of properties inherent within bacterial biofilms indicate that their gene expression is different from that of planktonic bacteria. Two of the possible important genes are *rpoS* and *bolA*. The *rpoS* gene has been known as the alternative sigma (σ) factor, which controls the expression of a large number of genes, which are involved in responses to a varied number of stresses, as well as transition to stationary phase from exponential form of growth. Morphogene *bolA* response to stress environment leads to round morphology of *E. coli* cells, but little is known about its involvement in biofilms and its development or maintenance. The purpose of this study was to understand and analyse the responses of *rpoS* and *bolA* gene to sudden change in the environment. In this study, *E. coli* K-12 MG1655, *rpoS*, and *bolA* mutant strains were used and gene expression was studied. Results show that both genes contribute to the ability to respond and adapt in response to various types of stresses. *RpoS* response to various stress environments was somehow constant in both the planktonic and biofilm phases, whereas *bolA* responded well under various stress conditions, in both planktonic and biofilm mode, up to 5–6-fold change in the expression was noticed in the case of pH variation and hydrogen peroxide stress (H_2O_2) as compared with *rpoS*.

Keywords *E. coli* · Biofilm · Stress environment · *rpoS* · *bolA* · Relative quantification

Introduction

Bacteria form biofilms as an adaptive mechanism in challenging environments. These can exist wherever surface contact is available to bacteria in naturally occurring fluids [1]. Biofilms are pervasive and problematic because they are more resistant to antibiotics, hydrodynamic shear forces, UV light, and chemical biocides; increased rates of genetic exchange, altered biodegradability, and increased secondary metabolite production than their planktonic counterparts [2, 3]. It is difficult to understand mechanisms of biofilm formation, as biofilms are heterogeneous in the environment and industrial settings and are composed of complex microbial communities [4].

It has been estimated that 65% of the infections are biofilm-associated [5, 6]. Reduced susceptibility of the biofilm bacteria to antimicrobial agents is a vital problem in the treatment of chronic infections [5, 6]. Single-species biofilm might exist in a variety of infections and on the surfaces of indwelling medical implants. The mechanism of biofilm formation can be better understood at the molecular level by studying single-species biofilm under controlled conditions.

Recently, research into the genetic control of biofilm formation has gained importance. Various intrinsic properties within bacterial biofilms indicate that their gene expression is different to their planktonic counterparts and numerous genes have been proposed to be important in

M. Adnan (✉) · G. Morton · S. Hadi
School of Forensic and Investigative Sciences,
University of Central Lancashire, Maudland Building,
PR1 2HE Preston, Lancashire, United Kingdom
e-mail: madnan@uclan.ac.uk

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biofilm formation. Vast arrays of genes are implicated in biofilm formation [7, 8]. Two of the possibly important genes are *rpoS* (RNA polymerase sigma factor) and morphogene *bolA*. *RpoS* is a sigma subunit of RNA polymerase in *E. coli* that is induced and can replace vegetative sigma factor *rpoD* to some extent, under several stress conditions. Consequently, transcription of numerous σ^S-dependent genes is activated [1].

Morphogene *bolA* was first described to be involved in adaptation to the stationary growth phase [9]. However, its function is still not fully understood. Its expression might be induced by different forms of stresses that result in the high-level expression of *bolA* mRNA and the formation of biofilms. It also has a major effect on the bacterial envelope and, therefore, may be implicated in cellular protection under adverse growth conditions [10]. Even though the significance of the *rpoS* gene in biofilm development has been suggested, the role of *rpoS* and *bolA* gene in the formation of biofilm and its expression under different types of stresses has not been investigated.

Stress may be defined as any detrimental factor that adversely affects the growth or survival of microorganisms. Outcomes of stresses applied to microorganisms vary. Sublethal levels of stress reduce or stop the growth of the microorganism and do not result in viability loss [11]. In the case of moderate stress environments, the outcome leads to loss in cell viability and stops the growth of microorganism. Acute or extreme stress is lethal to cells and causes the death of the mainstream of the population. The increase in resistance of an organism to one stress, after application of a different and unrelated sublethal stress, is known as cross-protection [12]. Stress responses are extremely imperative to microorganisms as their habitats are subject to continuous change [11].

In response to changes in their environment, bacteria have the ability to regulate the expression of genes that control their growth and physiology quickly [13]. Because bacterial gene expression is strongly regulated at the transcriptional level [14] and prokaryotic RNAs have short half-lives [15], transcriptional profiling has been widely used in characterization of bacterial responses to various environmental conditions [14, 16]. Reverse transcription followed by quantitative real-time PCR (qRT–PCR) is a sensitive tool to quantitatively analyze RNA levels transcribed from a relatively large number of genetic regions. In addition, it can quantify low-abundance RNAs and, with slight modification, can be applied to measure all categories of RNAs [17]. Moreover, direct measurement of RNA levels from a set of responsive genes that either get induced or repressed under a specific environmental condition can reveal information about bacterial responses and be critical to understanding conditions in microenvironments around bacteria at the time of expression profiling.

Materials and methods

Bacterial strains and growth conditions

E. coli K-12 MG1655 wild type (WT) and mutant strains (Δ) have been used in this study and were kindly provided by National Institute of Genetics, Japan. The WT strain was *E. coli* K-12 MG1655 and the mutants were *E. coli* K-12 MG1655 *rpoS* mutant (Δ*rpoS*) and *E. coli* K-12 MG1655 *bolA* mutant (Δ*bolA*). Cells were grown in LB (Luria–Bertani) medium. Samples were taken at $OD_{600} = 1.0$ and was considered as exponential growth phase, whereas $OD_{600} = 2.2$ was considered to be stationary growth phase.

Inoculum preparation

A bacterial suspension was prepared by gently removing bacteria from the solid medium using a sterile nichrome loop to inoculate the bacteria into a 500 ml flask containing 200 ml of sterile nutrient medium. This bacterial suspension was incubated at 37°C with agitation at 120 rpm for 18 h to have bacteria in the exponential phase of growth.

Stress response experiment

Heat shock, cold shock, pH stress, and H_2O_2 stress

A volume of 0.1 ml of *E. coli* K-12 MG1655 culture (WT, Δ*bolA*, and Δ*rpoS*) was withdrawn at 2 min intervals and plated out directly to determine the viable cell numbers. Percentage survival was defined as the percentage change in the CFU counts per ml obtained after incubation onto LB medium for 15 min following a sudden shift from optimal growth conditions, i.e., heat shock temperatures (42 and 46°C), cold shock temperatures (5 and 20°C), pH stress levels (pH 5, 6, 8, and 9), and different concentrations of H_2O_2 (3, 4, and 5 mM). This was done to check the rapid change in expression level of *rpoS* and *bolA* genes.

Glycogen assay

To confirm the *rpoS* mutant status, both *E. coli* WT and Δ*rpoS* strains were streaked on LB agar plates and incubated overnight at 37°C. After incubation, plates were left at 4°C for 24 h before they were flooded with concentrated iodine solution. Glycogen-deficient Δ*rpoS* gave a negative-staining reaction (*white colonies*), whereas the WT glycogen-excess strains generated a positive-staining reaction (*dark brown colonies*) [18].

Catalase activity

Cultures were also tested qualitatively for catalase activity by applying 6% (wt/vol) H_2O_2 directly onto colonies on Luria agar plates. Vigorous bubbling indicated WT *rpoS* activity and positive reaction to hydrogen peroxide.

Biofilm formation assay: crystal violet staining

A biofilm formation assay was performed using a microtitre plate. A volume of 20 µl aliquots of an overnight culture with OD_{600} of 1.0 were inoculated into 200 µl medium in a PVC microtitre plate. After 72 h incubation, the medium was removed from wells, which were then washed five times with sterile distilled water, and unattached cells were removed. Plates were air-dried for 45 min and each well with attached cells were stained with 1% crystal violet (CV) solution in water for 45 min. After staining, plates were washed with sterile distilled water five times. At this point, biofilms were visible as purple rings formed on the side of each well. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. About 100 µl from each well was transferred to a new microtiter plate, and the level (OD) of the crystal violet present in the destaining solution was measured at 595 nm.

Experimental replication

Data from all experiments, including control treatments for both the planktonic and biofilm phase, represent the averages of three or more independent experiments.

Isolation of RNA

RNA was routinely isolated using the RNeasy® Protect™ Bacteria Mini Kit (Qiagen Ltd., UK), which comprises two steps: (i) immediate stabilization of bacterial RNA and (ii) subsequent isolation and purification of total RNA.

Analysis of RNA integrity

The integrity of total RNA samples was determined by using denaturing (formaldehyde) agarose gel electrophoresis. RNA samples, used for RT-PCR analysis, were routinely checked using this method for the presence of two clear sharp bands of 16S and 23S *E. coli* ribosomal RNA, which are indicative of intact RNA.

cDNA synthesis for real-time two-step RT-PCR

Messenger RNA was reverse transcribed into cDNA using the QuantiTect® Reverse Transcription kit (Qiagen Ltd., UK). RNA was converted to cDNA with 15 min incubation at 42°C and 3 min inactivation at 95°C. The cDNA was subjected to real time PCR using ABI 7500 (Applied Biosystems). Reactions were performed in a 12.5 µl reaction volume.

Primer designing

Specific primers for *rpoS*, *bolA*, and 16S rRNA (housekeeping gene) were designed using Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Table 1). Primers were ordered from Invitrogen™ life technologies, UK. On receipts, all primers were rehydrated in nuclease-free water and dispensed into 10 µM aliquots of working stock solution before storage at −20°C.

Optimization of the PCR

Optimal PCR conditions were determined using Veriti Thermal Cycler (Applied Biosystems). The optimum concentrations of magnesium chloride and primers for both sets of *rpoS* and *bolA* primers were found to be 1.5 mM and 0.3 µM, respectively. These concentrations were subsequently used in all real-time RT-PCR experiments to maintain reaction stringency. The optimum annealing temperature for the amplification of *rpoS* and *bolA* was determined to be 60°C.

Real-time quantitative RT-PCR

QuantiTect™ SYBR® Green I PCR (Qiagen) assays were run on ABI 7500 Real-Time PCR machine for quantitative analysis of *rpoS* and *bolA* mRNA. Initial assays were carried out according to the reaction conditions recommended by the manufacturer in conjunction with the optimum parameters determined using standard PCR.

Melting curve analysis

The identity of PCR products was confirmed by melting curve analysis, which was performed after the amplification stage of every experiment.

Analysis of gene expression using $2^{-\Delta\Delta CT}$ method (relative quantification)

The polymerase chain reaction is an exponential process whereby the specifically amplified product ideally doubles each cycle. As such, the measured Ct (cycle threshold) value is a logarithmic value that needs to be converted into a linear relative quantity [19]. The average Ct was calculated for both the target genes and 16S rRNA, and the ΔCt

Table 1 List of primer sequences for *rpoS*, *bolA*, and 16S rRNA (housekeeping gene)

Primer	Sequence	Length (bp)	Annealing temperature (°C)
16S rRNA (forward)	AGGCCTTCGGGTTGTAAAGT	20	55
16S rRNA (reverse)	CGGGGATTTCACATCTGACT	20	55
rpoS (forward)	GATGACGTCAGCCGTATGCTT	21	59
rpoS (reverse)	GAGGCCAATTTCACGACCTAC	21	59
bolA (forward)	CCGTATTCCTCGAAGTAGTGG	21	59
bolA (reverse)	GCAACCCTTCCCACTCCTTAA	21	59

(delta threshold) was determined as (the mean of the triplicate Ct values for the target gene) minus (the mean of the triplicate Ct values for 16S rRNA). The ΔΔCT (delta delta threshold) represented the difference between samples. The expression levels of the gene of interest were normalized by dividing it by the relative expression level for the housekeeping gene for the same sample. The fold-change in gene expression was calculated by dividing the normalized expression level for the experimental sample by the normalized number for the control sample.

Results

Growth curve was plotted to check the differences in the growth rate of *+rpoS/−bolA*, *+bolA/−rpoS*, and WT. It was found that *E. coli* with Δ*rpoS* and Δ*bolA* gene can grow at the same rate as WT does in planktonic cells (Fig. 1).

The analysis of integrity of RNA was routinely checked using formaldehyde agarose gel electrophoresis (Fig. 2). The product sizes for *rpoS*, *bolA*, and 16S rRNA were 273, 216, and 201 bp, respectively (Fig. 3). Throughout in this study, ribosomal gene 16S rRNA was used as a reference gene.

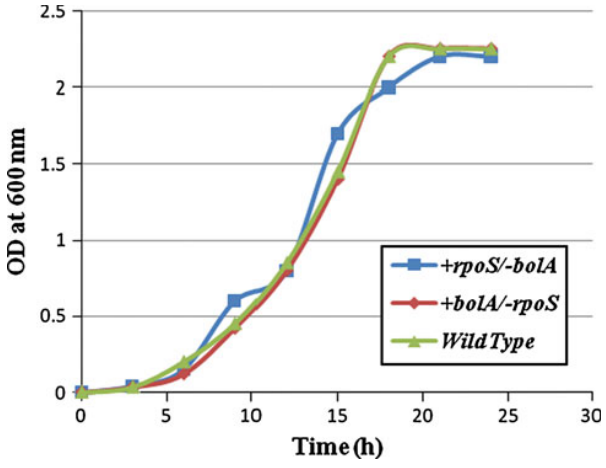

Fig. 1 Planktonic growth curve of wild type (*WT*), *rpoS* mutant (*filled square*, *rpoS*), and *bolA* mutant (*filled diamond*, *bolA*) strains in LB media. Optical density was measured at A_{600}. $OD_{600} = 1.0$ (exponential growth phase) and $OD_{600} = 2.2$ (stationary growth phase). The data used are an average of three individual experiments

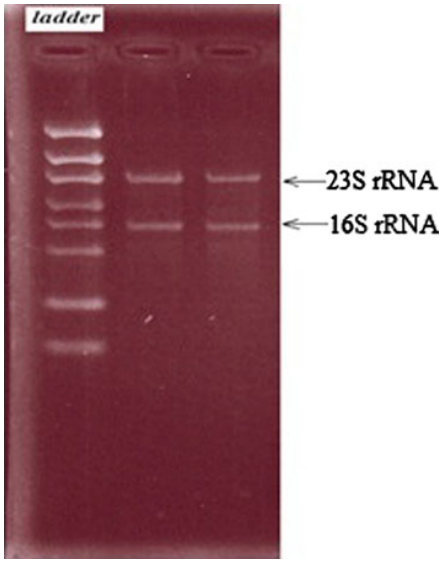

Fig. 2 The analysis of the integrity of RNA by formaldehyde agarose (1.5% w/v) gel electrophoresis, from the total RNA samples extracted from exponentially growing *E. coli* K-12 MG1655 cells. The size of 16S rRNA and 23S rRNA was 1.5 and 2.9 kbp, respectively

Preparation of DNA standards and a standard curve for quantification using real-time PCR

Relative quantification was employed for determination of the relative level of expression of the genes of interest and the housekeeping gene for all experimental samples. Absolute quantification was also performed to generate the Ct values for relative quantification. The advantage of absolute quantification is the quality of results, which provide information on actual levels of a given mRNA, in this case *rpoS* and *bolA* mRNA. Furthermore, the results can be compared as independent results, and are not linked to parameters specific to the experiment. The calibration curve was obtained during the runs performed with the DNA standards, and the original screenshot of a standard curve generated during the experiment was taken as an

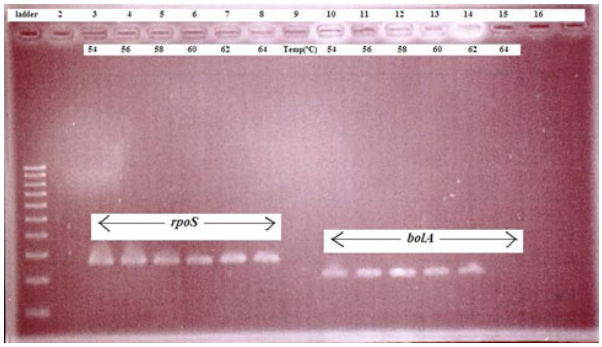

Fig. 3 Agarose gel showing optimised primers for *rpoS* and *bolA* genes at different temperatures with a product size of 273 and 216 bp

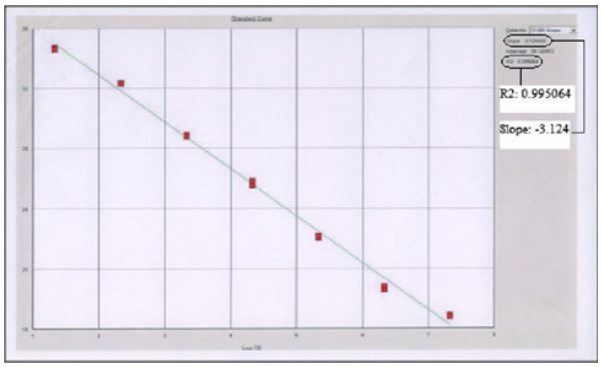

Fig. 4 Illustrated example of the calibration curve generated from the average Ct values, for each standard, obtained from all real-time RT-PCR determinations performed for analysis of *rpoS* and *bolA* mRNA transcription

example (Fig. 4). The PCR amplification efficiency can be determined from the slope of the calibration curve. A slope equal to −3.3 indicates 100% efficiency. It should be noted that absolute quantities of each template are calculated based on individual calibration curves generated during individual PCR runs. The optimal baseline and threshold setting for each experiment was set to manual Ct (i.e., threshold 0.02). Ct values were generated for preparation of standard curve for each standard using seven independent experiments.

The melting temperature of the specific product amplified from the initial 16S rRNA, *rpoS*, and *bolA* mRNA template had a predicted melting temperature of 83, 84, and 80°C (Fig. 5). From the melting curve plot, it could be deduced that no primer dimers or secondary products were formed because only one peak was seen, which corresponds to the desired product. The products of all real-time PCR experiments presented in this report were confirmed using melting curve analysis and by agarose gel electrophoresis analysis.

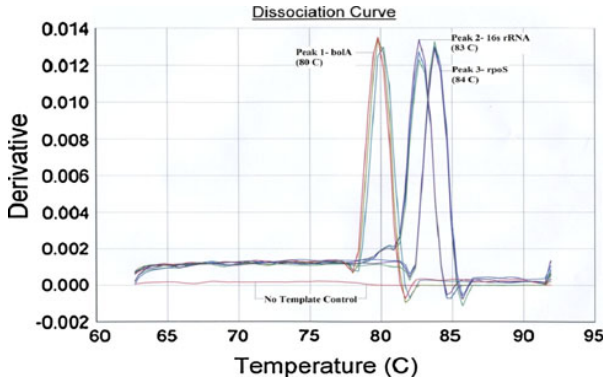

Fig. 5 The graph illustrates data from a typical real-time RT-PCR experiment with melting curve analysis. Two-step RT-PCR was carried out according to the optimised protocol. It illustrates the calculated plot of fluorescence against temperature. Using this plot, the melting temperature of the amplification product can be determined, which in this case is 83, 84, and 80°C for 16S rRNA, *rpoS*, and *bolA*. The data collected also include no template control

Analysis of *rpoS* and *bolA* gene expression using the relative quantification method under heat, cold, pH, and oxidative stress

A noticeable difference in gene expression of *rpoS* and *bolA* gene under various stress-induced environments in both the planktonic and biofilm phases was seen. In this study, the data are presented as the fold change in target gene expression in various stress-induced environments normalized to the internal control gene (16S rRNA) and relative to the normal control. The *N*-fold differential expression in the target gene of a stress-induced samples compared with the normal sample counterpart was expressed as $2^{-\Delta\Delta CT}$ in this study. The *rpoS* and *bolA* gene expression level was seen higher in biofilms than the exponential planktonic cells. Expression analysis of mRNA of *rpoS* and *bolA* genes under various stress environments was performed using relative quantification method. Results showed the *N*-fold change in the expression of both *rpoS* (Fig. 6) and *bolA* (Fig. 7) genes under heat shock temperatures (42 and 46°C), cold shock temperatures (5 and 20°C), pH stress levels (pH 5, 6, 8, and 9), and different concentrations of H_2O_2 (3, 4, and 5 mM).

Discussion

Earlier studies on *rpoS* and *bolA* genes have investigated long-term stress conditions and biofilm formation under several forms of stress, including nutrient starvation at stationary phase, where the increased level of expression has been seen. This study assessed whether *rpoS* and *bolA*

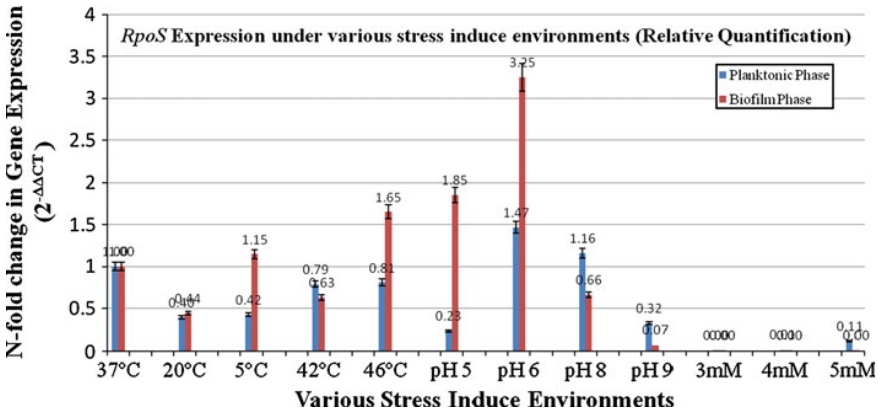

Fig. 6 *Bar graph* represents the expression of *rpoS* gene under various stress conditions in planktonic and biofilm phase. The cultures were grown overnight in LB at 37°C and percent survival was calculated. The values shown are the means of three independent experiments and the *error bars* indicate the range. Increased mRNA expression was defined as *N*-fold > 1.0, "normal" expression (control) was an *N*-fold = 1, and decreased mRNA expression was *N*-fold < 1.0

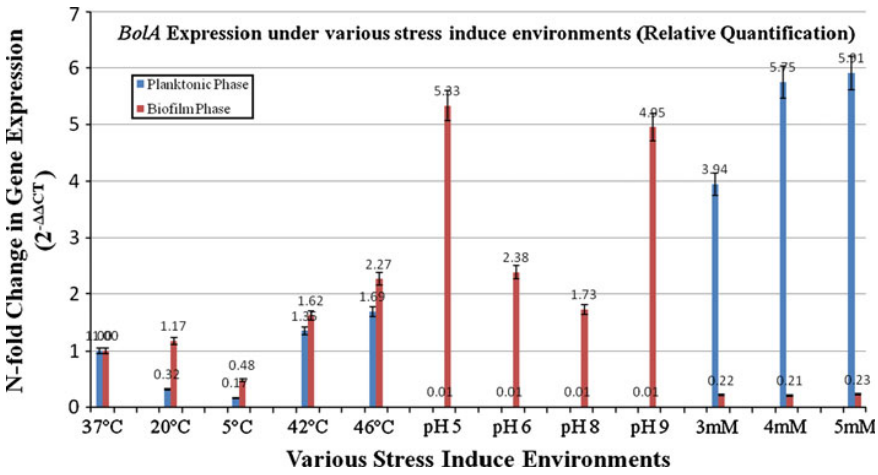

Fig. 7 *Bar graph* represents the expression of *bolA* gene under various stress conditions in planktonic and biofilm phase. The cultures were grown overnight in LB at 37°C and percent survival was calculated. The values shown are the means of three independent experiments and the *error bars* indicate the range. Increased mRNA expression was defined as *N*-fold > 1.0, "normal" expression was an *N*-fold = 1 (control), and decreased mRNA expression was *N*-fold < 1.0

gene could express under suddenly changing stress conditions, i.e., 15 min intervals from optimal condition to the various stress-induced conditions (i.e., heat, cold, pH fluctuation, and oxidative stress) in both planktonic and biofilm phase. Morphogene *bolA* is known to express in the stationary phase. Its expression in the biofilm phase at exponential level of growth and the possible role of *bolA* gene under sudden change in environment was therefore investigated.

E. coli frequently encounters various types of stresses in natural and man-made environments. In this study, real-time RT-PCR was performed to investigate the expression profiles of *rpoS and bolA* genes in response to similar stresses. The stress-induced conditions used in this study were chosen to represent some scenarios that this bacterium may encounter during natural shifts. These results indicate that the *bolA* and *rpoS* respond to different conditions quite distinctly, and have distinct expression patterns under various stress conditions.

RpoS is a conserved stress regulator that plays a significant role in survival under stress conditions in *E. coli.* The *rpoS* mutation had a pronounced effect on gene expression in stationary phase, and more than 1,000 genes were differentially expressed. Even in exponential phase when *rpoS* is expressed at low levels, mutation in *rpoS* affects the expression of a large set of genes [20]. On the other hand, *bolA* expression is also confined to stationary phase. Its involvement in biofilm formation and expression under stationary phase is two different events, which are related to stress. So the purpose here was to analyse the expression of *rpoS* and its dependent gene *bolA* under biofilm mode of growth, as a sudden response to stress.

Expression of *rpoS* and *bolA* in various stress conditions

No activity of *rpoS* was found under oxidative stress, which suggests that cells in mature biofilms do not require expression of the *rpoS* gene under oxidative stress in either the planktonic or in biofilm phases (Fig. 6). *RpoS* might be able to respond in later stages/higher concentration (H_2O_2) to oxidative stress but not suddenly (in this study). An interesting result was seen in the case of *bolA*, which showed a 5–6-fold increase in expression under oxidative stress in the planktonic phase when compared with *rpoS* expression. Decreased expression of *bolA* in the biofilm phase is seen under oxidative stress when compared with the planktonic phase, which shows that cells can respond well in the planktonic phase in presence of *bolA* but not in biofilms, whereas *rpoS* cannot respond in either phase. The data indicate that gene expression within biofilm is different from that observed in standard planktonic growth cultures. Nearly, 1.6-fold increase in the expression of *rpoS* and 2.2-fold increase in the expression of *bolA* were seen after 15 min of heat stress, i.e., shift from 37 to 46°C, under the biofilm mode of growth. In the planktonic phase, a minor change was seen after the shift to 42 and 46°C from 37°C (Fig. 6). Sudden decrease in the expression of *rpoS* and *bolA* both under cold shock condition suggests that low temperature does not induce the expression of both genes, or it can be said that *rpoS* and *bolA* cannot respond suddenly to the cold shock condition, whereas on the other hand, variation in the pH change induces the expression of *rpoS* and *bolA* up to 3.5- and 5.5-fold increase under biofilm mode of growth, which in turn shows the necessity for both genes when the pH is changed. It also hypothesizes that cells in biofilms were in stress conditions and requires the expression of *rpoS* and *bolA* as a sudden response to environmental change.

Overall, results from this study suggest a new phenotype for the *bolA* and *rpoS* gene. In addition to its ability to produce round cell morphology, *bolA* is implicated in biofilm development [21]. The fact that *bolA* is expressed under unfavorable conditions (i.e., stress and stationary phase) suggests that biofilm formation is a mode of action by which the bacteria protect themselves against the environment. The expression of *bolA* is under the transcriptional control of σ^S (encoded by *rpoS*). The presence or absence of σ^S has an impact on biofilms [22]. In *rpoS* mutant strains, the biofilm cell density is reduced by 50%, and there are differences in biofilm structure [23]. Interestingly, deletion of *bolA* also reduces biofilm formation by *E. coli* K-12 MG1655. Considering the fact that the levels of *bolA* depend on σ^S, we can still hypothesize that *bolA* may facilitate the biofilm development. As the expression level of *bolA* was higher than that of *rpoS* alone shows that the sudden change in environment could increase the expression of *bolA*. This might indicate that σ^S may act through *bolA* to facilitate biofilm development.

The study showed that both *rpoS* and *bolA* genes can respond and express under sudden change in environment. Change in pH suggests the importance of *rpoS* and *bolA* and their response to the pH fluctuation is constructive, which may lead to increased *bolA* and *rpoS* mRNA levels resulting in biofilm formation and development. In general, the study demonstrated that temperature, pH, and hydrogen peroxide have a dramatic effect on gene expression, signifying that adaptation to various environmental change conditions requires a coordinated multifunctional response. This study concludes that *rpoS* gene and its coordinated expression with *bolA* gene possibly play a major role in biofilm development.

Acknowledgments We gratefully acknowledge the supports from the National Bio Resource Project (NIG, Japan): *E. coli* for providing bacterial strains for use during this project and the Society for Applied Microbiology (sfam) for a research grant to complete this project.

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Appendix 5

Poster presented at Society for Experimental Biology workshop, Lancaster University, Lancaster, United Kingdom.

An Investigation of the Genetic Control of Biofilm Formation in Bacteria (*E. coli* K12 MG1655)

M. Adnan[a], D. Greenway, G. Morton, S. Hadi
School of Forensic and Investigative Science, University of Central Lancashire, Preston, PR1 2HE, UK

Introduction

Genetic adaptation is one of the key features of bacteria which enables them flexible to survive in any type of environment. One of the clinically relevant example of bacterial adaptation is the ability of bacteria to grow as sessile community which is referred to as "Biofilm"(1). Biofilms are therefore a major health concern.

A number of studies of bacterial biofilms indicate that gene expression in biofilm bacteria is different from planktonic bacteria. In biofilm bacteria stress response genes are induced whenever a cell needs to adapt to survive under adverse growth conditions (2). Many genes have been implicated as major players in biofilm formation and propagation. Two of the possible important genes are *rpoS* and *bolA* gene. The fact that *bolA* is expressed under unfavourable conditions which suggests that biofilm formation is a mode of action by which the bacteria protect themselves against environmental stress.

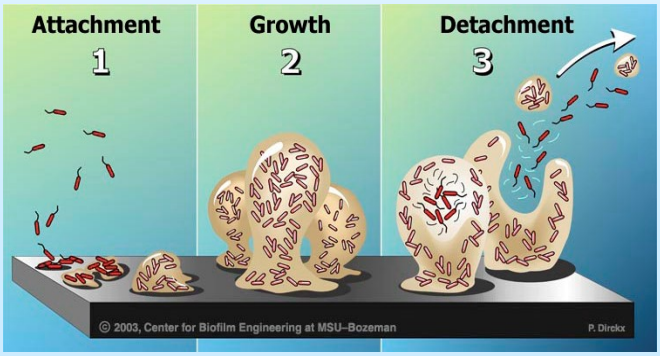

Figure 1: Biofilm life cycle in three steps (4)

Biofilm and Infections

Biofilms are implicated in a wide range of health concerns.

- Catheters, contact lenses, artificial joints and many other medical devices implanted in the body are prone to biofilm growth.
- Biofilms have an ability to grow on living tissues and involve in human diseases like endocarditis, cystic fibrosis and middle ear infections.
- Bacterial colonisation of indwelling medical devices in hospitalised patients is a common source of nosocomial infections.

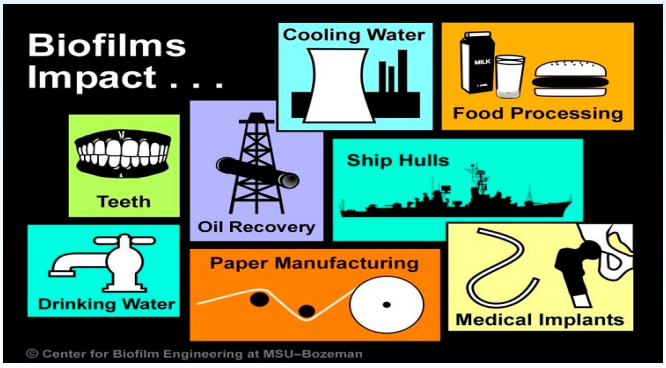

Figure 2: Schematic representation of the diversity of biofilms (4)

Aim and Objectives

- This study will investigate the role of *rpoS* and *bolA* genes in biofilm formation in diverse stress conditions (acid, hydrogen peroxide, cadmium, heat shock, and cold shock stresses).
- *E. coli* K12 MG1655, *rpoS* and *bolA* mutant strain will be used, and biofilm development and gene expression will investigated.
- Wild type (WT) and mutant strain of *E. coli* K12 MG1655 will be cultured to produce biofilms using microtitre plate technique. The biofilms will be studied using confocal microscopy and architectural differences will be noticed.
- Real time quantitative PCR will be used to determine the differences of gene expression in WT and mutant strain..

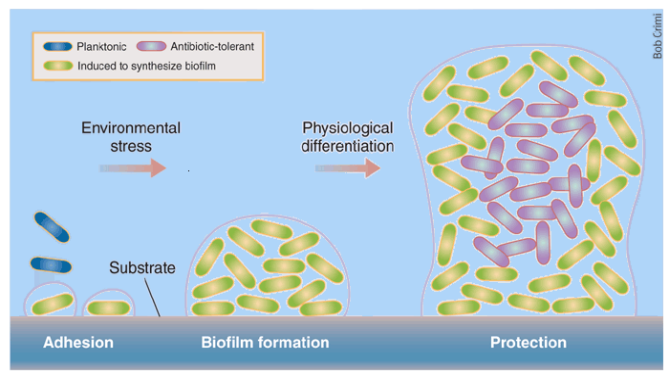

Figure 3: Biofilm Formation in Stress condition (5)

Expected Outcomes

The outcome of this study will reveal the role of *rpoS* and *bolA genes* in Biofilm formation under diverse stress conditions. It will also reveal the correlation patterns of the two genes during Biofilm development.

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4. www.erc.montana.edu.
5. www.nature.com

a. *e-mail address* : madnan@uclan.ac.uk

Appendix 6

Poster presented at University of Central Lancashire Annual research conference, Preston, United Kingdom

An Investigation of the Genetic Control of Biofilm Formation in Bacteria (*E. coli* K-12 MG1655)

M. Adnan[a], G. Morton, S. Hadi
School of Forensic and Investigative Sciences, University of Central Lancashire, Preston, PR1 2HE, UK

Introduction

Genetic adaptation is one of the key features of bacteria affording flexibility to survive in different types of environments. One of the clinically relevant examples of bacterial adaptation is the ability of bacteria to grow as sessile community which is referred to as "Biofilm"(1). Biofilms are therefore a major health concern.

A number of studies indicate that gene expression in biofilm bacteria is different from planktonic bacteria and stress response genes are induced whenever a cell needs to adapt to survive under adverse growth conditions (2). Many genes have been implicated as major players in biofilm formation and propagation. Two of the possible important genes are *rpoS* and *bolA* gene. The fact that *bolA* is expressed under unfavourable conditions suggests that through biofilm formation bacteria protect themselves against environmental stresses.

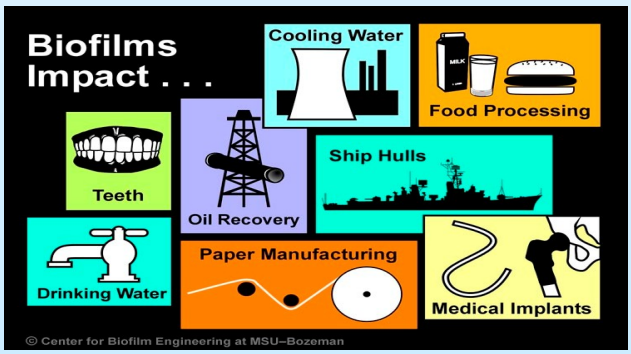

Figure 1: Schematic representation of diversity of biofilms (3)

Aim and Objectives

- This study aims to investigate the role of *rpoS* and *bolA* genes in biofilm formation under diverse stress conditions (acid, hydrogen peroxide, cadmium, heat shock, and cold shock stresses).
- *E. coli* K12 MG1655, *rpoS* and *bolA* mutant strains have been selected for the investigation of gene expression during biofilm development.
- Wild type (WT) and mutant strain of *E. coli* K12 MG1655 will be cultured to produce biofilms using microtitre plate technique.
- Oxygen consumption in Biofilm by *E.coli* K12 MG1655 will be studied using Respirometry technique.
- Real time quantitative PCR will be used to determine the differences of gene expression in WT and mutant strain.

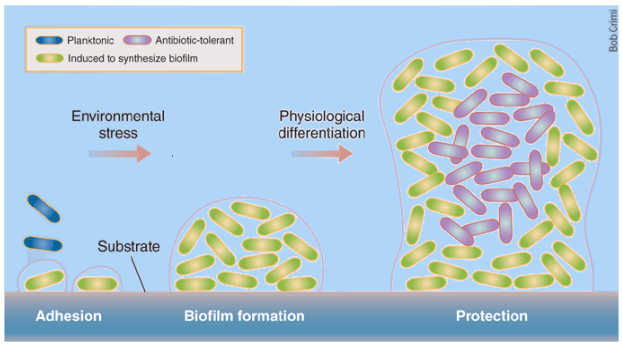

Figure 2: Biofilm Formation in Stress condition (3)

Methods

Bacterial strains were kindly provided by National Institute of Genetics, Japan. Cultures were grown in LB media (Luria-Bertani) with aeration.

RNA Extraction: Bacterial cells were harvested and resuspended into RNAprotect® Bacteria Reagent (Qiagen, UK) and RNA was extracted using Qiagen RNeasy® kit according to manufacturer's instructions. RNA quality was checked by electrophoresis on 2% native agarose gel with a molecular marker RiboRuler™ High Range RNA ladder 200-6000bp (Fermentas® Life Sciences, UK).

RT-PCR: cDNA was synthesized using standard reverse transcriptase reaction with Omniscript and Sensiscript Reverse Transcriptase enzyme. In order to monitor DNA contamination of the RNA extracts, controls were prepared as parallel reactions without adding any RT enzyme.

PCR Primer Design: For *rpoS*, *bolA* and 16s rRNA genes, the most conserved regions of the genes were selected for PCR primer design. The amplicon length using these primers was 273bp for *rpoS* 216bp for *bolA* and 201bp for 16s rRNA genes.

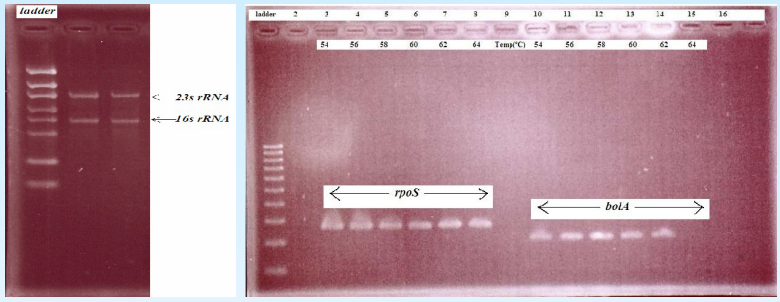

Figure 3: Agarose gel showing 16s and 23s rRNA in gel-1 and optimised primers in gel-2

Results

PCR primers for *rpoS* and *bolA* genes were optimised and the amplicon length for *rpoS* is 273bp and *bolA* is 216bp. Gel-1 (Figure 3)shows the 16s and 23s ribosomal RNA bands which are 1.5 kb and 2.9 kb and gel-2 (Figure 3) shows the optimised primers at different temperatures (54°C-64°C).

References

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3. www.erc.montana.edu

a. *e-mail address* : madnan@uclan.ac.uk

Appendix 7

Accepted abstract for oral presentation at 2nd International conference of biological and environmental sciences, Mansoura University, Egypt

Importance of *rpoS* and *bolA* gene in adaptation of *Escherichia coli* in stress environments and grow in Biofilms.

Mohd Adnan[a*], Glyn Morton[a], Sibte Hadi[a]

[a]**Address:** School of Forensic and Investigative Sciences, University of Central Lancashire, PR1 2HE, Preston, United Kingdom.

* Corresponding author

E-mail address: madnan@uclan.ac.uk

Fax: +44(0)1772894895

Tel: +44(0)1772984395

Abstract

Microorganisms were initially characterized as freely suspended cells and described on the basis of their morphology and growth characteristics. Scientists have recently realized that more than 99% of all bacteria exist as biofilms which is defined as a collection of microorganisms that are irreversibly attached to a surface and enclosed in an extracellular matrix allowing growth and survival in sessile environment. This is due to the fact that biofilm organisms transcribe genes which planktonic organisms do not.

Flexibility and adaptation of bacteria through systemized gene expression permits its survival in various environments. Stress responses enable its survival under more severe conditions, enhance resistance and/or enhance virulence. Many genes have been implicated as major players in biofilm formation and propagation. In *E. coli* two of the possible important genes are *rpoS* and *bolA* gene. *RpoS* is also called as a master regulator of general stress response. Even though many studies have revealed the importance of *rpoS* in

planktonic cells but little is known about the functions of *rpoS* in biofilms. *BolA* which is a morphogene in *E.coli* is overexpressed under stressed environments and causes round morphology. The hypothesis is *bolA* could be implicated in biofilm development. This study aims to investigate the role of *rpoS* and *bolA* genes in biofilm formation under diverse stress conditions (acid, hydrogen peroxide, cadmium, heat shock, and cold shock stresses) and understanding the stress tolerance response of *E. coli*. Real time quantitative PCR is used to determine the differences of gene expression in WT and mutant strains.

Knowledge of the genetic regulation of biofilm formation leads to the understanding of the factors that drive the bacteria to switch to the biofilm mode of growth.

Acknowledgement

We gratefully acknowledge support from the National Bio Resource Project (NIG, Japan): *E. coli* for providing bacterial strains for use during this project and Society for Applied Microbiology (sfam) for providing a hardship research grant to complete this project successfully.

Appendix 8

Poster presented at European Congress for Clinical Microbiology and Infectious Diseases (ECCMID 2010), Vienna, Austria

Morphogene *BolA*: Its role in biofilm formation and respiration of *E.coli* K-12 MG1655

M. Adnan[1*], M. Pereira[2], I. Machado[2], G. Morton[1], S. Hadi[1]

[1]School of Forensic and Investigative Sciences, University of Central Lancashire, Preston, PR1 2HE, UK

[2]Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Introduction

One important and clinically relevant example of *E.coli* adaptation through systematized gene expression is their ability to grow in biofilms. Stress response genes are induced whenever a cell needs to survive under adverse growth conditions (1). One of the possible gene having an influence on this process is morphogene *bolA*. It was first described to be involved in adaptation to stationary phase. However, its function is still not fully understood, and its expression may be induced by different forms of stresses which results in high level of expression of *bolA* and might lead to the formation of biofilms (2). To survive within stressed environments, overexpression of *bolA* leads to the round morphology to render the cell shorter and rounder (Fig. A). Respiratory activity measured by oxygen uptake rate due to glucose oxidation has already been used to assess the potential of antimicrobial agents against planktonic bacteria and their biofilm counterparts (3). However, no reports are available concerning the use of this technique in validating the gene and its involvement in respiration or metabolic activity. Therefore, this work aims to understand the stress tolerance response and oxygen consumption of *E.coli* in relation with *bolA* gene under different environmental stress conditions.

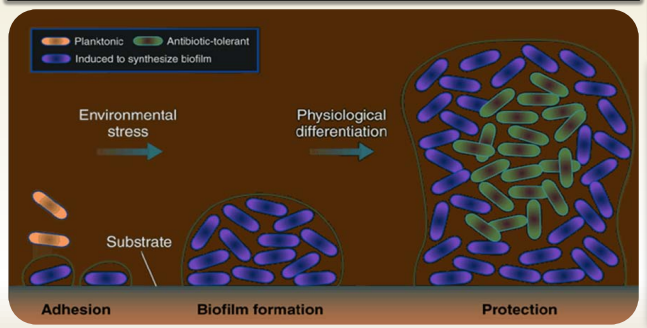
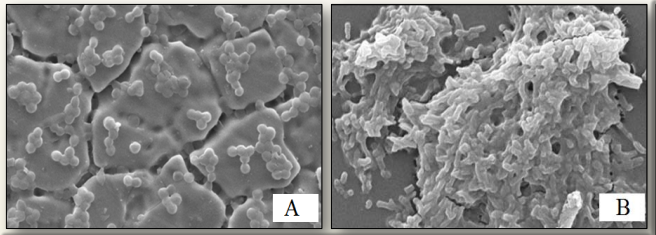


Figure 1: Biofilm formation in stress condition



Methods

RNA Extraction: Bacterial cells were harvested and resuspended into RNAprotect® Bacteria Reagent (Qiagen, UK) and RNA was extracted using Qiagen RNeasy® kit. RNA quality was assessed by agarose gel electrophoresis.

RT-PCR: Real time RT-PCR was used to examine the expression level of *bolA* gene in biofilms and planktonic cells. Total RNA was extracted, converted to cDNA and processed using real time PCR (ABI PRISM 7500, Applied Biosystems). Reactions were performed in a 25 µl reaction volume. *BolA* specific primers were developed in house for this purpose.

Assessment of bacterial respiratory activity:

The involvement of *bolA* gene in *E.coli* respiration was observed by checking its glucose metabolism and oxygen uptake rate under various environmental stress conditions in biofilm suspensions. It was evaluated by measuring oxygen uptake rate using biological oxygen monitor (BOM).

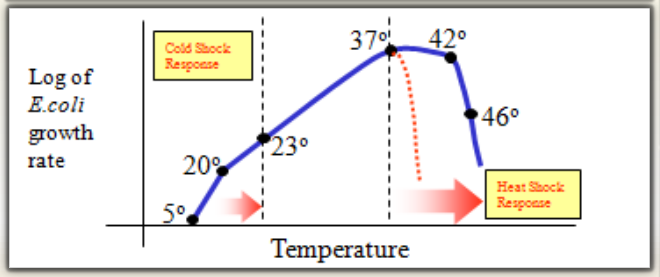
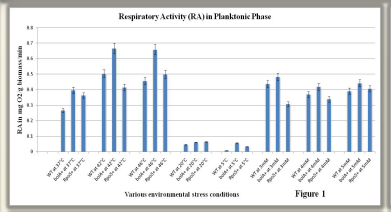
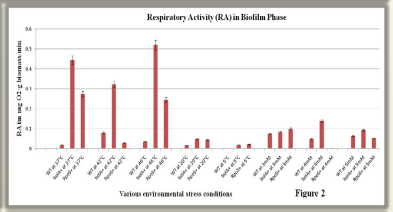


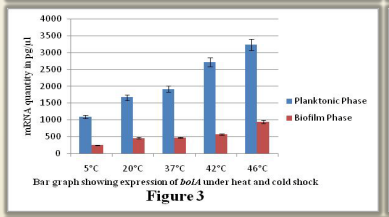
Figure 2: Experimental design for heat and cold shock conditions

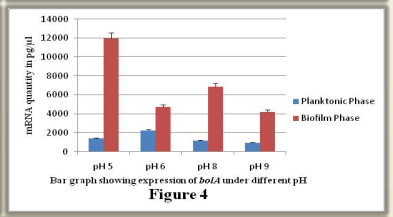
Results

Figure 1 and 2 below, shows the respiratory activity of *E.coli* K-12 MG1655 in planktonic and biofilm phase in presence and absence of *bolA* gene. Figure 3 and 4 explains the expression of *bolA* under heat, cold, acidic and alkaline stress conditions in both planktonic and biofilm phase. Expression level was measured after sudden shift from optimal growth conditions to various stress conditions.









Conclusion

Consistently high expression of *bolA* under different stress conditions and higher respiratory activity in the presence of *bolA* shows that it has a role in responding to stress environments and in formation of biofilms. Change in expression was seen in planktonic and biofilm form of growth, but the level of expression was less compared to planktonic phase in heat and cold shock conditions. This might be due to slow/no growth rate in biofilm phase. Sudden increase in expression of *bolA* was seen in response to pH fluctuations in biofilm phase.

References

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[*]*e-mail address* : madnan@uclan.ac.uk