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Research article

Effect of heterologous protein expression on *Escherichia coli* biofilm formation and biocide susceptibility

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Abstract: *Escherichia coli* is recognized as an excellent model for biofilm studies and one of the favourite hosts for recombinant protein expression. This work assesses the influence of heterologous protein production on biofilm formation and susceptibility to chemical treatment. Biofilm formation by two *E. coli* strains was compared using a flow cell system. One strain contained the commercial pET28A plasmid and the other a plasmid derivative with the same backbone but containing the enhanced green fluorescent protein (eGFP) gene. The susceptibility of biofilms to the biocide benzyldimethyldodecylammonium chloride (BDMDAC) was also assessed. It was found that the eGFP-expressing strain formed thicker biofilms with a higher cell density than the non-producing strain. Biofilms of both strains were neither completely inactivated nor removed by biocide treatment. Similar inactivation efficiencies were obtained, although biofilm cohesion was higher for the non-producing strain.

Keywords: biofilm; *Escherichia coli*; plasmid; heterologous protein expression; flow cell system; biocide treatment

1. Introduction

Escherichia coli has been extensively used for recombinant protein production [1,2,3] due to its fast growth at high cell densities, minimal nutrient requirements and well-characterized genetics, and due to the availability of a large number of cloning vectors and host strains [4]. This bacterium has the ability to accumulate recombinant proteins to at least 20% of the total cell protein [5] and has a good track record in drug regulatory authorities for the manufacturing of recombinant therapeutic

proteins [3]. Recombinant protein expression in *E. coli* is usually achieved by inserting the gene coding for the protein of interest into a multicopy plasmid under the transcriptional control of either a constitutive or inducible promoter [1].

The proximity of bacterial cells in biofilms provides an excellent environment for the exchange of genetic material carried by the plasmid [6]. Thus, the effect of *E. coli* plasmids (especially conjugative plasmids) on biofilm formation by their hosts has been described in numerous studies and most of these plasmids have been found to enhance biofilm formation [7–14]. Ghigo [7] was the first author to show that natural conjugative plasmids induced the biofilm formation of different *E. coli* K-12 strains. His work suggests that the conjugative pili responsible for the horizontal transfer of the plasmid may act as adhesins, connecting the cells and stabilizing the biofilm structure in hydrodynamic systems [7]. Reisner's results [8] also support and expand the idea that conjugative plasmids encode an important pathway for *E. coli* biofilm development, showing that the absence of biofilm growth was related with conjugative plasmids repressed for pili expression. Yang et al. [11] observed that mature biofilms harbouring an F-like conjugative plasmid (R1*drd*19) were thicker than biofilms not bearing that plasmid. Interestingly, the presence of R1*drd*19 plasmid increased the expression of numerous chromosomal genes related to envelope stress, motility and other genes known to be involved in biofilm formation [11]. May and Okabe [15] also demonstrated that the F pilus caused increased colonic acid and curli production during biofilm development, promoting cell-surface adherence [16]. Other conjugative plasmids of *E. coli* (pOLA52 and pMAS2027) have been shown to enhance biofilm formation through type 3 fimbriae [6,14].

Although non-conjugative plasmids are commonly used for recombinant protein production in *E. coli*, few studies have addressed their effect on biofilm formation. Gallant et al. [17] noted that plasmids encoding the common β-lactamase marker TEM-1 reduced cell adhesion and biofilm formation. Conversely, *E. coli* O157:H7 cells carrying a 92kb virulent and non-conjugative plasmid (pO157) [18,19] influenced biofilm formation and architecture [20]. Under smooth flow conditions, the pO157 plasmid promoted biofilm development through increased EPS production and generation of hyperadherent variants [20]. Concerning the impact of expression plasmids, Huang et al. [21,22,23] studied the production of β-galactosidase in *E. coli* DH5α cells carrying a non-conjugative plasmid containing the *tac* promoter. These authors found that plasmid-bearing cells formed biofilms with a higher cell density when compared to non-transformed cells [21]. Furthermore, the recombinant protein was successfully produced in biofilm cells, but at a lower level than in planktonic cells [21,23]. Later, O'Connell et al. [24] have described the first system for high level heterologous protein production in *E. coli* biofilm cells using a pUC-based vector for the expression of enhanced green fluorescent protein (eGFP). These authors showed that the biofilm environment enhanced the eGFP production when compared to planktonic cells. Despite the enormous potential of biofilms as an expressing system, this subject remains largely unexplored.

In a previous study, our research group has shown that the presence of the non-conjugative plasmid pET28A in *E. coli* JM109 (DE3) cells increased biofilm formation under turbulent flow conditions when compared to non-transformed cells [25]. The *eGFP* gene was cloned into this plasmid (resulting in plasmid pFM23) [26] and it was shown that heterologous protein expression increased the spatial heterogeneity of the biofilms [27]. Since the expression of the *eGFP* gene increases the metabolic drain on the host cells, it is important to assess if it also affects the process of biofilm formation and resistance. The aim of this work was to assess the effects of heterologous protein production by comparing the biofilm formation of *E. coli* cells harboring plasmid pET28A

(devoid of the expression gene) or plasmid pFM23 (for the intracellular expression of eGFP), as well as the biofilm susceptibility to the biocide benzyldimethyldodecylammonium chloride (BDMDAC). This quaternary ammonium compound has already demonstrated strong antibacterial activity [28] and is often used in industrial applications [29].

2. Materials and Methods

2.1. Bacterial strain and plasmids

The *E. coli* strain JM109 (DE3) from Promega (USA) was used in this work because it is recommended for protein expression with the pET system [30], besides it has shown good biofilm-forming ability in turbulent flow conditions [25].

Competent *E. coli* cells were transformed by heat shock [31] with the control plasmid pET28A (Novagen, USA; Figure 1A) or with the expression plasmid pFM23 (Figure 1B), which was obtained by cloning the *eGFP* gene into the pET28A vector as previously described by Mergulhão et al. [26]. Transformants were selected on lysogeny broth (LB-Miller, Sigma, USA) agar supplemented with kanamycin (Eurobio, France).

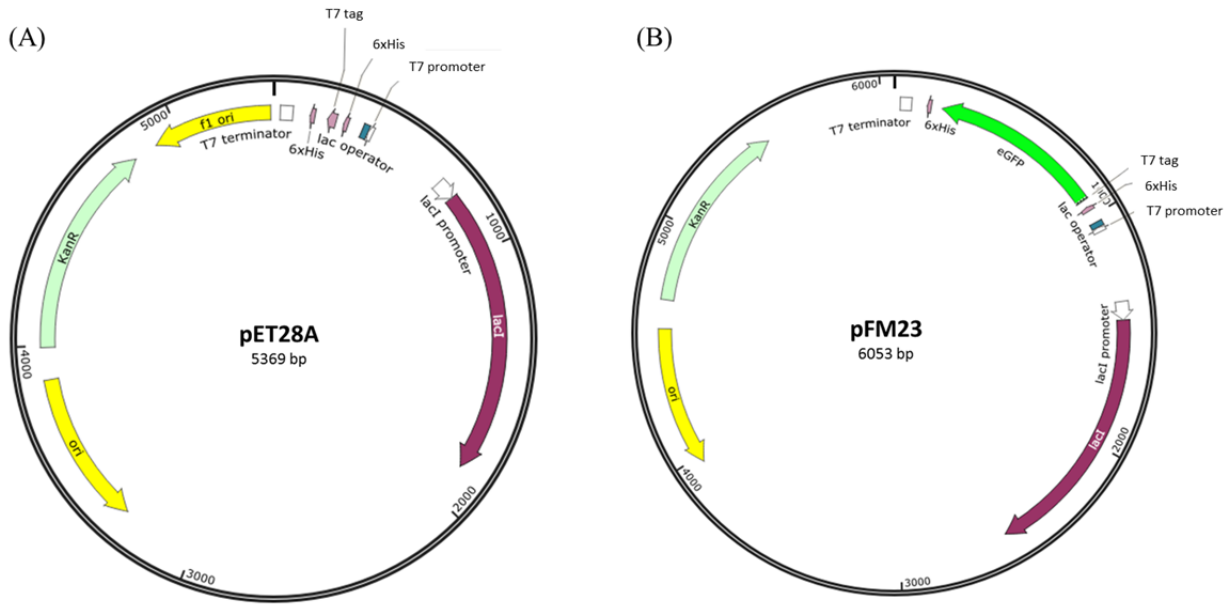

Figure 1. Plasmid maps of (A) pET28A and (B) pFM23. Both plasmids harbour (a) a pMB1 origin of replication (ori), (b) a repressor for the *lac* promoter (lacI), (c) a transcriptional promoter from the T7 phage (T7 promoter), (d) a lactose operator (*lac* operator), (e) an affinity purification tag (6 × His), (f) a T7 transcriptional terminator (T7 terminator), and (g) a kanamycin resistance gene (*Kan*R). Plasmid pFM23 contains the *eGFP* gene (eGFP).

2.2. Biofilm formation system and sampling

In order to assess the effect of heterologous protein expression on the biofilm-forming and resistance capacity of *E. coli* cells, the experimental apparatus presented in Figure 2 was used. It includes a recirculating tank where planktonic cells grow and a vertical flow cell for biofilm formation. Peristaltic and centrifuge pumps allow the circulation of the bacterial suspension in the system. The flow cell is composed by a semicircular Perspex duct (3.0 cm diameter and 1.2 m length, corresponding to a hydraulic equivalent diameter of 1.8 cm) with 20 apertures on its flat wall to fit removable rectangular pieces of Perspex (coupons). Polyvinyl chloride (PVC) slides (2 × 1 cm) were glued onto the coupons and were in contact with the bacterial suspension circulating in the flow cell reactor. This system allows individual sampling of each coupon without disturbing the biofilm formed on the others [32].

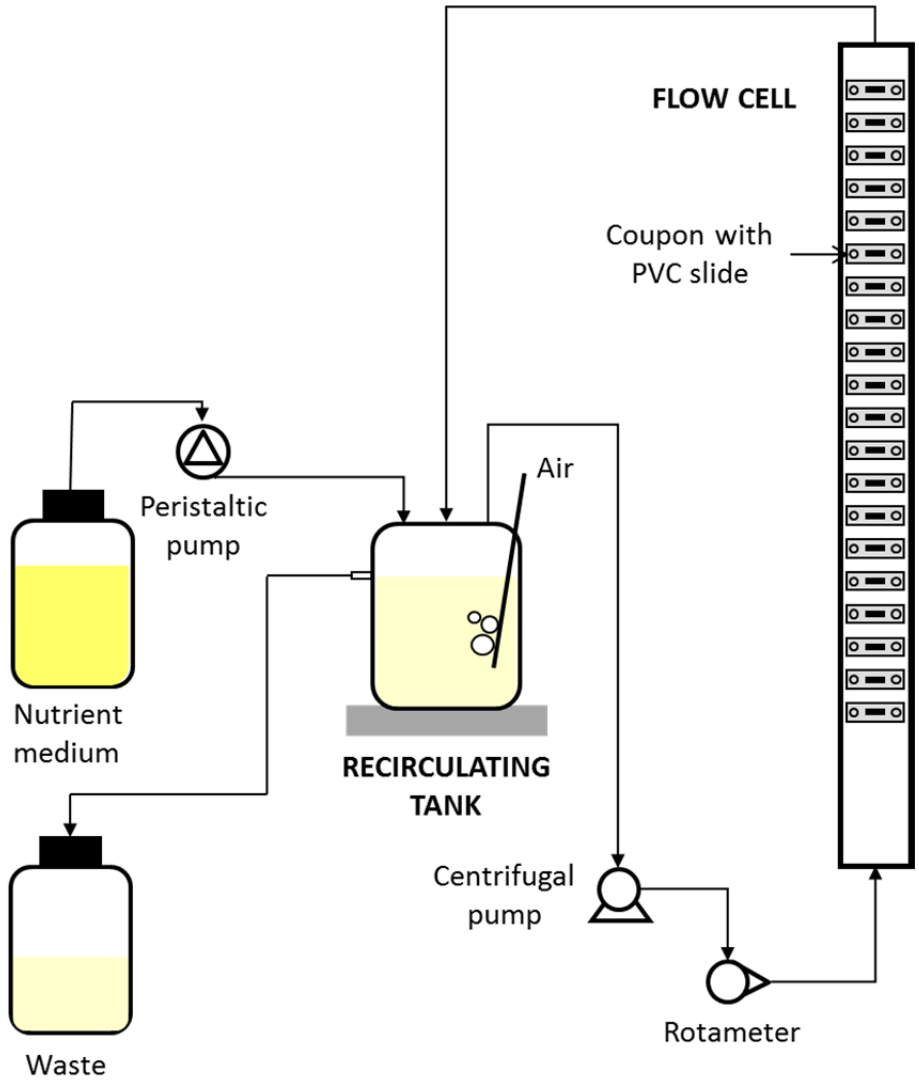

Figure 2. Schematic representation of the flow cell system.

E. coli cells containing the pET28A or pFM23 plasmid were grown by recirculating the bacterial suspension at 30 ºC during 12 days under a turbulent flow with a Reynolds number of 4600 (flow rate of 255 l h^{-1}) [25]. The recirculating tank of 1 l was continuously fed at a flow rate of 0.025 l h^{-1} with the nutrient medium consisting of 0.55 g l^{-1} glucose, 0.25 g l^{-1} peptone, 0.125 g l^{-1} yeast extract and phosphate buffer (0.188 g l^{-1} KH_2PO_4 and 0.26 g l^{-1} Na_2HPO_4), pH 7.0 [25]. For maintenance of selective pressure, the growth and feeding media were supplemented with 20 μg ml^{-1} kanamycin.

For biofilm sampling, the system was stopped every day to allow coupon removal and carefully started again maintaining the same flow conditions. Day 1 corresponds to the inoculation of the reactor system and the sampling was initiated on day 2 of the experiment.

2.3. Biofilm and planktonic cell analysis

Biofilm thickness was determined using a digital micrometer [32]. The needle connected to the digital micrometer was applied on the top of the biofilm and penetrated the sample to the bottom of the biofilm. The thickness was obtained by the difference between the readings made at the top and bottom of the sample. Ten measurements were made at random points on each coupon and the average value was determined. Then, the biofilm was resuspended and homogenized in 25 ml of 8.5 g l^{-1} NaCl solution to assess the total cell number by staining with 4'-6-diamidino-2-phenylindole (DAPI) [33] during the steady-state period (between days 7 and 12).

Regarding planktonic cells, optical density (OD) at 610 nm [32] and total cell number were assayed. Planktonic and biofilm culturability were calculated by dividing the number of colony forming units (CFU) by the total cell number obtained by DAPI counts. For assessing CFU, planktonic and biofilm samples were serial-diluted (into 9 ml of 8.5 g l^{-1} NaCl solution) and spread on top of solid growth medium (PCA; Merck, Portugal) supplemented with kanamycin (20 μg ml^{-1}). Enumeration of CFU was carried out after 24 h incubation at 30 ºC.

Specific eGFP production was assessed by the fluorometric method described in Mergulhão and Monteiro [34] during the steady-state period. A sample volume corresponding to an equivalent $OD_{610\ nm}$ = 1 was used to harvest the cells by centrifugation (3202 g for 10 min). The pellet was resuspended in 100 μL of Buffer I (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) and added to a 96-well microtiter plate (Orange Scientific, USA) already containing 100 μl of Buffer I. Fluorescence was measured using a microtiter plate reader (SpectraMax M2E, Molecular Devices, Inc., UK) with the excitation filter of 488 nm and the emission filter of 507 nm.

2.4. Biofilm susceptibility

The quaternary ammonium compound BDMDAC (Sigma, USA) was used to assess the biocide susceptibility of biofilms formed by both *E. coli* strains. For these experiments, biofilms were developed for 3 days and the biocide was applied as previously described by Teodósio et al. [25]. The biofilm cells were exposed to the biocide for 9 h and a coupon was sampled every hour to determine the biofilm thickness (using a digital micrometer), the total number of cells (obtained by DAPI staining) and the number of culturable cells (corresponding to the CFU counts).

Time zero corresponds to the moment when the biocide was introduced on the recirculating tank and the flow was re-established using the same conditions as for biofilm formation.

To evaluate the contribution of EPS to the biofilm resistance, the biofilm matrix was extracted using a Dowex resin (50 × 8, Na^+ form, 20–50 mesh; Fluka Chemika, Switzerland) and quantified in terms of protein and polysaccharide content [33]. Protein and polysaccharide assays were performed using biofilm suspensions before EPS extraction (total constituents) and with EPS (matrix constituents) after extraction.

2.5. Statistical analysis

The results presented in Figures 3 and 4 originated from averages of triplicate data sets obtained in independent experiments. Average standard deviations (SDs) on the triplicate sets were calculated for all analysed parameters. For biofilm formation (Figure 3), the following averages were obtained: SD < 13% for OD and SD < 27% for biofilm thickness. Concerning biofilm susceptibility (Figure 4), the following averages SDs were obtained: SD < 22% for biofilm thickness, SD < 5% for biofilm total cells, and SD < 14% for biofilm culturable cells.

Paired *t*-test analysis was performed based on a confidence level of 90% (differences reported as significant for *P* values < 0.1 and marked with *) and 95% (differences reported as significant for *P* values < 0.05 and marked with $^{*}_{*}$).

3. Results

3.1. Biofilm formation

The effect of heterologous protein expression on the biofilm-forming capacity of *E. coli* JM109 (DE3) cells harbouring the pFM23 plasmid was assessed by comparison with a strain bearing the plasmid backbone (pET28A) but devoid of the *eGFP* gene (Figure 3).

Although higher optical density values were registered for the eGFP-expressing cells (on average 20%) in most experimental points ($P < 0.05$ between days 5 and 12), the growth profiles of both *E. coli* strains were similar (Figure 3A). Planktonic cells grew up to day 5 and then the culture cell density decreased (about 40%) until the end of the experiment. The steady state concentration of planktonic cells was 1.6×10^8 cell ml^{-1} for the pET28 strain, whereas this concentration was 71% higher for the pFM23 strain.

The biofilm results (Figure 3B) revealed that the eGFP-expressing strain formed thicker biofilms than the pET28A strain. The thickness of the biofilms formed by the two strains was similar at the beginning of the experiment (about 150 μm), but a 74% increase was detected for the pFM23 strain between days 3 and 4, whereas the biofilm of the pET28A strain maintained its thickness ($P > 0.05$). Afterwards, biofilm thickness increased for both strains with the eGFP-producing strain forming 52% thicker biofilms. Regarding biofilm cells, the total cell number was 3.5×10^8 cell cm^{-2} for the pET28A strain in the steady-state period and this number was 92% higher for the producing strain.

Considering planktonic and biofilm culturability, values were lower (on average 55% in steady state) for eGFP-expressing cells when compared to the non-producing strain.

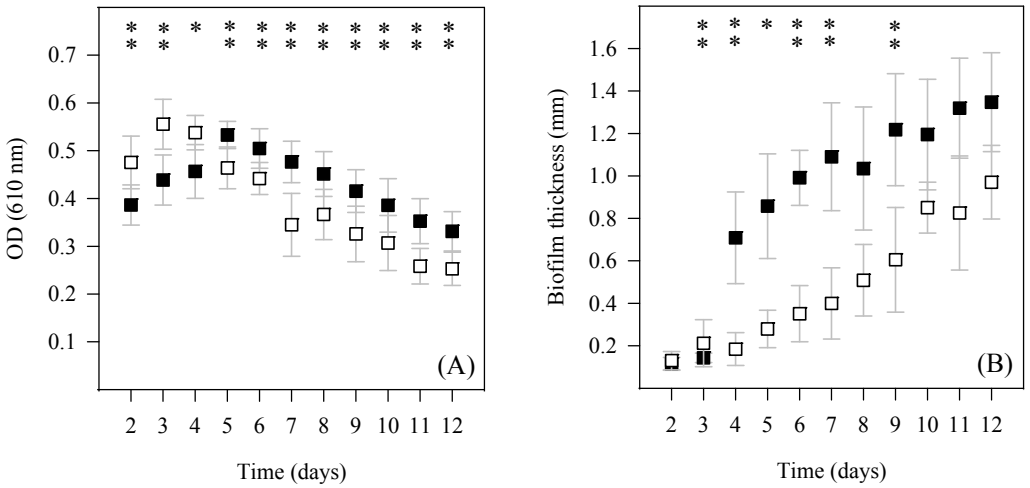

Figure 3. Time-course evolution of (A) OD and (B) biofilm thickness for *E. coli* JM109 (DE3) + pFM23 (■) and *E. coli* JM109 (DE3) + pET28A (□). The means ± SDs for three independent experiments are illustrated. Statistical analysis corresponding to each time point is represented with * for a confidence level greater than 90% ($P < 0.1$) and with ** for a confidence level greater than 95% ($P < 0.05$).

Fluorescence analysis has shown that eGFP was produced in both planktonic and sessile states, but that a higher production was obtained in biofilm cells (5.8 fg cell^{-1} versus 0.18 fg cell^{-1} for planktonic cells in steady state).

3.2. Biofilm susceptibility

Biofilms formed by each of the strains were subjected to a 9 h-biocide treatment and the biofilm susceptibility is shown in Figure 4.

Figures 4A and B show that the biofilm formed by the eGFP-producing strain was reduced significantly in the first two hours of biocide action (a thickness reduction of about 46% was observed accompanied by a reduction of 53% in the total cell number), but thereafter the thickness and cell number remained approximately constant. For the pET28A strain, the cell number was relatively constant during the BDMDAC treatment (around 7.4 log cell cm^{-2}), but the thickness of the biofilm was reduced by 47% in the first 3 h. Therefore, the biofilm was compressed during this period and apparently had a higher cohesion since cell removal was lower than for the eGFP-expressing strain. Both biofilms reached approximately the same thickness at the end of the treatment ($P > 0.05$ at 9 h). The number of biofilm culturable cells (Figure 4C) decreased for both strains, although a faster inactivation of pET28A-carrying cells was observed at the beginning of the biocide treatment. A 3 log reduction on cell culturability was observed for both types of biofilms after 9 h of exposure to the biocide, but they were neither completely inactivated (Figure 4C) nor removed (Figure 4A). The most important period for the antibiofilm action of BDMDAC was the first two hours of treatment.

In order to evaluate the role of EPS on the biofilm susceptibility, the matrix of the biofilms was characterized in terms of protein and polysaccharide contents. Although biofilms formed by the

eGFP-producing strain had higher total protein content (78% above than the non-producing strain), the mass percentage of proteins contained within the matrix was similar for both strains (28% for the eGFP-producing strain and 25% for the pET28A-bearing strain). The eGFP-expressing strain also produced biofilms with more polysaccharides (76% increase), however these biofilms only have 18% of the total polysaccharides in the matrix when compared to 53% in the pET28A strain.

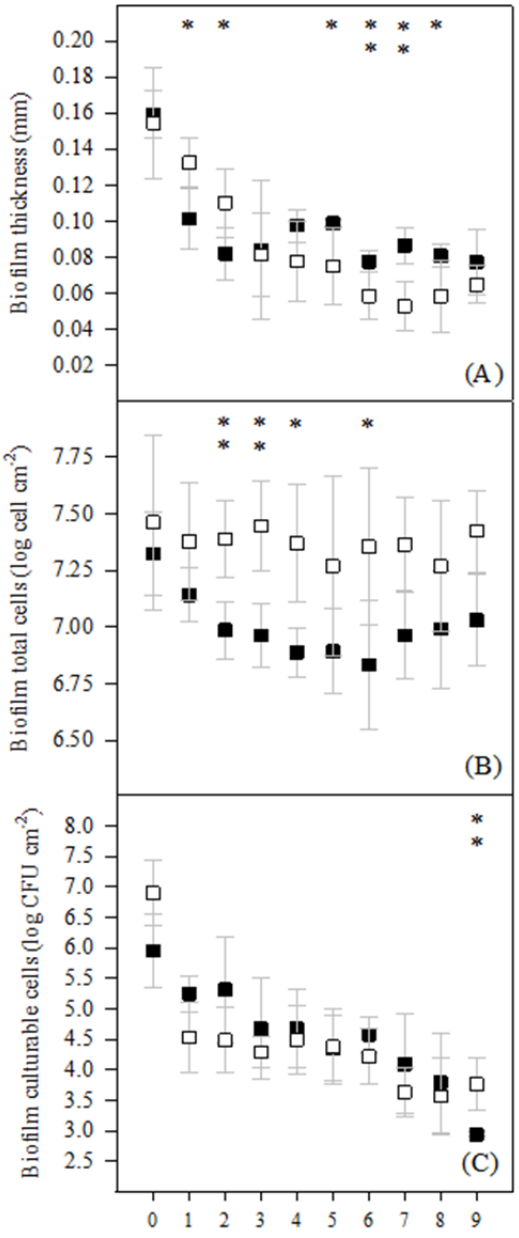

Figure 4. Biofilm susceptibility experiments to biocide treatment: (A) biofilm thickness, (B) biofilm total cells, (C) biofilm culturable cells. *E. coli* JM109 (DE3) + pFM23 (■), *E. coli* JM109 (DE3) + pET28A (□). The means ± SDs for three independent experiments are illustrated. Statistical analysis corresponding to each time point is represented with * for a confidence level greater than 90% ($P < 0.1$) and with ** for a confidence level greater than 95% ($P < 0.05$).

4. Discussion

4.1. Biofilm formation

Biofilm production was enhanced in *E. coli* cells bearing the pFM23 plasmid for heterologous protein expression when compared to the strain harbouring the plasmid without the expression cassette (pET28A vector). Several studies suggest that both conjugative and non-conjugative plasmids can promote biofilm formation [7,10,20,21,25]. In a previous work [25], it was shown that *E. coli* JM109 (DE3) cells transformed with plasmids pET28A and pUC8 formed thicker biofilms with a higher cell concentration than the non-transformed cells. In the present study, it is shown that heterologous protein production, which is known to increase the metabolic drain on the host cell [35,36], intensifies this effect. Sørensen and Mortensen [36] reported that the stress response of maintenance of a plasmid in the host cell is increased when a target protein is highly expressed. Environmental stress situations like starvation, amino acid depletion and heat shock resemble the stress response caused by a plasmid. Although the plasmid copy number influences the host stress response, the major cause for this metabolic burden is commonly the expression of plasmid-encoded genes such as antibiotic resistance or recombinant protein genes [36,37,38]. It is well known that heterologous protein expression induces the production of stress proteins, elevated respiration rates and high-energy requirements [36,39]. Since stress conditions can favour biofilm formation [40], the increased metabolic burden associated with heterologous protein expression may have stimulated biofilm formation by the eGFP-producing strain.

The metabolic stress imposed by the production of eGFP in both planktonic and biofilm cells may have a detrimental effect on cell viability, as observed by Kurland and Dong for bacterial suspensions [41] and by Gomes et al. [27] for biofilms grown under the same hydrodynamic conditions used in this work. Recombinant protein production significantly affects cell metabolism by channelling resources towards producing foreign proteins [36,42,43,44]. In particular, an increased protease activity and decreased growth rate and cell viability are some stress signals that can be induced during recombinant protein synthesis [35,41,45,46]. In this work, planktonic cell growth is not decreased in eGFP-producing cells probably because their production levels are much lower than in biofilm cells.

Our results show that the biofilm state enhance recombinant protein production. This corroborates the results obtained by O'Connell et al. [24] using *E. coli* ATCC 33456 containing the plasmid pEGFP (a pUC family vector) grown in a chemostat and in a parallel plate flow cell. Furthermore, it is shown that continuous biofilm cultures for recombinant protein production are beneficial for plasmid maintenance when compared to chemostats [24] since sessile cells tend to grow more slowly than planktonic cells [47], leading to fewer divisions and correspondingly less plasmid segregation. Since the results indicate that heterologous protein production is up to 30-fold higher in biofilm cells than in planktonic cells, it is possible that a biofilm reactor system may be devised for industrial protein production. In such a reactor, the biofilm would have to be retrieved from an appropriate surface to initiate downstream processing. Besides the reactor itself, there are other surfaces (like tubing, pumping equipment, etc.) that need to be cleaned after each production cycle using standard cleaning in place (CIP) procedures. These CIP protocols often include biocides and disinfectants like BDMDAC and therefore it is useful to understand if the biofilm susceptibility to this type of agent changed upon heterologous protein production.

4.2. Biofilm susceptibility

The effects of biocide treatment on eGFP-producing and non-producing biofilms were assessed regarding thickness, total cell number and culturable cell number. Results show that the treatment was not sufficient for complete biofilm removal nor inactivation. In a previous study [48], the same concentration of benzalkonium chloride (BAC)-a quaternary ammonium compound that includes BDMDAC in its composition-was also ineffective in removal of *Pseudomonas* biofilms. A more recent report [49] showed that the percentages of killing and removal of *P. fluorescens* biofilms by BAC were only 15.5% and 13.8%, respectively. Simões et al. [48] demonstrated that the presence of BAC increases biofilm mechanical stability. On the other hand, it is known that the antimicrobial mode of action of certain cationic surfactants (like QACs) has been attributed to their positive charge, which promotes an electrostatic interaction with negatively charged sites on cell membrane [50]. Therefore, it is conceivable that the electrostatic interactions can increase cell-to-cell cross-linking, preventing cell removal and resulting in more compact biofilms.

During biocide treatment, the biofilm formed by the pET28A-bearing strain was more compacted than the one obtained with the eGFP-producing strain and this may be related to a higher mass percentage of polysaccharides localized at the matrix [51]. However, the same extent of cell killing was achieved for both strains, which indicates that mass transfer within the biofilm was not controlling biofilm susceptibility. Araújo et al. [49] tested the penetration ability of biocides BAC and BDMDAC in *Pseudomonas* biofilms and concluded that the ability of an antimicrobial agent to penetrate a biofilm is not correlated with its killing and removal efficiencies. This reinforces the fact that antimicrobial susceptibility in biofilms is multifactorial and that mass transport limitations are part of the problem but should not be considered alone.

5. Conclusion

This study shows that heterologous protein expression can have a positive effect on *E. coli* biofilm development. It was also demonstrated that biofilm cells can produce a model recombinant protein at much higher levels than planktonic cells. Apart from affecting recombinant protein production, the physiological changes associated with the transition from planktonic to biofilm growth can impact the biofilm susceptibility to biocides. A better understanding of these processes is important for the manipulation of biofilms for biotechnological applications such as the high-level production of heterologous proteins.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

References

1. Mergulhão FJM, Monteiro GA, Cabral JMS, et al. (2004) Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*. *J Microbiol Biotechnol* 14: 1–14.
2. Sanchez-Garcia L, Martín L, Mangues R, et al. (2016) Recombinant pharmaceuticals from microbial cells: a 2015 update. *Microb Cell Fact* 15: 1–7.
3. Overton TW (2014) Recombinant protein production in bacterial hosts. *Drug Discov Today* 19: 590–601.
4. Baneyx F (1999) Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol* 10: 411–421.
5. Pines O, Inouye M (1999) Expression and secretion of proteins in *E. coli*. *Mol Biotechnol* 12: 25–34.
6. Ong CL, Beatson SA, McEwan AG, et al. (2009) Conjugative plasmid transfer and adhesion dynamics in an *Escherichia coli* biofilm. *Appl Environ Microbiol* 75: 6783–6791.
7. Ghigo JM (2001) Natural conjugative plasmids induce bacterial biofilm development. *Nature* 412: 442–445.
8. Reisner A, Höller BM, Molin S, et al. (2006) Synergistic effects in mixed *Escherichia coli* biofilms: conjugative plasmid transfer drives biofilm expansion. *J Bacteriol* 188: 3582–3588.
9. Reisner A, Haagensen JA, Schembri MA, et al. (2003) Development and maturation of *Escherichia coli* K-12 biofilms. *Mol Microbiol* 48: 933–946.
10. May T, Okabe S (2008) *Escherichia coli* harboring a natural IncF conjugative F plasmid develops complex mature biofilms by stimulating synthesis of colanic acid and curli. *J Bacteriol* 190: 7479–7490.
11. Yang X, Ma Q, Wood TK (2008) The R1 conjugative plasmid increases *Escherichia coli* biofilm formation through an envelope stress response. *Appl Environ Microbiol* 74: 2690–2699.
12. Król JE, Nguyen HD, Rogers LM, et al. (2011) Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. *Appl Environ Microbiol* 77: 5079–5088.
13. Norman A, Hansen LH, She Q, et al. (2008) Nucleotide sequence of pOLA52: a conjugative IncX1 plasmid from *Escherichia coli* which enables biofilm formation and multidrug efflux. *Plasmid* 60: 59–74.
14. Burmølle M, Bahl MI, Jensen LB, et al. (2008) Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in Enterobacteriaceae strains. *Microbiology* 154: 187–195.
15. May T, Ito A, Okabe S (2009) Induction of multidrug resistance mechanism in *Escherichia coli* biofilms by interplay between tetracycline and ampicillin resistance genes. *Antimicrob Agents Chemother* 53: 4628–4639.
16. Castonguay MH, van der Schaaf S, Koester W, et al. (2006) Biofilm formation by *Escherichia coli* is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria. *Res Microbiol* 157: 471–478.

17. Gallant CV, Daniels C, Leung JM, et al. (2005) Common β-lactamases inhibit bacterial biofilm formation. *Mol Microbiol* 58: 1012–1024.
18. Lim JY, Yoon J, Hovde CJ (2010) A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *J Microbiol Biotechnol* 20: 5–14.
19. Burland V, Shao Y, Perna NT, et al. (1998) The complete DNA sequence and analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Res* 26: 4196–4204.
20. Lim JY, La HJ, Sheng H, et al. (2010) Influence of plasmid pO157 on *Escherichia coli* O157:H7 Sakai biofilm formation. *Appl Environ Microbiol* 76: 963–966.
21. Huang CT, Peretti SW, Bryers JD (1993) Plasmid retention and gene expression in suspended and biofilm cultures of recombinant *Escherichia coli* DH5α (pMJR1750). *Biotechnol Bioeng* 41: 211–220.
22. Huang CT, Peretti SW, Bryers JD (1994) Effects of inducer levels on a recombinant bacterial biofilm formation and gene expression. *Biotechnol Lett* 16: 903–908.
23. Bryers JD, Huang CT (1995) Recombinant plasmid retention and expression in bacterial biofilm cultures. *Wat Sci Tech* 31: 105–115.
24. O'Connell HA, Niu C, Gilbert ES (2007) Enhanced high copy number plasmid maintenance and heterologous protein production in an *Escherichia coli* biofilm. *Biotechnol Bioeng* 97: 439–446.
25. Teodósio JS, Simões M, Mergulhão FJ (2012) The influence of nonconjugative *Escherichia coli* plasmids on biofilm formation and resistance. *J Appl Microbiol* 113: 373–382.
26. Mergulhão FJ, Taipa MA, Cabral JM, et al. (2004) Evaluation of bottlenecks in proinsulin secretion by *Escherichia coli*. *J Biotechnol* 109: 31–43.
27. Gomes LC, Carvalho D, Briandet R, et al. (2016) Temporal variation of recombinant protein expression in *Escherichia coli* biofilms analysed at single-cell level. *Process Biochem* 51: 1155–1161.
28. Ferreira C, Pereira AM, Pereira MC, et al. (2011) Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on *Pseudomonas fluorescens*. *J Antimicrob Chemother* 66: 1036–1043.
29. Ferreira C, Pereira AM, Melo LF, et al. (2010) Advances in industrial biofilm control with micro-nanotechnology, In: Méndez-Vilas A, editor. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. Badajoz: Formatex, 845–854.
30. Yanischperron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* 33: 103–119.
31. Sambrook J, Russell DW (2001) Molecular Cloning: a Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.
32. Teodósio JS, Simões M, Melo LF, et al. (2011) Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient conditions and turbulent flow. *Biofouling* 27: 1–11.
33. Gomes LC, Silva LN, Simões M, et al. (2015) *Escherichia coli* adhesion, biofilm development and antibiotic susceptibility on biomedical materials. *J Biomed Mater Res A* 103: 1414–1423.
34. Mergulhão FJ, Monteiro GA (2007) Analysis of factors affecting the periplasmic production of recombinant proteins in *Escherichia coli*. *J Microbiol Biotechnol* 17: 1236–1241.
35. Bentley WE, Mirjalili N, Andersen DC, et al. (1990) Plasmid-encoded protein: The principal factor in the "metabolic burden" associated with recombinant bacteria. *Biotechnol Bioeng* 35: 668–681.

36. Sørensen HP, Mortensen KK (2005) Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J Biotechnol* 115: 113–128.
37. Cunningham DS, Koepsel RR, Ataai MM, et al. (2009) Factors affecting plasmid production in *Escherichia coli* from a resource allocation standpoint. *Microb Cell Fact* 8: 1475–2859.
38. Hoffmann F, Weber J, Rinas U (2002) Metabolic adaptation of *Escherichia coli* during temperature-induced recombinant protein production: 1. Readjustment of metabolic enzyme synthesis. *Biotechnol Bioeng* 80: 313–319.
39. Hoffmann F, Rinas U (2004) Stress induced by recombinant protein production in *Escherichia coli*, In: Enfors S-O, editor. *Physiological Stress Responses in Bioprocesses*, Berlin: Springer-Verlag Berlin Heidelberg, 73–92.
40. Landini P (2009) Cross-talk mechanisms in biofilm formation and responses to environmental and physiological stress in *Escherichia coli*. *Res Microbiol* 160: 259–266.
41. Kurland CG, Dong H (1996) Bacterial growth inhibition by overproduction of protein. *Mol Microbiol* 21: 1–4.
42. Glick BR (1995) Metabolic load and heterologous gene expression. *Biotechnol Adv* 13: 247–261.
43. Xia XX, Qian ZG, Ki CS, et al. (2010) Native-sized recombinant spider silk protein produced in metabolically engineered *Escherichia coli* results in a strong fiber. *Proc Natl Acad Sci USA* 107: 14059–14063.
44. Yang YX, Qian ZG, Zhong JJ, et al. (2016) Hyper-production of large proteins of spider dragline silk MaSp2 by *Escherichia coli* via synthetic biology approach. *Process Biochem* 51: 484–490.
45. Dong H, Nilsson L, Kurland CG (1995) Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J Bacteriol* 177: 1497–1504.
46. Georgiou G, Shuler ML, Wilson DB (1988) Release of periplasmic enzymes and other physiological effects of β-lactamase overproduction in *Escherichia coli*. *Biotechnol Bioeng* 32: 741–748.
47. Williams I, Venables WA, Lloyd D, et al. (1997) The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*. *Microbiology* 143: 2407–2413.
48. Simões M, Pereira MO, Vieira MJ (2005) Effect of mechanical stress on biofilms challenged by different chemicals. *Water Res* 39: 5142–5152.
49. Araújo PA, Mergulhão FJM, Melo LF, et al. (2014) The ability of an antimicrobial agent to penetrate a biofilm is not correlated with its killing or removal efficiency. *Biofouling* 30: 675–683.
50. Cloete TE, Jacobs L, Brözel VS (1998) The chemical control of biofouling in industrial water systems. *Biodegradation* 9: 23–37.
51. Ahimou F, Semmens MJ, Haugstad G, et al. (2007) Effect of protein, polysaccharide, and oxygen concentration profiles on biofilm cohesiveness. *Appl Environ Microbiol* 73: 2905–2910.

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