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Effects of antibiotic concentration and nutrient medium composition on *Escherichia coli* biofilm formation and green fluorescent protein expression

Luciana C. Gomes† and Filipe J. Mergulhão*†

LEPABE – Department of Chemical Engineering, Faculty of Engineering, University of Porto, 4200-465 Porto, Portugal

*Corresponding author: Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal. Tel: (+351) 225081668; Fax: (+351) 5081449; E-mail: filipem@fe.up.pt

One sentence summary: Increased recombinant protein production in *Escherichia coli* biofilms is achieved by using LB medium supplemented with a low antibiotic concentration.

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†Luciana C. Gomes, http://orcid.org/0000-0002-8992-1097

†Filipe J. Mergulhão, http://orcid.org/0000-0001-5233-1037

ABSTRACT

Recombinant protein production processes have to maximise yield while minimising cost, which involves balancing plasmid maintenance with cell growth and protein expression. The aim of this study was to analyse the influence of two factors on heterologous protein production in *Escherichia coli* biofilm cells—the concentration of antibiotic used to maintain the selective pressure and the nutrient medium composition. *Escherichia coli* JM109(DE3) cells transformed with plasmid pFM23 for enhanced green fluorescent protein (eGFP) expression and containing a kanamycin resistance gene were used. They were exposed to 20 or 30 μg mL^{-1} kanamycin during biofilm growth in two different culture media, a diluted medium (DM) or the lysogeny broth (LB). The higher antibiotic concentration increased the specific eGFP production in planktonic cells, whereas no increase was detected in biofilm cells. Biofilm formation was increased in DM when compared to LB. Nevertheless, bacteria grown in LB had higher eGFP production than those grown in DM in both planktonic and sessile states (20-fold and 2-fold, respectively). Therefore, among the conditions tested, LB supplemented with 20 μg mL^{-1} kanamycin was the most advantageous medium to obtain the highest specific eGFP production in biofilm cells.

Keywords: Biofilm; *Escherichia coli*; plasmid; green fluorescent protein; kanamycin; lysogeny broth

INTRODUCTION

Escherichia coli is one of the preferred hosts for recombinant protein production (Mergulhão, Summers and Monteiro 2005), and *E. coli* plasmids have been traditionally used as expression vectors (Mergulhão *et al*. 2004). Since engineered plasmids are often lost in culture (Summers 1998), it is essential to impose a selective pressure to ensure plasmid stability, which is often achieved by adding antibiotics to the culture medium.

In suspended cultures, it is well documented that plasmids impose a metabolic burden on the host cell due to plasmid replication and consumption of precursor metabolites (Bentley *et al*. 1990; Glick 1995), being the expression of antibiotic resistance genes the major cause for this burden (Cunningham *et al*. 2009). The resistance agent is often an enzyme that will act upon reducing or eliminating the antibiotic activity. Therefore, protein synthesis is involved and precursors such as amino acids and energy are consumed. Studies involving non-expressing

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plasmids showed that the marker protein can represent up to 20% of total cellular protein (Birnbaum and Bailey 1991; Rozkov *et al.* 2004), exceeding the levels required for plasmid maintenance. Most publications addressing the metabolic load of plasmids were performed in planktonic conditions as recombinant protein production in *E. coli* is commonly performed in this state. However, it has been demonstrated that biofilm cells can have higher recombinant production levels than planktonic cells (O'Connell, Niu and Gilbert 2007) and therefore it is interesting to explore the capabilities of *E. coli* biofilms in recombinant bioprocesses. Concerning the particular effect of antibiotic markers, it is known that they can affect bacterial biofilm formation (Bagge *et al.* 2004; Gallant *et al.* 2005; May, Ito and Okabe 2009; Teh, Wang and Dykes 2014). Some studies refer that the presence of the β-lactamase gene on a plasmid can reduce the amount of biofilm formed by *E. coli*, while the presence of other antibiotic resistance genes may not impair biofilm formation (Bagge *et al.* 2004; Gallant *et al.* 2005). The antibiotic marker also affects the recombinant protein production due to its contribution to plasmid stability, since maintaining a high concentration of plasmid-bearing cells may favour expression (Marini *et al.* 2014).

Another important variable in both biofilm formation and recombinant protein expression is the nutrient composition of culture medium. Several studies have evaluated the effect of nutrient levels on biofilm formation (Peyton 1996; Teodósio *et al.* 2011; Gomes *et al.* 2014). Some authors suggested that an increase in nutrient concentration enhances biofilm growth (Peyton 1996; Rochex and Lebeault 2007), while others have shown that a high nutrient media inhibits biofilm formation (Dewanti and Wong 1995; Eboigbodin, Ojeda and Biggs 2007). Regarding recombinant protein expression in suspended cultures, it was found that production levels are dependent on the nutrient composition of culture medium (Broedel, Papciak and Jones 2001). Culture media may influence plasmid segregational stability (Matsui *et al.* 1990; O'Kennedy and Patching 1997; Goyal, Sahni and Sahoo 2009) and some authors argue that the use of complex nitrogen sources such as yeast extract or tryptone results in increased plasmid stability (Matsui *et al.* 1990).

The main goal in the production of plasmid-derived recombinant proteins is to achieve the maximum yield of product using minimum resources. Thus, it is important to understand the influence of culture conditions such as the antibiotic concentration and nutrient medium composition on the expression of recombinant proteins in *E. coli* biofilms. For this purpose, *E. coli* JM109(DE3) cells harbouring the plasmid pFM23, an expression vector for enhanced green fluorescent protein (eGFP), was used. This vector contains a kanamycin resistance gene and cells were exposed to different concentrations of this antibiotic during biofilm development in two culture media, a diluted medium (DM) and lysogeny broth (LB).

MATERIALS AND METHODS

Bacterial strain

Escherichia coli JM109(DE3) (Promega, Madison, USA) was transformed with plasmid pFM23 (constructed from pET28A, Novagen, Madison, USA) for the cytoplasmic production of eGFP (Mergulhão and Monteiro 2007) under the control of the T7 promoter.

Biofilm system and experimental conditions

A flow cell reactor system described by Gomes *et al.* (2016) was operated for 12 days at feed flow rate of 0.025 L h^{-1} with DM containing either 20 or 30 μg mL^{-1} kanamycin (Eurobio, Courtaboeuf, France). DM contains 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. This was the first culture medium to be tested since the *E. coli* strain previously demonstrated a good biofilm formation capacity in the same growth medium (Teodósio *et al.* 2011; Teodósio, Simões and Mergulhão 2012). The concentrations of kanamycin used are in the range recommended for selection of plasmids containing the kanamycin resistance gene (Sambrook and Russell 2001). A kanamycin concentration of 20 μg mL^{-1} is sufficient to maintain the pFM23 plasmid stability since the number of colony forming units (CFU) on selective plates containing 20 μg mL^{-1} kanamycin was similar to that of non-selective plates (data not shown). Although 30 μg mL^{-1} kanamycin is the working concentration indicated for plasmids derived from pET vectors (Novagen 2005; Carapuça *et al.* 2007), this 50% higher concentration may increase the risk of antibiotic resistance spread in a large-scale process (Rosano and Ceccarelli 2014). The other medium tested was LB supplemented with 20 μg mL^{-1} kanamycin. This commercial medium is composed of 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl (LB-Miller, Sigma, St. Louis, USA), and it is commonly used for recombinant protein expression with the pET system (Novagen 2005).

Analytical methods

On each experimental day, a coupon was removed from the flow cell and biofilm wet weight and thickness were determined as previously described (Teodósio *et al.* 2011). Then the biofilm was resuspended and homogenised by vortexing as indicated by Gomes *et al.* (2015, 2016) in 25 mL of 8.5 g L^{-1} NaCl solution for cell and eGFP quantifications.

The sample was diluted to an appropriate cell density to yield >10 and <300 CFU per plate of solid growth medium (PCA, Merck, Algés, Portugal) supplemented with 20 or 30 μg mL^{-1} kanamycin. Colony enumeration was carried out after 24-h incubation at 30°C and the final values of culturable cells were expressed as log CFU cm^{-2} of coupon area. Biofilm total cells (viable plus non-viable) were determined with the Live/Dead® BacLight bacterial viability kit (Invitrogen Life Technologies, Alfagene, Carcavelos, Portugal) as indicated by Gomes *et al.* (2016) (see supplementary material, Fig. S1).

The eGFP expression in biofilm cells was analysed as described by Mergulhão and Monteiro (2007). A volume of biofilm detached cells corresponding to an equivalent optical density (OD) of 1 at 610 nm was centrifuged. 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, Braine-l'Alleud, Belgium) containing 100 μl of Buffer I. Fluorescence was measured with the excitation filter of 488 nm and the emission filter of 507 nm (SpectraMax M2E, Molecular Devices, Inc., Berkshire, UK). Calibration curves were constructed with purified eGFP standards and the final values were presented as specific eGFP production (fg $cell^{-1}$) considering that an OD of 1 corresponds to a cellular concentration of 7.6×10^8 cells mL^{-1} (Teodósio *et al.* 2011).

For planktonic cells, OD at 610 nm, culturable cell number (log CFU mL^{-1}) and specific eGFP production (fg $cell^{-1}$) were assessed.

For the experiments with DM, glucose consumption in the whole system was determined as an indicator of metabolic activity (Teodósio *et al.* 2011).

After 7 days of biofilm development, the content of EPS (proteins and polysaccharides) was assessed for both DM and LB

media supplemented with 20 μg mL^{-1} kanamycin. Matrix proteins and polysaccharides from biofilms were separated from the cells using a Dowex resin (50 × 8, Na^+ form, 20–50 mesh from Fluka Chemika, Buchs, Switzerland) and quantified as described by Gomes *et al.* (2015).

Calculations and statistical analysis

The results presented in Table 2 and Figs 1 and 2 originated from averages of triplicate sets obtained in independent experiments for each culture condition (DM with 20 or 30 μg mL^{-1} kanamycin and LB with 20 μg mL^{-1} kanamycin). Table 1 shows the averages of eGFP production values obtained between days 7 and 12 (steady state) and presented as individual time points in panels E and F of Figs 1 and 2.

The following average standard deviations (SDs) were obtained for planktonic and biofilm parameters (Figs 1 and 2): SD < 15% for OD, SD < 32% for biofilm wet weight, SD < 7% for planktonic and biofilm culturable cells, SD < 11% for planktonic and biofilm eGFP production, SD < 12% for glucose consumption and SD < 33% for biofilm thickness.

In order to ascertain the statistical significance, one-way ANOVA was performed using Statgraphics v6.0 software (Manugistics, USA) based on a confidence level of 90% (differences reported as significant for P values < 0.1 and marked with asterisk $*$) and 95% (differences reported as significant for P values < 0.05 and marked with double asterisks $^{*}_{*}$).

RESULTS

Effect of antibiotic concentration

The effect of antibiotic concentration on *Escherichia coli* biofilm formation and eGFP expression is presented in Fig. 1 where two concentrations of kanamycin (20 and 30 μg mL^{-1}) in DM are compared.

Analysis of the planktonic condition (Fig. 1A and C) indicates that there were few differences in *E. coli* growth for both antibiotic concentrations and that the number of culturable biofilm cells (Fig. 1D) was also similar (6 out of 10 time points have P value > 0.1). Regarding the biofilm wet weight and thickness (Fig. 1B and H), there was a strong increase (of about 85%) between days 3 and 8 and a sharp decrease after that for the highest antibiotic concentration. Cell detachment may have occurred, as confirmed by a decrease in the total cell number (see supplementary material, Fig. S1).

Recombinant eGFP production in planktonic cells (Fig. 1E) was higher for the highest antibiotic concentration during the 12-day experiment and an average production value of 0.7 fg cell^{-1} was reached in this condition. For the lowest kanamycin concentration, the specific production was reduced 10-fold after day 5. For biofilm cells (Fig. 1F), the specific protein production was similar in both antibiotic concentrations (5 out of 10 time points have P value > 0.1) and average values around 6 fg cell^{-1} were obtained. Although the total number of cells has decreased after day 8 for the highest kanamycin concentration (Fig. S1), the specific eGFP production was similar between day 8 and day 12 (Fig. 1F). Since the specific eGFP production was determined taking into account the total number of cells, this means that there was a decrease in the volumetric production of the biofilm.

Figure 1G presents the glucose consumption profiles in the whole system (suspended plus biofilm cells). Higher glucose consumption values (on average 18%) were obtained in DM containing 20 μg mL^{-1} kanamycin, with statistically significant differences in most time points (7 out of 10, $P < 0.05$ or $P < 0.1$).

Effect of nutrient medium composition

Since the variation in antibiotic concentration had no effect on specific eGFP production in biofilm cells, a different medium (LB) was tested. This medium was supplemented with the lowest concentration of kanamycin tested (20 μg mL^{-1}) in order to maintain the selective pressure without increasing the use of antibiotic in the bioprocess.

Comparing the DM with LB, higher OD values (on average 33%) were registered for LB (Fig. 2A). The concentration of planktonic culturable cells (Fig. 2C) was similar between growth media (5 out of 10 time points have P value > 0.05) and throughout the experimental time in the case of LB medium ($P = 0.22$). For biofilm culturable cells (Fig. 2D), a higher cell number was obtained in DM in most experimental points. Biofilm wet weight (Fig. 2B) was also higher in DM, whereas in LB the biofilm reached a constant value 5-fold lower than that obtained in DM after day 7. The evolution of biofilm thickness (Fig. 2G) was similar to the wet weight and a 10-fold difference was obtained between DM and LB.

By analysing the specific eGFP production (Fig. 2E and F), it can be seen that cells grown in LB produced more eGFP than those grown in DM in both planktonic and sessile states. When the system was in steady state, the planktonic eGFP production in LB was 20-fold higher than in DM, whereas the biofilm expression was 2-fold higher than in DM (Table 1). In LB medium and DM supplemented with 30 μg mL^{-1} of kanamycin, the specific eGFP production from biofilm cells was on average 10-fold higher than in planktonic cells. In DM supplemented with 20 μg mL^{-1} of kanamycin, a 95-fold higher production was obtained in biofilm cells when compared to their planktonic counterparts (Table 1).

The influence of medium composition on EPS production was also assessed (Table 2). The matrix of *E. coli* biofilms formed in LB with 20 μg mL^{-1} kanamycin had a lower protein content (about 3-fold) compared to the biofilms developed in DM containing the same antibiotic concentration. On the contrary, the amount of matrix polysaccharides produced in LB was about 12-fold higher than in DM.

DISCUSSION

Effect of antibiotic concentration

When the antibiotic concentration was changed from 20 to 30 μg mL^{-1}, there was no change in the growth curves of planktonic cells. Identical results were reported by Marini *et al.* (2014), who studied the effect of kanamycin (in 10–50 μg mL^{-1} concentration range) on *Escherichia coli* cell growth during the expression of recombinant pneumolysin. In the present work, the number of biofilm culturable cells was also similar in both antibiotic conditions during most of the assay, although cell detachment occurred at a later stage for the highest antibiotic concentration.

Increasing the antibiotic concentration increased the specific eGFP production from planktonic cells, whereas no comparable benefit was detected in biofilm cells. It is possible that mass transfer limitations within the biofilm may have created a microenvironment where the effective antibiotic concentration was similar in both situations (Stewart 2003). Planktonic cells are not subjected to this type of mass transfer limitations (Moreira *et al.* 2015) and therefore the increase in kanamycin

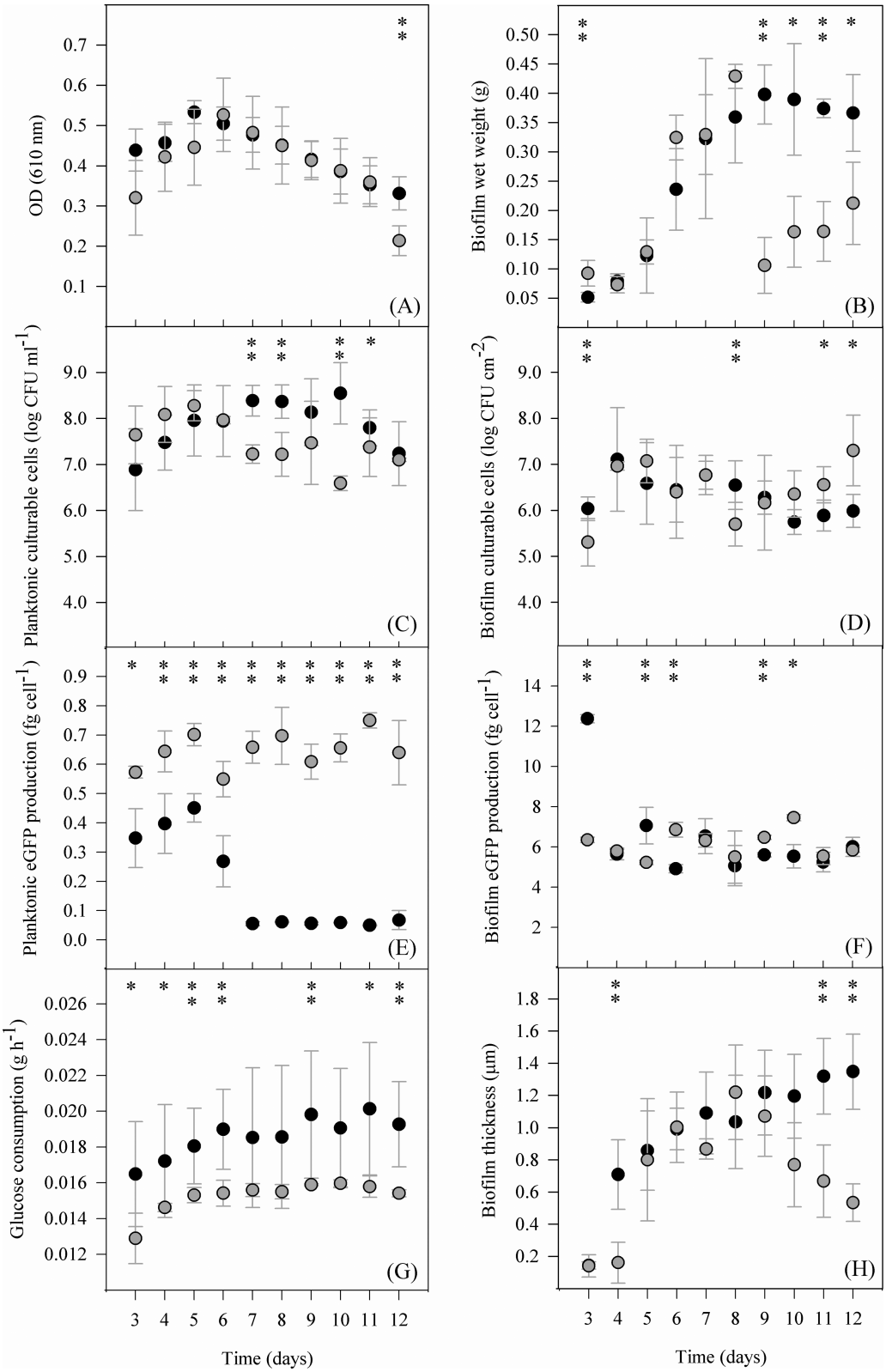

Figure 1. Time course of planktonic and biofilm parameters in DM: (**A**) OD in the recirculating tank, (**B**) biofilm wet weight, (**C**) planktonic culturable cells, (**D**) biofilm culturable cells, (**E**) planktonic-specific eGFP production, (**F**) biofilm-specific eGFP production, (**G**) glucose consumption in the system, (**H**) biofilm thickness. Kanamycin concentration of 20 μg mL^{-1} (dark filled circles) and 30 μg mL^{-1} (light filled circles). The means ± SDs for three independent experiments are illustrated. Statistical analysis corresponding to each time point is also represented with asterisk for a confidence level greater than 90% ($P < 0.1$) and with double asterisks for a confidence level greater than 95% ($P < 0.05$).

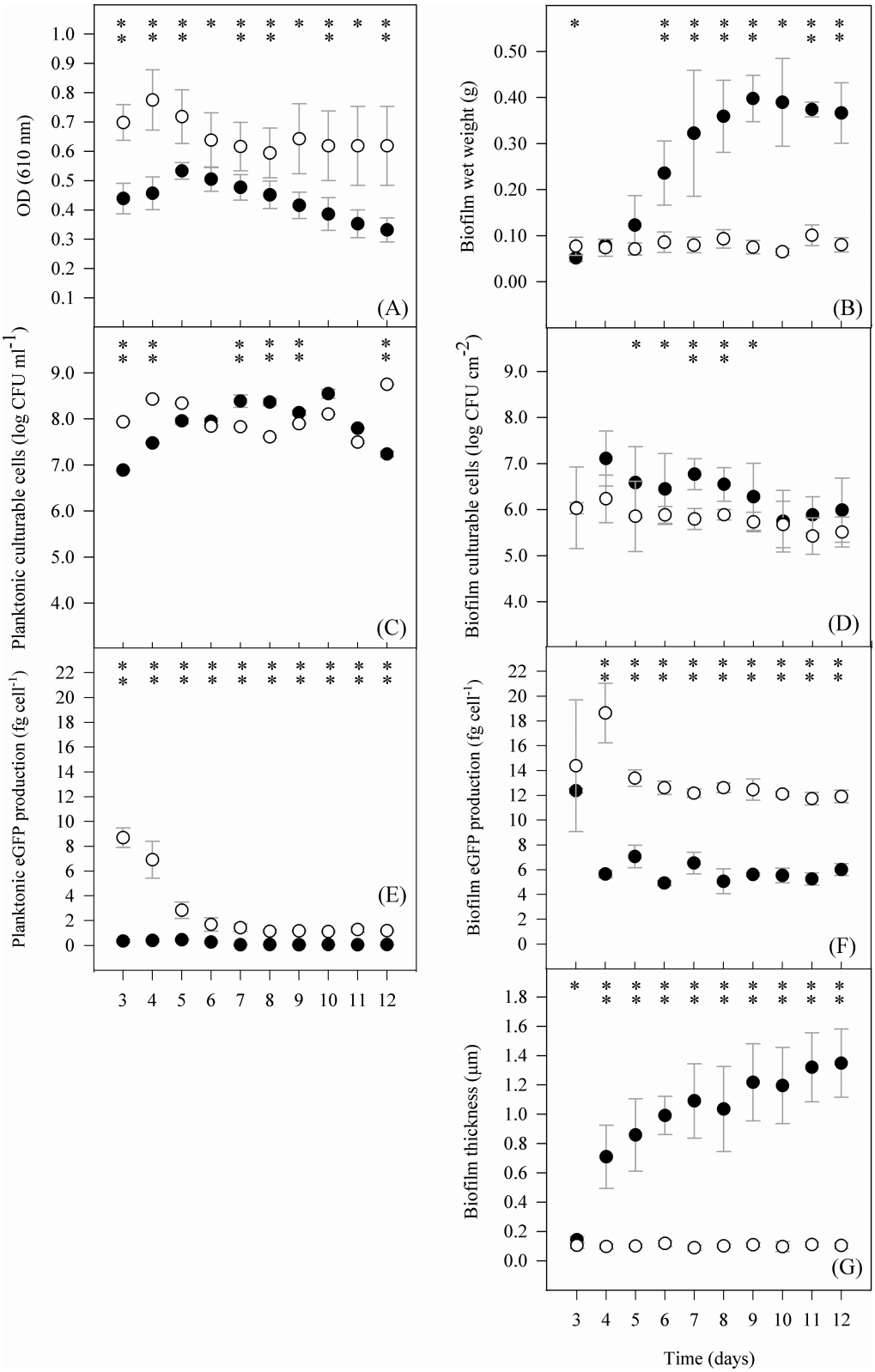

Figure 2. Time course of planktonic and biofilm parameters: (**A**) OD in the recirculating tank, (**B**) biofilm wet weight, (**C**) planktonic culturable cells, (**D**) biofilm culturable cells, (**E**) planktonic-specific eGFP production, (**F**) biofilm-specific eGFP production, (**G**) biofilm thickness. DM with 20 μg mL^{-1} kanamycin (dark filled circles), LB with 20 μg mL^{-1} kanamycin (light filled circles). The means ± SDs for three independent experiments are illustrated. Statistical analysis corresponding to each time point is also represented with asterisk for a confidence level greater than 90% ($P < 0.1$) and with double asterisk for a confidence level greater than 95% ($P < 0.05$).

Table 1. Specific eGFP production for planktonic and biofilm conditions in different growth media: DM with 20 and 30 μg mL^{-1} kanamycin and LB with 20 μg mL^{-1} kanamycin. The results presented were obtained under steady state (between days 7 and 12).

Medium	Kanamycin concentration (μg mL^{-1})	Specific eGFP production (fg cell^{-1})	
		Planktonic	Biofilm
DM	20	0.060	5.7
DM	30	0.67	6.2
LB	20	1.2	12

Table 2. Analysis of 7-day-old biofilms formed in DM and LB with 20 μg mL^{-1} kanamycin.

Biofilm characteristics	DM	LB
Biofilm mass (mg$_{biofilm}$ cm^{-2})	8.00 ± 1.77	4.50 ± 0.870
Log cellular density (cells cm^{-2})	9.09 ± 0.740	7.76 ± 0.460
Matrix proteins (mg g$^{-1}_{biofilm}$)	27.4 ± 2.01	9.40 ± 1.70
Matrix polysaccharides (mg g$^{-1}_{biofilm}$)	19.2 ± 1.68	231 ± 35.0

concentration may have exerted a higher selective pressure on these cells, which may have increased the plasmid copy number and thus the production of recombinant protein. Yazdani and Mukherjee (2002) conducted continuous cultures for the expression of recombinant streptokinase in *E. coli* and showed that using high dosage of antibiotic helped to increase the plasmid stability. To the best of our knowledge, the only work about the effect of antibiotic concentration on heterologous protein production in biofilms was published by O'Connell *et al.* (2007). Contrary to our findings, these authors showed that low concentrations of antibiotic enhanced protein production in biofilms of *E. coli* (strain ATCC 33456 containing the plasmid pEGFP) developed in a flow cell reactor. These conflicting results seem to indicate that the profile of plasmid stability and recombinant protein expression is different for different host–vector systems.

Effect of nutrient medium composition

Using LB instead of DM favoured planktonic growth as indicated by the higher values of culture OD. LB contains higher levels of peptone and yeast extract, which often lead to higher cell culture densities (Studier 2005; Teodósio *et al.* 2011).

Higher specific eGFP production was obtained in LB for both planktonic and biofilm conditions. Matsui *et al.* (1990) reported that the use of complex nitrogen sources such as tryptone found in LB resulted in higher plasmid stability. Furthermore, LB is a complex medium that provides an abundance of amino acids to support the protein expression in *E. coli* (Donovan, Robinson and Glick 1996). On the other hand, it was reported that the addition of yeast extract increases the non-induced background expression from the *lac* promoter (Doran *et al.* 1990; Solaiman and Somkuti 1991). In the expression system used in this work, the *eGFP* gene is transcribed by the T7 RNA polymerase whose expression is controlled by the *lac* promoter. It is foreseeable that if yeast extract induces T7 expression from the chromosome, a stronger transcription of the *eGFP* gene may have occurred and thus higher expression levels were obtained.

Besides enhancing the specific production of eGFP, *E. coli* biofilms grown in LB also presented lower concentration of proteins within the extracellular matrix than the biofilms formed in DM. This observation is probably a consequence of the channelling of more resources normally involved in EPS-associated protein synthesis (such as amino acids, nucleotides and metabolic energy) towards the recombinant protein expression. It is known that the T7 RNA polymerase is much more active than the *E. coli* RNA polymerase and that the stability of the mRNA it produces is higher than the host mRNA (Studier and Moffatt 1986). Both effects can contribute to a rapid saturation of *E. coli* translational machinery so that the synthesis of other proteins (such as matrix proteins) may have been reduced in LB medium.

Compared to biofilms developed in DM, the amount of matrix polysaccharides in biofilms formed in LB increased with the simultaneous reduction of protein content. It is known that EPS composition is important in biofilm cohesion and adhesion to surfaces (Flemming and Wingender 2010), wherein higher polysaccharide concentrations are believed to contribute to biofilm cohesion (Ahimou *et al.* 2007). It is possible that biofilm cells grown in LB try to compensate for the low protein level in the matrix with a higher production of polysaccharides in order to maintain the mechanical stability of the biofilm.

In this work, increasing the antibiotic concentration from 20 to 30 μg mL^{-1} (50% increase) only had a modest effect on the specific eGFP production of biofilm cells. Therefore, using the lowest antibiotic concentration may be the best option if DM is chosen, not only due to the cost of antibiotic, but also because of the dissemination of antibiotic resistance, particularly in large-scale cultures (Rosano and Ceccarelli 2014). Specific recombinant production was always higher in biofilm cells and the highest levels were obtained in LB. However, if the volumetric production values are analysed, a 60% increase can be obtained by using DM instead of LB due to the increased cell density of biofilms formed in DM.

This work highlights the potential of biofilm cells to be used as microbial cell factories not only for the synthesis of simple molecules like ethanol, butanol or lactic acid, but also for the production of complex proteins with particular folding requirements. Although other microbial hosts have shown their merits in this regard, the productive potential of *E. coli* biofilms has barely been explored. A deeper understanding of the operational conditions that maximise production is crucial to harness the power of these productive biofilms.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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