Role of *bolA* and *rpoS* genes in biofilm formation and adherence pattern by *Escherichia coli* K-12 MG1655 on polypropylene, stainless steel and silicone surfaces

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Running headline: Biofilm formation and adherence pattern by *E. coli*

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Abstract

Escherichia coli (E. coli) has developed sophisticated means to sense, to respond and to adapt in stressed environment. It has served as a model organism for studies in molecular genetics and physiology since the 1960s. Stress response genes are induced whenever a cell needs to adapt and survive under unfavorable growth conditions. 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 various stress factors, as well as transition to stationary phase from exponential form of growth. Morphogene *bolA* response to stressed environment leads to round morphology of *E. coli* cells, but little is known about its involvement in biofilms and its development or maintenance. This study has been undertaken to address the adherence pattern and formation of biofilms by *E. coli* on stainless steel, polypropylene and silicone surfaces after 24 hours of growth at 37°C. Scanning electron microscopy (SEM) was used for direct examining the cell attachment and biofilm formation on various surfaces and it was found that, in presence of *bolA*, *E. coli* cells were able to attach to the stainless steel and silicone very well. By contrast, polypropylene surface was not found to be attractive for *E. coli* cells. This indicates that *bolA* responded and can play a major role in presence and absence of *rpoS* in cell attachment.

Keywords: *E. coli*; *bolA*; *rpoS*; biofilms; microbial adhesion

Introduction

Bacteria commonly grow in surface-attached densely packed communities known as biofilms [1]. In other words, aggregates of cells embedded in a gluey matrix, the extracellular polymeric substance (EPS) [2]. Present view on biofilm formation is much more intricate, and is considered a well-controlled phenomenon [3, 4]. Stress may be defined as any damaging factor that adversely affects the growth or survival of microorganisms [5]. Outcomes of stresses applied to microorganisms differ. In response to changes in their surroundings, bacteria have the ability to regulate the expression of genes that control their growth and physiology quickly [6]. In *E. coli*, two of the possible important genes for biofilm growth are *rpoS* and *bolA*. *RpoS* is also called as a master regulator of general stress response [2]. Even though many study have revealed the importance of *rpoS* and it's regulated genes in planktonic cells under stress conditions, but less is known about the functions of *rpoS* in biofilms. In contrast, *bolA*, which is a morphogene in *E. coli* is known for over expressing under stressed environments, resulting in round morphology [7, 8]. It was foremost described to be implicated in adaptation to stationary form of growth. However, its function is still not completely understood and is not only confined to stationary phase, but its expression might also 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 [9, 10]. *E. coli* seems to adhere and initiate biofilm development on specific and favorable surfaces, especially in response to specific environmental cues.

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 [11]. Bacteria are capable of sensing surfaces and adhesion occurs when microorganisms deposit and attach onto surfaces [12]. This attachment initiates a complex differentiation programme resulting in the synthesis of alginate and biofilm formation process. One study

revealed that, necessary and responsible genes for alginate production in *Pseudomonas aeruginosa* were shown to be up regulated within 15 minutes of contact with surface [13]. Various changes in gene regulation cause the biofilm cells to become phenotypically and metabolically different from their planktonic counterparts [3, 4, 14]. This difference has been persuasively shown in *E. coli, B. cereus, P. aeruginosa and P. putida. B. cereus* a well-known food-poisoning organism [15] that produces biofilms on stainless steel in protein-rich media such as milk [16].

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. Recent example of one such study, which has assessed the occurrence of biofilm formation by human anaerobic periodontopathogens like *Fusobacterium nucleatum*, *Eikenella corrodens* and *Capnocytophaga spp.* [17]. The adhesion of *Bacillus spp*. has also been extensively studied, 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 [18]. 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 [19]. The potential of bacteria to produce biofilms can be measured in the laboratory using microtiter plates. 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 Microscopy. The most common method for the enumeration and morphological observation of microorganism on various surfaces is microscopy [20]. Scanning electron microscopy is considered the most appropriate technique for evaluating the interaction of microorganisms in the biofilm matrix and was used for the investigation of biofilms on various substrates [20]. Involvement of *rpoS* and *bolA* genes in

biofilm formation under various stress-induced conditions has been shown and studied previously [2]. But, whether, these genes play an important role in attachment of *E. coli* to specific surfaces or not? is shown in this study.

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 OD600 = 1.0 and was considered as exponential growth phase, whereas OD600 = 2.2 was considered to be stationary growth phase.

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 37ºC, were stored at 4ºC. 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.

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.

Glycogen assay (Preliminary conformational test)

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 [21]. Glycogen phenotypes of WT *E. coli* and Δ *rpoS* strains reveal a functional and non-functional status of *rpoS* sigma factor (σ^S). Colonies were grown on LB agar plates overnight at 37°C and then stored at 4°C for another 24 h before they were flooded with concentrated iodine solution. 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 (WT) and (ii) white colonies indicate glycogen-deficient cells with non-functional σ^S (Δ).

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 OD600 of 1.0 was 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 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.

Scanning Electron Microscopy Observations

The scanning electron microscopy (SEM) observation was made of at least 15 fields of each biofilm covered slides. Prior to the SEM observations, the biofilm samples were steadily dehydrated in an absolute ethanol 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, modifying the real biofilm structure, as has been

documented by [22]. SEM observations were visualized through the acquisition of representative microphotographs.

Results

After 24 hours of growth at 37°C, adherence pattern of *E. coli* K-12 MG1655 on silicone, polypropylene and stainless steel was studied. It was found that mutation in *bolA* did not support the cells to attach to polypropylene surface (image not shown). While, in presence of *bolA*, *E. coli* cells were somehow able to attach to the polypropylene surface. Very well attachment pattern was seen on stainless steel surface in presence of *bolA* (Fig. 1). It can possibly form biofilms too under various environmental stress conditions on stainless steel. All three strains were able to attach to silicone surface very well (Fig. 3, 5 and 7). WT strain (i.e. in presence of both *rpoS* and *bolA* genes) was found to form a thick biofilm mass on silicone surface, which shows that silicone is not a suitable surface for manufacturing and designing medical implants. In other words, it is the best surface for *E. coli* to attach and grow as biofilms without any stress conditions. This again proves the importance of *rpoS* and *bolA* genes in *E. coli* attachment and biofilm formation. It also indicates that *bolA* responds and get regulated by some other genes in absence of *rpoS*, which is also stated by Adnan *et. al.* in *bolA* expression studies in planktonic and biofilm phase in both presence and absence of *rpoS* gene (3). Polypropylene was not found to be the core surface for *E. coli* cells to attach and grow as biofilms (Fig. 2 and 6). Some unusual result was found in case of WT strain of *E. coli* on stainless steel surface. No attachment at all was seen on stainless steel by WT *E. coli* (image not shown). While, $bolA^{+}/rpoS^{-}$ (presence of *bolA* and absence of *rpoS*) and $rpoS^{+}/bolA^{-}$ (presence of *rpoS* and absence of *bolA*) strains were able to attach very well on stainless steel surface. This point is under investigation at molecular level and can be studied further to come up with a justification that what makes *E. coli* to attach to stainless steel with mutation in any one of these two genes, but not with WT.

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

Condition: P1: Plate 1

Strain: WT: Wild Type, B: *bolA*$^{+}$/*rpoS*$^{-}$, R: *rpoS*$^{+}$/*bolA*$^{-}$

Substrate: SI: Silicone, ACO: Stainless Steel, PP: Polypropylene

1/2: Magnification under 1000x (1) and 5000x (2).

Table 1: Condition used in this study with the designated names and remarks

Plate	Strain	Material	Name	Figure	Remarks
24 h @ 37 °C	*WT*	Stainless Steel	-	NA	Nothing Visible
		Silicone	P1WTSI 1/2	7	Thick biofilm
		Polypropylene	P1WTPP 1/2	6	Less attachment
	bolA^{+}/*rpoS*$^{-}$	Stainless Steel	P1BACO 1/2	1	Very well attached
		Silicone	P1BSI 1/2	3	Very well attached
		Polypropylene	P1BPP 1/2	2	Less attachment
	rpoS^{+}/*bolA*$^{-}$	Stainless Steel	P1RACO 1/2	4	Very well attached
		Silicone	P1RSI 1/2	5	Very well attached
		Polypropylene	-	NA	Nothing visible

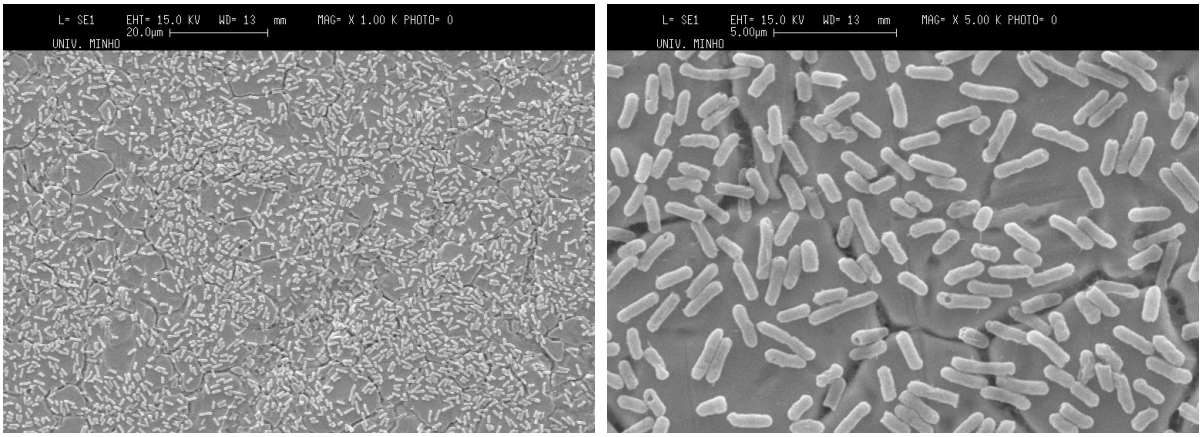

Figure 1 (P1BACO 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 (***bolA*$^{+}$*/rpoS*$^{-}$**)** after 24 hours at 37°C on stainless steel.

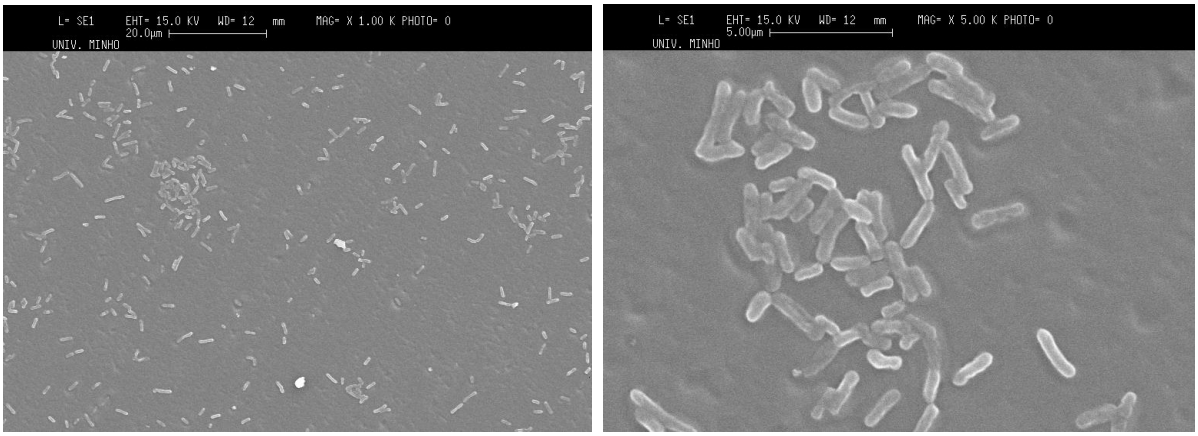

Figure 2 (P1BPP 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 (***bolA*$^{+}$*/rpoS*$^{-}$**)** after 24 hours at 37°C on polypropylene.

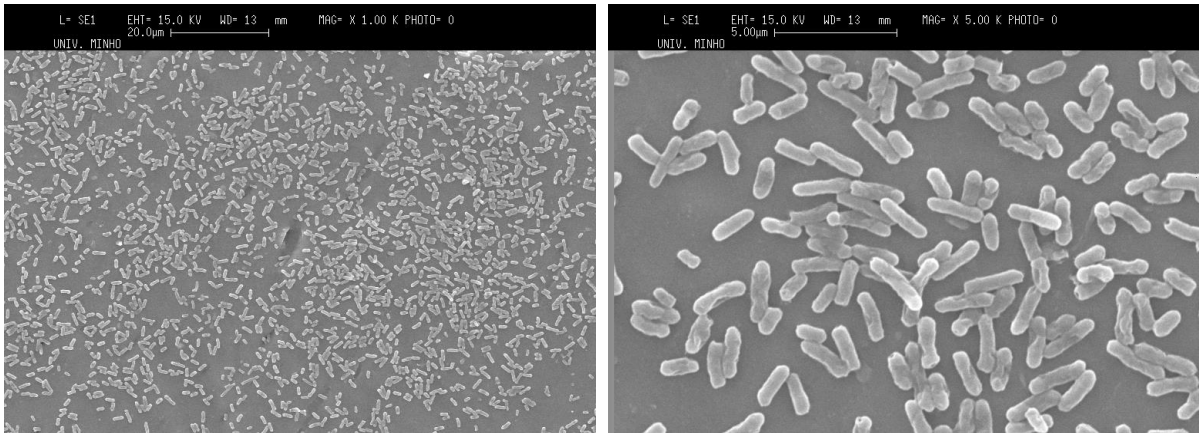

Figure 3 (P1BSI 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 **(*bolA*$^{+}$/*rpoS*$^{-}$)** after 24 hours at 37°C on silicone.

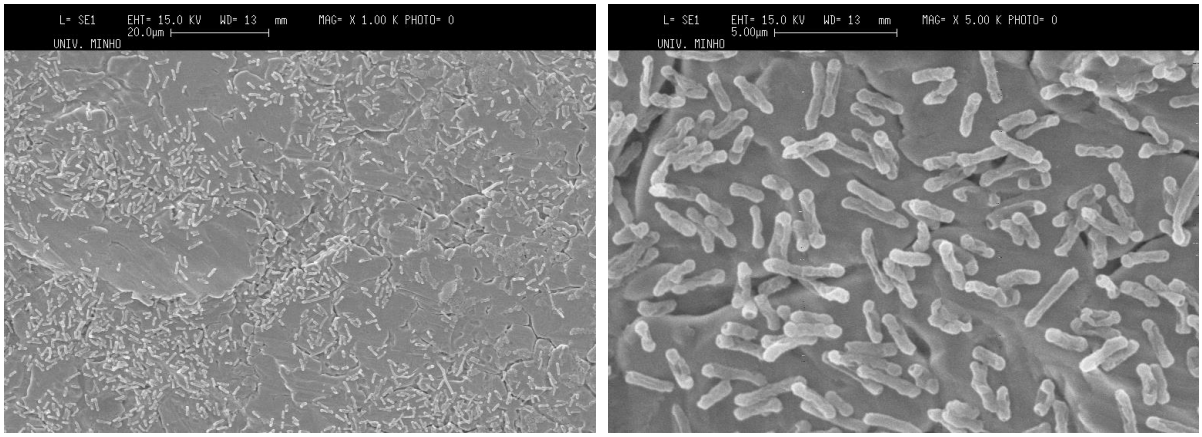

Figure 4 (P1RACO 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 ***(bolA*$^{-}$ */rpoS*$^{+}$*)*** after 24 hours at 37°C on stainless steel.

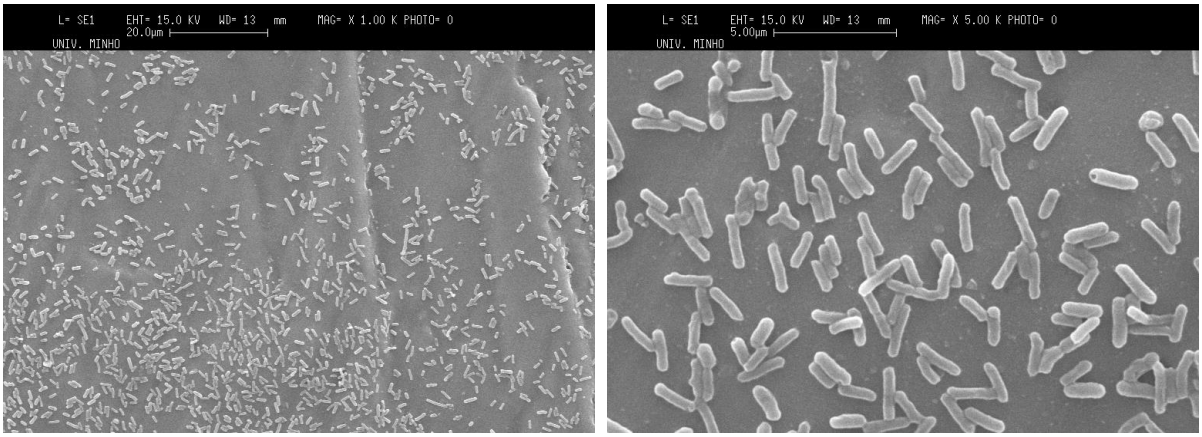

Figure 5 (P1RSI 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 ***(bolA*** $^{-}$ ***/rpoS*** $^{+}$ ***)*** after 24 hours at 37°C on silicone.

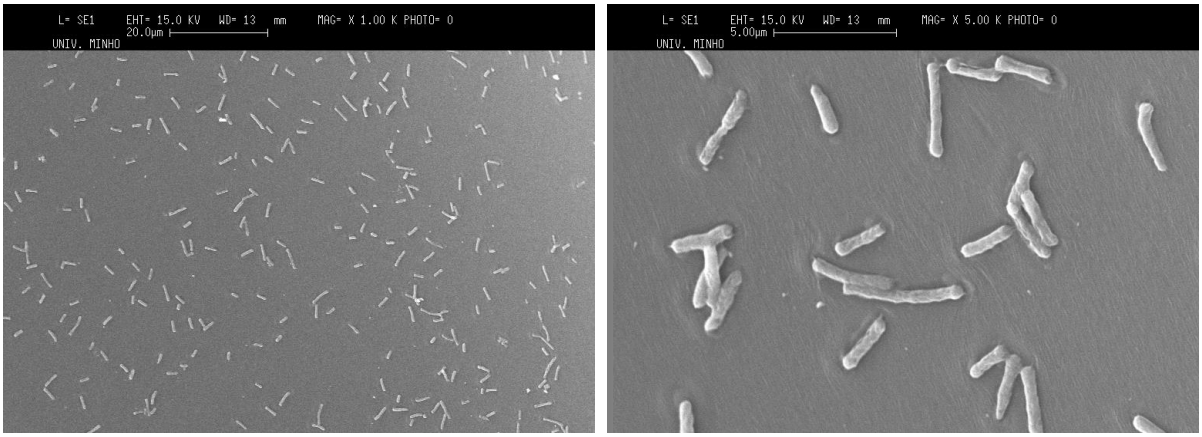

Figure 6 (P1WTPP 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 (WT) after 24 hours at 37°C on polypropylene.

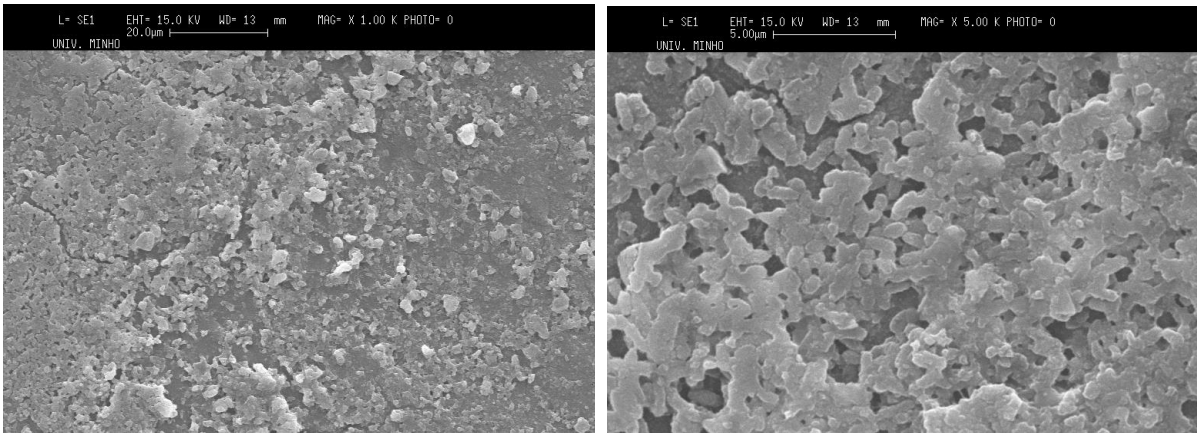

Figure 7 (P1WTSI 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 (WT) after 24 hours at 37°C on silicone.

Discussion

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 and biofilm formation on silicone surface, as compared to stainless steel and polypropylene surfaces. This observation suggests the ability of the *E. coli* cells to adhere to silicone is greater than their ability to adhere to stainless steel or polypropylene. Our study is also indicating, the role of *rpoS* and *bolA* in cell attachment and importance of silicone and stainless steel as the optimal surface for most bacterial biofilms to attach and develop without any induced stress conditions. The extent of biofilm accumulation on surfaces is controlled by the amount of nutrient available for cell replication and EPS production [23, 24]. A rich nutrient environment provides an ideal environment for bacteria to adhere, thus triggering biofilm formation through the secretion of EPS [25]. Bacteria do not form biofilms where the nutrients are lacking. They will leave the environment and revert back to the free-swimming life style [26]. Similarly, *E. coli* cells reach the highest population density when the nutrients in the environment are optimal. This study can be further experimented up to 72 hours with reduced nutrient availability, and various other

induced stress conditions. Variation in cell morphology under these environments and surfaces can also be studied. The scanning electron microscopic evidence provided by this study shows the attachment of *E. coli* on various substrates. SEM was also 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.

Acknowledgements

We gratefully acknowledge University of Hail, Saudi Arabia for their kind support and all the staff members and students of IBB, Centre of Biological Engineering, University of Minho for lending their expertise and assistance with Scanning Electron Microscopy.

Conflict of Interests

There is no conflict of interests.

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List of Table

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Plate	Strain	Material	Name	Figure	Remarks
24 h @ 37 °C	*WT*	Stainless Steel	-	NA	Nothing Visible
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		Polypropylene	P1BPP 1/2	2	Less attachment
	rpoS^{+}/*bolA*$^{-}$	Stainless Steel	P1RACO 1/2	4	Very well attached
		Silicone	P1RSI 1/2	5	Very well attached
		Polypropylene	-	NA	Nothing visible

Figure Legends

Figure 1 (P1BACO 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 **(*bolA*$^{+}$/*rpoS*$^{-}$)** after 24 hours at 37°C on stainless steel.

Figure 2 (P1BPP 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 **(*bolA*$^{+}$/*rpoS*$^{-}$)** after 24 hours at 37°C on polypropylene.

Figure 3 (P1BSI 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 **(*bolA*$^{+}$/*rpoS*$^{-}$)** after 24 hours at 37°C on silicone.

Figure 4 (P1RACO 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 ***(bolA*$^{-}$ */rpoS*$^{+}$*)*** after 24 hours at 37°C on stainless steel.

Figure 5 (P1RSI 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 ***(bolA*$^{-}$ */rpoS*$^{+}$*)*** after 24 hours at 37°C on silicone.

Figure 6 (P1WTPP 1/2): Scanning electron microphotograph of *E. coli* K-12 MG1655 (WT) after 24 hours at 37°C on polypropylene.

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