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Effect of External Iron Concentration on Biofilms Formed By Enteropathogenic *Escherichia coli*

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ABSTRACT

Microorganisms growing as collective communities called 'biofilms' are widely recognised today. Our understanding of this growth style of bacteria is however, still limited. Biofilms impact almost all aspects of human life: Personal, medical and industrial. *Escherichia coli* are model organisms for the study of biofilms. In this work, eight Entero Pathogenic *E. coli* (EPEC) were investigated for their biofilm forming abilities in iron limited and excess conditions. *E. coli* were able to form biofilms within a limited range of external iron concentration. This study observed a difference in iron requirements for planktonic and biofilms growth with biofilm bacteria needing higher concentrations of iron. Viable counts obtained from biofilms grown on the pegs modified Calgary Biofilm Device were found to be different and did not reveal any trends. This study underpins our understanding that external iron availability influences biofilms formed by these *E. coli* strains.

Keywords: Extrapolymeric; Kreb's cycle; Planktonic; Biofilm;*E. coli* strains; Morphology

INTRODUCTION

Escherichia coli commonly colonises the human intestine and exists as a biofilm *in-vivo*. These are a source of various extraintestinal infections such as urinary tract infections, infantile septicaemia, meningitis, urinary tract, gastrointestinal infections among others [1]. The infections spread through faecal-oral route. *E. coli's* ability to cause infections is attributed to its virulence factors, ability to invade, colonize and form biofilms [2]. As biofilms, microorganisms are irreversibly attached to a surface which can be biotic or abiotic and are enclosed in a matrix of extra polymeric substances that is produced by the microbes themselves [3]. These also exhibit an altered phenotype and high resilience to physical attack, desiccation, oxidative stress or attack by chemicals, biocides, immunological agents, antibiotics, bacteriophages and protozoa. The presence of biofilm matrix and slower growth rates play a crucial role in this resilience against antimicrobials making these difficult to eradicate.

It is suggested that external iron concentration may encourage microorganisms to transition from planktonic form to sessile biofilm form [4]. Iron plays an important role in multiple crucial biological processes such as photosynthesis, nitrogen fixation, respiration, Kreb's Cycle, DNA synthesis and other processes; therefore is essential for all life [5]. It is also the fourth most abundant element on Earth's crust; and exists as ferric (Fe+3) and ferrous (Fe+2) state. However, in nature, iron has limited availability; the most commonly available form of iron is its ferric form which has a solubility of 10^{-18} M at neutral pH 6 [6]. This is much less than the required concentration (10^{-7} to 10^{-5} M) for optimal growth of bacteria [7]. Also, the iron concentration needed to support the planktonic growth is different to those required for biofilm [8]. It has been reported that lower concentration is needed for planktonic growth and motility, whereas higher concentrations are needed for aggregation and biofilm formation. Entero Pathogenic *E. Coli* (EPEC) are members of group of *E. coli* referred to as attaching and effacing

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E. coli and can cause persistent diarrhoea which may result in death in severe cases [9]. These characteristically adhere to the host cell surface forming localised microcolonies. The study is aimed at investigating the effect of external iron concentration and its type on biofilm formation.

MATERIALS AND METHODS

Biofilm Formation

Luria Bertani without NaCl broth (LBNS) was prepared from ingredients. The dLBNS was prepared by addition of DIP® (Sigma-Aldrich, UK) to LBNS and autoclaving at 121°C at 1.5 atmospheric pressure for 30 minutes followed by holding the medium at room temperature for 24 h to allow full binding of iron to the chelating agent [10]. To the prepared dLBNS the following compounds were added, separately: Ferric ammonium citrate, $C_6H_8O_7.xFe_{3+}.yNH_3$ (Acros Chemicals, UK), ferrous sulphate (Sigma-Aldrich, UK), $FeSO_4$; ferric chloride, $FeCl_3$(Sigma-Aldrich, UK) and ammonium sulphate $(NH_4)_2SO_4$, ammonium citrate, $(NH_4)_2C_6H_6O_7$, sodium citrate, $N_aC_6H_7O_7$, ammonium chloride NH_4Cl and citric acid, $C_6H_8O_7$ (all from Sigma-Aldrichand UK) to give the final concentrations of these compound in dLBNS at 0.1 µM, 1 µM, 10 µM, 100 µM, 200 µM, 300 µM, 400 µM, 500 µM and 1000 µM. LBNS was positive control and dLBNS negative control [11]. The inoculum was prepared by inoculating LBNS with single colony (18 h-20 h) obtained on Tryptone Soya Agar (Sigma-Aldrich, UK) followed by sub-culturing (1:9) for 3 consecutive days at 37°C for 18 h-20 h. Cells obtained were washed twice in physiological saline (4000 rpm) and then washed once in sterile dLBNS and re-suspended in dLBNS [12]. The suspension was diluted 1:10 in respective test media to obtain final inoculum. Wells in the 96-well microtitre plates [Nunc, USA] were filled with 180 µl of test media to which 20 µl of inoculum was added, giving a concentration of 106 bacterial cells/ml [13]. The prepared microtitres were placed in a humidified chamber and incubated at 37°C ± 2°C for 20 h. Planktonic growth was recorded as optical density observed at 600 nm. Resulting biofilms were quantified by crystal violet binding assay [14]. Modified Calgary Biofilm Device (mCBD) and Adhesion Efficiency: A modification of the original calgary device consists of two components [15]. The top component consists of 96 pegs (transferable solid phase screening system, VMR, UK) and is designed to fit into the well of the 96-well microtitre plate, without the pegs touching the bottom or the sides [16]. Biofilms were grown in mCBD to determine the adhesion efficiency of strains for 60 h and pegs were removed from lids and washed once in sterile physiological saline followed by orbital shaking for 2 min in fresh saline, the cell suspension thus obtained was plated on TSA and incubated for 24 hours at 37°C [17]. Bacterial cell concentrations were determined upon colony counting for both pegs and the corresponding wells. The percentage of adhesion efficiency was determined by the ration between the number of biofilm cells and planktonic cells. This was determined for supplemented coumpounds at 500 µM concentration. All experiments were conducted in triplicate, thrice. Statistical significance was determined by analysis of variance at p=0.05. Visualisation of biofilms: Biofilms grown on pegs were visualised using LIVE/DEAD BacLight bacterial visibility kit (Molecular Probes, Eugene, Oregon, USA) according to manufacture's instructions and viewed in blue fluorescent light using F-View soft imaging system (Olympus) [18].

RESULTS

All *E. coli* strains were able to grow planktonically and form biofilms in LBNS and in dLBNS, all strains could grow planktonically albeit to a much lesser extent and it did not support formation of biofilm by any strain [19]. This shows the extent of reductions in planktonic growth and biofilms formed as a percentage when grown in dLBNS to LBNS [20]. Percentage of reduction in planktonic growth and biofilms formed were calculated as described in section 3.2.7 [21]. It is calculated in reference to LBNS (positive control), Percentage of Reduction=[(ODControl-ODTest)/ODControl] x 100%. OD for planktonic growth was measured at 600 nm and for biofilms at 570 nm. The values shown are mean of three experiments conducted in triplicate **(Table 1)**.

Table 1: Percentage of reduction in planktonic growth and biofilm formed by E. coli under iron chelated conditions.

	Percentage of reduction	
Strain	**Planktonic growth**	**Biofilm formed**
E. coli 54	96.8	86.4
E. coli 39/7	88.1	90.3
E. coli SK39	88.9	89.6
E. coli 555	96.6	85.9
E. coli 25922	75.9	87.4
E. coli 2637	92.6	80.1
E. coli O125	49.7	87.7
E. coli H1941	94.2	86.6

A gradual increase in planktonic growth was observed with increasing concentration of iron compounds; however, the same was not observed for biofilms formed [22]. The biofilm formation restored for all strains at much higher concentrations to planktonic growth restoration concentrations indicating extent of external iron concentration impacts biofilms formation in *E. coli* neither planktonic growth nor biofilm formation got restored for any strain upon addition of ammonium sulphate, ammonium citrate, sodium citrate, ammonium chloride or citric acid [23]. Each strain responded differently to iron concentration in the medium. It also differed with iron ion type added to the medium as shown in **Table 2**.

Table 2: Concentrations of added iron compounds at which biofilm formation re-occurred. Concentrations are in µg/mL. The values represent concentration of added iron compounds at which biofilm formation was first recorded. The experiments were performed three times in triplicate.

	Compounds		
Strains	**Ferric ammonium citrate**	**Ferrous sulphate**	**Ferric chloride**
E. coli 54	0.1	0.1	0.1
E. coli 39/7	500	200	100
E. coli SK39	0.1	1	0.1
E. coli 555	0.1	0.1	0.1
E. coli 25922	10	0.1	0.1
E. coli 2637	0.1	0.1	0.1
E. coli O125	200	300	200
E. coli H1941	200	0.1	200

No pattern could be discerned in terms of added iron concentration or nature of iron compound added [24]. For most strains biofilms restored at concentration range between 100 ug/ml-200 µg/ml, however the biofilms formed at these concentrations were not to the same extent as LBNS **(Table 3)**.

Table 3: Concentration of added iron compound at which biofilm formation was similar or greater than in LBNS (positive control). Concentrations are in µg/mL. The values represent concentration of added iron compounds at which biofilm formation was first recorded.

	Compounds		
Strains	**Ferric ammonium citrate**	**Ferrous sulphate**	**Ferric chloride**
E. coli 54	100	100	100
E. coli 39/7	400	100	100
E. coli SK39	100	300	300
E. coli 555	200	200	100
E. coli 25922	200	100	100
E. coli 2637	200	100	100
E. coli O125	200	100	10
E. coli H1941	200	100	100

These results indicate that iron is needed for both planktonic growth and biofilm formation by the *E. coli* strains tested [25]. Biofilm formation is restored when concentration of iron in the medium reaches a threshold and that this threshold is different for each strain [26]. The strains continued to produce biofilms until the next threshold was reached, beyond which biofilm formation reduces. It can, therefore, be interpreted that only a range of iron availability is favourable for biofilm formation by these *E. coli* strains [27]. The recovered adherent cells were similar to those obtained from biofilms grown in LBNS (positive control). However a similar trend was not observed for percentage of adhesion efficiency as shown in **Table 4**.

Table 4: Effect of iron compounds on percentage of adhesion efficiency of E. coli strains. Concentration of supplemented iron in iron-replete medium 500 µM. The percentage of adhesion efficiency was determined by ratio of the number of adherent bacteria to planktonic bacteria in the same well.

	Adhesion Efficiency (%)			
Strain	**LBNS**	**Ferric ammonium citrate**	**Ferrous sulphate**	**Ferric chloride**
E. coli 54	0.013	0.051	0.039	0.081
E. coli 39/7	0.222	0.017	0.018	0.004
E. coli SK39	0.159	0.006	0.015	0.005
E. coli NCIMB 555	0.022	0.042	0.036	0.005
E. coli CMCC 2637	0.061	0.017	0.023	0.007
E. coli ATCC 25922	0.019	0.009	0.005	0.002
E. coli O125 3563	0.004	0.002	0.005	0.02
E. coli H1941	0.326	0.06	0.014	0.012

Adhesion efficiencies increased only for two strains *E. coli* 54 and *E. coli* NCIMB 555; all other strains had reduced efficiencies upon addition of iron compounds to dLBNS [28]. Then shows the microscopic images of biofilms formed by strains in on pegs in LBNS and dLBNS supplemented with iron compounds [29]. The biofilms were grown in LBNS for 60 h at 37°C, using TSP system [30]. These images demonstrate that all strains are capable to growing as biofilms on polystyrene surface of pegs of TSP system [31]. Strains differed in the extent and style of colonisation on the peg surface. Some strains form blanket like biofilm on the surface others form tiny clusters of a few bacterial cells while other exhibit microcolony formation [32]. The biofilms were stained in LIVE/DEAD BacLight™ bacterial viability kit and visualised in blue light using F-view soft imaging system, Olympus [33]. Images were taken at 40 × magnification. Scale Bar=50 µm **(Figure 1)**.

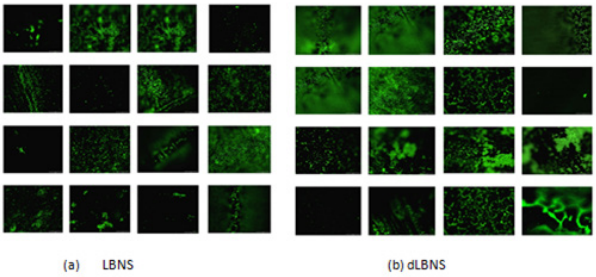

Figure 1: Microscopic images of E. coli biofilms formed in LBNS and dLBNS supplemented with added iron compounds.

Absorbance values for biofilm formation assay indicated dLBNS to be not a good supporter of biofilm formation by the strains [34]. This was further confirmed, as these were not visualised upon staining with LIVE/DEAD BacLight solution [35]. Presence of supplemented iron altered the biofilm morphology for strain. *E. coli* 54 formed dense clusters in presence of LBNS but formed lawn style growth in presence of ferric ammonium citrate and ferrous sulphate but formed small scattered clusters in presence of ferric chloride [36]. All strains showed such changes in the morphology of biofilms formed [37].

DISCUSSION

Iron is a limiting factor for many biological systems and important in biofilm formation [38]. And bacterial pathogenesis; the extent of iron availability can trigger the commensals in human body to enter pathogenic mode [39]. It is considered to be an important signal for bacteria to enter into biofilm growth style and sensed by bacteria to enter biofilm mode of growth [40]. Iron ions are also known to bind to the biofilm bacterial cells in greater numbers than to planktonic bacterial cells thus facilitating availability of iron to these microbial cells over their peers [41]. The objective of this study was to determine the effect of excess and limitation of external iron on the extent of biofilms formed by eight *E. coli* strains [42]. For the purpose DIP was used to chelate iron in the medium. This was intended to represent iron availability in mammalian hosts, where the concentration of extracellular free iron is as low as 10^{-18} M [43]. Its ready availability in the mammalian host is associated with reduction in resistance to infection and it is known to facilitate bacterial attachment to surfaces [44]. At this concentration, bacteria are unable to grow planktonically [45]. It was observed that availability of iron affected the biofilm formation for all strains tested [46]. Both biofilm and planktonic growth decreased significantly when iron was limited and was restored upon its addition. It has been reported that iron chelators contributed to restriction of biofilm formation [47]. The restoration biofilm formation upon addition to iron supplement was not influenced by any specific iron com-

pound, addition of all iron compounds led to biofilm formation being restored. However, the extent of biofilm formation was not in proportion to the concentration of iron used to supplemented medium [48]. Also, no such increase was observed on addition of any of the five control compounds (ammonium sulphate, ammonium chloride, sodium citrate and citric acid). These results indicate that iron depletion limits the ability of *E. coli* strains to form biofilm, while iron availability promotes biofilm formation. The presence of iron in the growth medium has been reported to facilitate bacterial attachment to surfaces. This may be the reason for the increased biofilm yield when iron was supplemented. These results are in contrast to other studies where researchers concluded that a lack of available iron induced biofilm formation in bacteria. This study observed a difference in iron requirements for planktonic and biofilms growth with biofilm bacteria needing higher concentrations of iron. Similar results were obtained in this study. These studies also reported a reduction in biofilms formed at concentrations higher than 500 µM [49-51]. This drop in biofilm production may occur due to the phenomenon of iron over load (resulting in toxicity to *E. coli*).

CONCLUSION

It is inferred that iron may enhance biofilm formation over a small range of concentrations only and iron concentrations below or above this range do not support biofilm formation. In experiments using mCBD (section 3.2.4), the concentration of adhering bacterial cells and percentage of adhesion efficiency did not show a consistent pattern. For most strains there was a decrease in the percentage of adhesion efficiency while some showed an increase. However, the decrease or increase was not proportionate to the amount added of the iron compound. Iron is also reported to influence other biofilm associated phenomena, e.g., quorum sensing and slime production (major constituents of extra cellular polymeric matrix). Addition of iron supplement like ferrous sulphate has been reported to restore biofilm formation. In the enteropathogenic *E. coli* strains studied here the biofilm formation reduced in iron reduced conditions and so did biofilm growth style associated characteristics of motility with an increased response when iron was re-supplied in the medium. The results of this study show that addition of ferric ammonium citrate, ferrous sulphate and ferric chloride support biofilm formation by *E. coli* over small concentration range, regardless of the iron compound used. It also affects the extent and the morphology of the biofilms formed.

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