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Temporal variation of recombinant protein expression in *Escherichia coli* biofilms analysed at single-cell level

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Highlights

- Epifluorescence microscopy and image processing enable single-cell expression analysis.
- *Escherichia coli* biofilm heterogeneity increased during biofilm development.
- Fluorescence heterogeneity was correlated with spatial heterogeneity.

Abstract

Bioprocesses based on surface-associated microorganisms are emerging in environmental and industrial areas owing to the physiological specificities and heterogeneities of biofilm cells. This study describes a simple and accurate method for evaluating recombinant protein expression at a single-cell scale during *Escherichia coli* biofilm development. The model recombinant protein used was enhanced Green Fluorescent Protein (eGFP), as its intrinsic fluorescence allows the quantification of expression at both population and single-cell levels. Specific cell fluorescence intensity sharply increased during the first 4 days of biofilm cultivation and thereafter decreased abruptly to reach a low-level plateau until the end of the experiment. During biofilm development, the population became increasingly heterogeneous with regard to eGFP expression. Three distinct biofilm types were observed along the experimental time: one with a homogeneous population (days 3–5), the second with a moderately heterogeneous population (days 6–8) and the third with a strongly heterogeneous population (days 9–11). Observation of *E. coli* biofilms by confocal laser scanning microscopy demonstrated marked spatial heterogeneity, with the cells actively producing eGFP restricted to the top layer of the biofilm. The proposed methodology allows a fine analysis of the recombinant protein expression within *E. coli* biofilms, and it may be used for optimizing the processing conditions.

Keywords: Biofilm; *Escherichia coli*; recombinant protein expression; fluorescence microscopy; single-cell scale; spatial heterogeneity

1. Introduction

The Gram-negative bacterium *Escherichia coli* is one of the most commonly used recombinant protein production hosts[1] due to its ability to grow rapidly and to high densities on inexpensive substrates, its well-known genetics and the availability of various systems for gene expression.[2]

Several bacteria such as *E. coli* naturally grow in a community attached to a substratum and not in liquid cultures. The biocatalytic potential of these bacterial communities, termed biofilms, can be attributed to their high cell density; the former feature is widely used for wastewater treatment[3] and also for the production of industrial chemicals such as ethanol, butanol and lactic acid.[4,5] Recombinant protein production in biofilms has been mostly studied in the context of waste biodegradation[6,7]; however, this strategy could also be advantageous in other processes such as the biosynthesis of pharmaceutical intermediates[8] and catalysts for the food industry. In fact, using a recombinant *Aspergillus niger* strain, which contained a gene encoding the glucoamylase–GFP fusion protein, Talabardon and Yang[9] showed that higher amounts of GFP (Green Fluorescent Protein) and glucoamylase were produced in immobilized cells than in suspension culture. Moreover, a previous study with *E. coli* ATCC 33456 containing the plasmid pEGFP showed that the biofilm environment enhanced plasmid maintenance and also the GFP concentrations.[10]

During recombinant protein production, it is important to not only monitor the total amount of proteins produced by the culture using bulk methods such as fluorometry or standard fluorescence microscopy but also evaluate the distribution of this protein in individual cells. For instance, knowing the fraction of protein-producing and non-producing cells may help in optimizing the operational parameters for maximum strain performance. Bacteria grown in biofilms are normally distributed in a heterogeneous manner as a consequence of exposure to the local environmental conditions that may vary

on the micrometre scale.[11] The concentration gradients of the chemicals dissolved in the interstitial fluid within the biofilm matrix promote differences in bacterial enzymatic activities in different areas of the biofilms[12,13] and can create variation at the gene and protein levels.[10,14-16]

In this work, a protocol using fluorescence imaging is proposed for quantifying the dynamics of protein expression within a biofilm population at both bulk and single-cell levels. It should be noted that the entire protocol required only an epifluorescence microscope and an open-source image analysis tool, both of which are available in most life science research and industrial laboratories.[17]

2. Materials and Methods

2.1 Biofilm-producing system and culture conditions

The enhanced Green Fluorescent Protein (eGFP) was chosen as a model protein for this study and the biofilms were grown on a flow cell reactor as described by Teodósio et al.[18]

The *E. coli* strain JM109(DE3) procured from Promega (USA) was transformed by heat shock[19] with plasmid pFM23 (constructed from pET28A, Novagen, WI, USA) for the cytoplasmic production of eGFP[20] under the control of the T7 promoter.

The flow cell consists of a semicircular Perspex duct (3.0-cm diameter and 1.2-m length) with 20 apertures on its flat wall to fit the removable rectangular pieces of Perspex (coupons). Polyvinyl chloride (PVC) slides (2 × 1 cm) were glued onto the Perspex pieces; the biofilms formed on the upper faces that were in contact with the bacterial suspension circulating through the system.

E. coli cells harbouring the pFM23 plasmid were grown by recirculating the bacterial suspension at 30 ºC for 11 days under a turbulent flow with a Reynolds number of 4600.[21]

This temperature was used since the *E. coli* strain JM109(DE3) had already demonstrated a good biofilm formation capacity at 30ºC in a similar culture medium and biofilm-producing system.[18,21] The recirculating tank of 1 L was aerated using an air pump (air flow rate 108 L h^{-1}) and continuously fed with 0.025 L h^{-1} of lysogeny broth (LB-Miller, Sigma, USA) supplemented with 20 µg mL^{-1} kanamycin (Eurobio, France) to maintain a selective pressure. The dissolved oxygen and pH of the recirculating culture were monitored and constant values of 2.0 mg L^{-1} (average standard deviation < 10%) and 8.2 (average standard deviation < 4%) were respectively obtained (data not shown).

2.2 Biofilm monitoring

For biofilm sampling, the system was stopped each day to allow coupon removal and carefully restarted, maintaining the same flow conditions as described by Teodósio et al.[21] Biofilm cell populations were resuspended and homogenized by vortexing as previously described[22] into 25 mL of 8.5 g L^{-1} NaCl solution for total and viable cell assessment and eGFP analysis.

2.2.1 Quantification of total and viable cells by epifluorescence imaging on detached biofilm populations

Biofilm total (viable plus non-viable) and viable cell counts were assessed using the Live/Dead® BacLight™ bacterial viability kit (Syto9/propidium iodide, Invitrogen Life Technologies, Alfagene, Portugal). Bacterial observations were performed after 10-min incubation with the fluorescent dyes in the dark[22] using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd, Switzerland). The optical filter combination for optimal viewing of the stained preparations consisted of a 515–560-nm excitation filter combined with a

dichromatic mirror at 580 nm and suppression filter at 590 nm. For capturing images, Leica IM50 Image Manager, Image Processing and Archiving software was used. Green cells labelled with Syto9 and red cells labelled with propidium iodide bacteria on each membrane were estimated from counts of a minimum of 20 fields of view. Both types of cells were automatically quantified using the image processing software ImageJ v1.48 (NIH, USA). After background subtraction, the green and red cells were segmented and counted. Results were expressed as the mean of triplicate samples obtained in three independent experiments measured as log cell cm^{-2}.

2.2.2 Quantification of eGFP expression by epifluorescence microscopy

Biofilm cells were filtered through a nucleopore-etched (Whatman Inc., NJ, USA) black polycarbonate membrane (pore size 0.2 mm) and images were acquired using a Leica DM LB2 epifluorescence microscope (Leica Microsystems Ltd) coupled with a Leica DFC300 FX camera (Leica Microsystems Ltd). A 450–490-nm excitation filter was used in combination with a dichromatic mirror at 510 nm and suppression filter at 515 nm. Fifteen fields of view were photographed for each sample. A total of three biofilm samples originating from three independent experiments were used for each time point. The images were analysed in batch mode using ImageJ v1.48 (NIH). For each RGB (red, blue and green) image, the green channel was processed with a sliding paraboloid to reduce uneven background. Cell segmentation was performed using an automatic black and white threshold after convolution with a Laplacian of Gaussian (9 × 9 kernel) filter. The resulting binary image was then redirected to a background-reduced duplicate, and each cell was analysed with the size and circularity intervals defined as 0.52–4.16 μm and 0.25–1.00, respectively. For each image, the mean fluorescence intensity was expressed as the mean of the values of individual cells, which was in turn obtained by averaging

each cell pixel intensity value. The mean fluorescence intensity was presented in arbitrary fluorescent units (A.F.Us.).

2.3 Deciphering spatial heterogeneity of eGFP expression within biofilm by Confocal Laser Scanning Microscopy

For visualization by CLSM, 3-day-old biofilms were formed on sterile PVC (polyvinyl chloride) coupons placed in a 24-well polystyrene, flat-bottomed TPP® tissue culture plate (Sigma-Aldrich, France) with lysogeny broth. The plate was incubated at 30 ºC under the defined shaking conditions to obtain the same average wall shear stress found in the sampling zone of the flow cell operating at a Reynolds number of 4600.[23,24] Bacteria from the biofilms were counterstained with 5 μM Syto61 (Invitrogen, France), a cell-permeant red fluorescent nucleic acid marker. The biofilms in the PVC coupons were observed using a Leica SP2 AOBS CLSM (Leica Microsystems, France) at the INRA MIMA2 microscopy platform. The coupons were scanned using a 40x water immersion objective lens at excitation wavelengths of 488 nm (argon laser) and 633 nm (helium–neon laser). The emitted fluorescence was recorded within the range of 500–580 nm to collect the eGFP emission fluorescence and 640–730 nm to collect the Syto61 fluorescence. Two-dimensional projections of the biofilm structures were reconstructed using the Section function of the IMARIS 7.0 software (Bitplane, Switzerland). The Stack Profile tool provided by the LCS software (Leica Microsystems) was used to trace the intensity values of both fluorescent signals with regard to the z-position.

2.4 Calculations and statistical analysis

The coefficient of variation was chosen to express the extent of heterogeneity in the expression of eGFP by a cell population (Fig. 3). For each image, the coefficient of

variation for the specific fluorescence intensity (%) corresponds to the variability of the fluorescence signal of each bacterial cell in relation to the mean of the population. The coefficients of variation were calculated for each of the 45 images analysed for each experimental day and represented in the form of a typical graph of frequency distribution.

One-way ANOVA was performed using the Statgraphics v6.0 software (Manugistics, Rockville, MD, USA) for comparing the coefficients of variation between each pair of experimental days and between groups of days (Fig. 3). Paired *t*-test analysis was also performed when appropriate. All tests were used based on a confidence level of 95% (differences reported as significant for P-values < 0.05).

3. Results

In this work, techniques based on epifluorescence microscopy were used to monitor the amount of recombinant protein expressed by biofilm cells and the physiological state of these cells during recombinant protein production (Fig. 1). Figure 1(a) presents the temporal eGFP expression profile in the biofilm during its development in the flow cell system. The specific fluorescence intensity increased from day 3 to day 4 having reached a maximum at this day. From this day onward, a drastic reduction of the specific cell fluorescence intensity (about 61%) was observed and the values stabilized by the end of the experiment at about half of the value measured on day 3.

Biofilm cell physiology was evaluated along the experimental time by quantifying the number of total, viable and eGFP-expressing cells (Fig. 1(b)). It can be seen that the total number of biofilm cells increased slightly between days 3 and 5 (39%) and remained stable until the end of the experiment. Concerning biofilm cell viability, it is possible to observe that during days 3 and 4, the number of viable cells followed the increase in the number of total cells, corresponding to a viability percentage of 74%. From day 4, the

fraction of viable cells decreased by 45% and was practically constant until the end of the experiment ($P < 0.05$). The number of eGFP-expressing cells followed the evolution of total and viable cells until day 4 and it was observed that most of the total cells (82%) expressed eGFP. However, between days 4 and 6, a strong reduction (of about 70%) in the number of eGFP-expressing cells was observed and by the end of the experimental time, the eGFP-expressing cells represented only 21% of the total cells. Figure 1(b) shows a gap between the biofilm cell viability (*i.e.*, cells with intact cell membrane) and the sessile cells expressing eGFP, with a statistically significant difference between both curves at days 6, 7, 8, 9 and 11 ($P < 0.05$). This difference may be associated with the existence of a slow growth population of viable but non-expressing (VBNE) cells. The onset of this biofilm population was noticeable from day 6 onward, and the fraction of VBNE cells remained almost constant until day 9.

The overall results of Fig. 1 show some similarity between the curves for viable and eGFP-producing cells (Fig. 1(b)) and the temporal evolution of recombinant protein production (Fig. 1(a)). Both curves show a maximum at day 4 followed by a decrease in the next 24 h, which was more pronounced in the case of eGFP-expressing cells and specific fluorescent intensity.

Figure 2 shows illustrative images obtained by epifluorescence microscopy to quantify the total biofilm cells (Fig. 2(a), (b) and (c)) and the green fluorescence signal of each eGFP-expressing cell (Fig. 2(d), (e) and (f)) at selected days along the biofilm development in the flow cell system. Qualitatively, these sequences of epifluorescence images confirm the results presented on Fig. 1. Although the total amount of cells forming the biofilm was not statistically different between days 3, 7 and 11 ($P > 0.05$), the second sequence of images displays the reduction in the number of eGFP-expressing cells from day 3 to days 7 and 11 (Fig. 2(d), (e) and (f)). The number of eGFP-expressing cells as

well as the fluorescence intensity of each expressing cell decreased. In addition, the cell population was more homogeneous on day 3 with regard to eGFP production as there was a lower variability in the intensity of the fluorescent signal emitted by each cell.

Besides analysing the bulk eGFP expression levels obtained with biofilm cells (Fig. 1(a)), analysis of the eGFP expression at a single-cell level was possible by image analysis. Figure 3 presents the coefficients of variation of the fluorescence intensity determined for each microscopic image obtained on each experimental day (a total of 45 images were obtained per day). The larger the coefficient of variation (shown in Fig. 3 as vertical black line segments), the greater the difference in the eGFP levels among the individual cells within the same image. As the experiment progressed, a larger variation in cell fluorescence was obtained indicating that the biofilm became increasingly heterogeneous during development. The temporal evolution of fluorescence heterogeneity shows three distinct groups: one with a homogeneous population (days 3–5), the second with a moderately heterogeneous population (days 6–8) and the third with a strongly heterogeneous population (days 9 to 11). The statistical test performed revealed no significant differences in the average coefficients of variation within each group ($P > 0.05$), but the groups are statistically different from each other ($P < 0.05$).

Considering the average of coefficients of variation represented in Fig. 3 for each experimental day, a good linear correlation was found between these two entities ($\boldsymbol{r = 0.92}$) with most of the experimental points included within the 95% confidence interval (Fig. 4). This clearly indicates that the biofilm heterogeneity increases over time.

In an effort to elucidate the rationale for this population heterogeneity, a confocal microscopy analysis was performed on a 3-day-old biofilm obtained in the same surface, with the same culture medium and at a similar shear stress and incubation temperature. A representative confocal image shows sharply stratified patterns of eGFP expression (Fig.

5(a)). A zone of bright green fluorescence was observed at the liquid interface of the biofilm, while the interior regions of the biofilm lack eGFP-expressing cells (non-expressing cells were marked in red). The qualitative assessment of the eGFP distribution in the verticality of the biofilm (Fig. 5(a)) was confirmed by the quantitative results extracted from the z-stack acquisition. While the bottom layer of the biofilm (40 μm of dimension) consisted predominantly of non-expressing cells that emitted the red signal, the eGFP-expressing cells are predominantly located in the upper 90 μm of the biofilm (Fig. 5(b)).

4. Discussion

This study demonstrates that epifluorescence microscopy is a powerful tool for assessing the features of recombinant protein expression in *E. coli* that are not routinely measured. Biofilm viability can be quantified with a simple staining procedure, and the cells associated with recombinant protein can be quantified in relative terms. Moreover, we report the application of an image analysis tool to fluorescence microscopy for the single-cell evaluation of recombinant protein expression during biofilm growth. The developed technique provides information about population heterogeneity concerning eGFP production, which may be of great relevance in bioprocess monitoring, especially while using biofilms that are known to be heterogeneous and contain several subsets of cells in different physiological states.[11] The temporal single-cell measurements showed that the biofilm heterogeneity increases over time. O' Connell et al.[10] studied the dynamics of fluorescence during biofilm development by flow cytometry and detected three populations of *E. coli* cells with differing levels of GFP expression in 24-h biofilms. These authors also found changes in the distribution of GFP fluorescence along time,[10] in agreement with the results obtained in this study. However, while O' Connell et al.[10]

reported the total absence of non-fluorescent cells after 48 h of flow cell operation, in this work an increase in the number of such cells was detected between days 4 and 6, representing on average 79% of the total population from this moment until the end of the experiment. The different results presented by O' Connell et al.[10] are likely to be explained by the use of a high-copy-number plasmid (pUC19-based vector) and different cultivation and hydrodynamic conditions for biofilm growth in a parallel plate flow chamber.

Fluorometry is an easy, fast and common way of measuring GFP fluorescence of a bacterial population.[25] However, it measures the total sum of fluorescence intensities of all bacterial cells, which is not a good indicator of single-cell gene expression in the heterogeneous populations. By contrast, an epifluorescence microscope or a flow cytometer can be used to quantify the fluorescence of individual cells. The standard flow cytometers can rapidly determine the fluorescence of a large number of cells.[26,27] Nevertheless, using a microscope equipped with a high numerical aperture lens and a standard cooled charge-coupled device (CCD) camera, like the one used in this work, it is possible to detect cellular fluorescence with much greater sensitivity and precision when compared to a flow cytometer.[26] This is consequently due to the efficient collection of the emitted photons and the long duration of exposure that can be achieved for a static field of cells on the microscope stage.[26,28] Conversely, epifluorescence microscopes are more common in the laboratory and industrial environments than the flow cytometers[27] due to their lower cost.

It should be highlighted that the low A.F.U. values and the presence of a high fraction of non-expressing cells in the biofilm after day 5 are unlikely to be associated with plasmid loss as the presence of antibiotics throughout the flow cell system ensured a strong selective pressure. In fact, it was found that the number of colonies on selective

plates was similar to that of the non-selective plates, indicating that the plasmid is stable in the flow cell system (data not shown). Furthermore, it is known that continuous biofilm cultures for recombinant protein production are advantageous in retaining plasmid-bearing cells[10] as cells in the biofilms tend to grow more slowly than their planktonic counterparts,[29] leading to fewer divisions and hence less plasmid partitioning. The strong decline in the A.F.U. values after day 4 may be due to the loss of the pre-existing eGFP molecules from cells due to lysis. This phenomenon may be the reason for the decrease in the amount of viable cells after 4 days of biofilm development. Lowder et al.[30] reported a strong correlation between cell death and leakage of GFP from cells due to the loss of membrane integrity. The decrease in biofilm viability was probably a result of the metabolic stress imposed by the high eGFP production levels[31] in the first days of bioreactor operation. It is well documented that the production of recombinant proteins significantly affects cell metabolism by channelling resources toward the production of the foreign proteins, thereby imposing a metabolic burden and stress to the host cells.[32-35] 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.[31,36-38] It has been shown that biofilm formation by itself is accompanied by the overexpression of many stress genes[15,39,40] and it is therefore likely that high-level recombinant protein expression may increase this cellular stress. The low fluorescence intensity values registered from day 5 may be due to the simultaneous effect of the drop in viability and in the number of eGFP-producing cells. In fact, a significant percentage of the total biofilm cells were stained with propidium iodide (considered as nonviable cells) and therefore did not further participate in cell growth and eGFP formation.

Although confocal microscopes are not available in many biofilm research laboratories, a confocal microscopy analysis was introduced in this work for determining

the association between the single-cell variations found within *E. coli* biofilms and the spatial distribution of eGFP-expressing cells inside biofilms. Gradients of pH, oxygen, nutrients and waste products are established at different depths in the biofilms, and it is widely recognized that such chemical heterogeneity can lead to cells in the biofilm exhibiting different metabolic activities.[11,39] In this work, staining with the Syto61 red fluorescent dye revealed that sessile cells expressing eGFP are confined to a single band at the top of the biofilm. These results are consistent with those of Werner et al.[12] and Lenz et al.[14] who observed spatially non-uniform patterns of GFP expression in *Pseudomonas aeruginosa* colony biofilms and in biofilms grown under continuous flow conditions. It was shown that GFP synthesis occurred in a relatively narrow zone (approximately 30–60 μm wide) at the interface between the biofilm and the source of oxygen,[12] and that the *gfp* mRNA levels correlated with this zone of active GFP fluorescence.[14] Rani et al.[16] also demonstrated a stratified pattern of protein synthetic activity in *Staphylococcus epidermidis* drip-flow and capillary biofilms, with a single band of bright green fluorescence detected along the biofilm–fluid interface and rings of green fluorescence located at the periphery of cell clusters, respectively. We hypothesize that the heterogeneous pattern of eGFP expression inside the *E. coli* biofilms may be a result of oxygen limitation. Oxygen limitation in *E. coli* K-12 biofilms was demonstrated by Prigent-Combaret et al.[41] by random insertion mutagenesis. This result was further corroborated by Schembri et al.[42] who have shown the induction of several genes that are commonly expressed during oxygen-limiting conditions in *E. coli* biofilm cells. Oxygen is the only requirement for GFP fluorescence, apart from gene expression by the host cells, which is necessary for the final stage of protein folding for the formation of fluorescent chromophore.[40] Therefore, in this biofilm formation system, deeper biofilm zones may not be fluorescent due to the lack of oxygen, which is required for eGFP

maturation, or by mass transfer limitation of other nutrients.[11,39] In a previous study, we have shown that the density of biofilms formed with this strain is affected by the nutrient load of the system.[18] This raises the possibility of optimizing the nutrient load for obtaining a more porous biofilm and therefore facilitate the access to fresh nutrients of this bottom layer, which could then be shifted to a productive state.

The image analysis technique described in this work can be used not only to assess the expression levels of a fluorescent protein in order to optimize production yields but it can also be used with different fluorescent proteins to study time-dependent processes using timer fluorescence proteins (with time-dependent chromophore maturation), for promoter tracking purposes (using split fluorescent proteins) or even to monitor physicochemical changes in microenvironments, among many other applications.[43,44] However, a limitation of this methodology is that it relies on the expression of fluorescent proteins. If in a particular process, the target protein is not fluorescent a translational fusion with a fluorescent protein tag may be employed for quantification purposes.[45] This strategy may require additional processing steps like tag cleavage in order to obtain the native protein.[46] A different strategy is to use a transcriptional fusion between the protein of interest and fluorescent protein[45] enabling determination of protein expression levels without further processing steps. Additionally, if the target protein is displayed on the cell surface it can be detected and quantitated using fluorescently-labelled antibodies.[47]

In conclusion, epifluorescence microscopy and the corresponding image analysis can be regarded as a further valuable tool for determining certain production parameters that cannot be obtained by bulk methods such as fluorimetry, namely the distribution of fluorescent protein production within a cell population. The information extracted with such single-cell techniques, combined with the biofilm physiological data, can be used

for monitoring the protein expression in biofilm cells and for further determining the best processing conditions for recombinant protein production in these types of cells.

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References

[1] F.J.M. Mergulhão, G.A. Monteiro, J.M.S. Cabral, M.A. Taipa, Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*, J. Microbiol. Biotechnol. 14 (2004), 1-14.

[2] F. Baneyx, Recombinant protein expression in *Escherichia coli*, Curr. Opin. Biotechnol. 10 (1999), 411-421.

[3] C. Nicolella, M.C.M. van Loosdrecht, J.J. Heijnen, Wastewater treatment with particulate biofilm reactors, J. Biotechnol. 80 (2000), 1-33.

[4] N. Qureshi, H. Brining, L. Iten, B. Dien, N. Nichols, B. Saha, M. Cotta. Adsorbed cell dynamic biofilm reactor for ethanol production from xylose and corn fiber

hydrolysate. The 36th Great Lakes Regional Meeting of the American Chemical Society. 2004.

[5] N. Qureshi, B.A. Annous, T.C. Ezeji, P. Karcher, I.S. Maddox, Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates, Microb. Cell Fact. 4 (2005), 24-24.

[6] S. Venkata Mohan, C. Falkentoft, Y. Venkata Nancharaiah, B.S.M. Sturm, P. Wattiau, P.A. Wilderer, S. Wuertz, M. Hausner, Bioaugmentation of microbial communities in laboratory and pilot scale sequencing batch biofilm reactors using the TOL plasmid, Bioresour. Technol. 100 (2009), 1746-1753.

[7] J.D. Bryers, C.-T. Huang, Recombinant plasmid retention and expression in bacterial biofilm cultures, Wat. Sci. Tech. 31 (1995), 105-115.

[8] S. Panke, M. Wubbolts, Advances in biocatalytic synthesis of pharmaceutical intermediates, Curr. Opin. Chem. Biol. 9 (2005), 188-194.

[9] M. Talabardon, S.-T. Yang, Production of GFP and glucoamylase by recombinant *Aspergillus niger*: effects of fermentation conditions on fungal morphology and protein secretion, Biotechnol. Prog. 21 (2005), 1389-1400.

[10] H.A. O'Connell, C. Niu, E.S. Gilbert, Enhanced high copy number plasmid maintenance and heterologous protein production in an *Escherichia coli* biofilm, Biotechnol. Bioeng. 97 (2006), 439-446.

[11] P.S. Stewart, M.J. Franklin, Physiological heterogeneity in biofilms, Nat. Rev. Micro. 6 (2008), 199-210.

[12] E. Werner, F. Roe, A. Bugnicourt, M.J. Franklin, A. Heydorn, S. Molin, B. Pitts, P.S. Stewart, Stratified growth in *Pseudomonas aeruginosa* biofilms, Appl. Environ. Microbiol. 70 (2004), 6188-6196.

[13] C.-T. Huang, K.D. Xu, G.A. McFeters, P.S. Stewart, Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation, Appl. Environ. Microbiol. 64 (1998), 1526-1531.

[14] A.P. Lenz, K.S. Williamson, B. Pitts, P.S. Stewart, M.J. Franklin, Localized gene expression in *Pseudomonas aeruginosa* biofilms, Appl. Environ. Microbiol. 74 (2008), 4463-4471.

[15] C. Beloin, J. Valle, P. Latour-Lambert, P. Faure, M. Kzreminski, D. Balestrino, J.A.J. Haagensen, S. Molin, G. Prensier, B. Arbeille, J.-M. Ghigo, Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression, Mol. Microbiol. 51 (2004), 659-674.

[16] S.A. Rani, B. Pitts, H. Beyenal, R.A. Veluchamy, Z. Lewandowski, W.M. Davison, K. Buckingham-Meyer, P.S. Stewart, Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states, J. Bacteriol. 189 (2007), 4223-4233.

[17] D.J. Webb, C.M. Brown, Epi-fluorescence microscopy, Methods Mol. Biol. 931 (2013), 29-59.

[18] J.S. Teodósio, M. Simões, L.F. Melo, F.J. Mergulhão, Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient conditions and turbulent flow, Biofouling 27 (2011), 1-11.

[19] J. Sambrook, D.W. Russell, Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York, 2001.

[20] F.J. Mergulhão, G.A. Monteiro, Analysis of factors affecting the periplasmic production of recombinant proteins in *Escherichia coli*, J. Microbiol. Biotechnol. 17 (2007), 1236–1241.

[21] J.S. Teodósio, M. Simões, F.J. Mergulhão, The influence of nonconjugative *Escherichia coli* plasmids on biofilm formation and resistance, J. Appl. Microbiol. 113 (2012), 373-382.

[22] L.C. Gomes, L.N. Silva, M. Simões, L.F. Melo, F.J. Mergulhão, *Escherichia coli* adhesion, biofilm development and antibiotic susceptibility on biomedical materials, J. Biomed. Mater. Res. A 103 (2015), 1414-1423.

[23] J.M.R. Moreira, J.S. Teodósio, F.C. Silva, M. Simões, L.F. Melo, F.J. Mergulhão, Influence of flow rate variation on the development of *Escherichia coli* biofilms, Bioprocess Biosyst. Eng. (2013), 1-10.

[24] M.M. Salek, P. Sattari, R. Martinuzzi, Analysis of fluid flow and wall shear stress patterns inside partially filled agitated culture well plates, Ann. Biomed. Eng. 40 (2012), 707-728.

[25] R.J. Bongaerts, I. Hautefort, J.M. Sidebotham, J.C. Hinton, Green fluorescent protein as a marker for conditional gene expression in bacterial cells, Methods Enzymol. 358 (2002), 43-66.

[26] T. Miyashiro, M. Goulian, Single-cell analysis of gene expression by fluorescence microscopy, Methods Enzymol. 423 (2007), 458-475.

[27] S. Carneiro, A.L. Amaral, A.C. Veloso, T. Dias, A.M. Peres, E.C. Ferreira, I. Rocha, Assessment of physiological conditions in *E. coli* fermentations by epifluorescent microscopy and image analysis, Biotechnol. Prog. 25 (2009), 882-891.

[28] H.M. Shapiro, Microbial analysis at the single-cell level: tasks and techniques, J. Microbiol. Methods 42 (2000), 3-16.

[29] I. Williams, W.A. Venables, D. Lloyd, F. Paul, I. Critchley, The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*, Microbiology 143 (1997), 2407-2413.

[30] M. Lowder, A. Unge, N. Maraha, J.K. Jansson, J. Swiggett, J.D. Oliver, Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506, Appl. Environ. Microbiol. 66 (2000), 3160-3165.

[31] C.G. Kurland, H. Dong, Bacterial growth inhibition by overproduction of protein, Mol. Microbiol. 21 (1996), 1-4.

[32] B.R. Glick, Metabolic load and heterologous gene expression, Biotechnol. Adv. 13 (1995), 247-261.

[33] H.P. Sørensen, K.K. Mortensen, Advanced genetic strategies for recombinant protein expression in *Escherichia coli*, J. Biotechnol. 115 (2005), 113-128.

[34] X.-X. Xia, Z.-G. Qian, C.S. Ki, Y.H. Park, D.L. Kaplan, S.Y. Lee, Native-sized recombinant spider silk protein produced in metabolically engineered *Escherichia coli* results in a strong fiber, Proc. Natl. Acad. Sci. USA 107 (2010), 14059-14063.

[35] Y.-X. Yang, Z.-G. Qian, J.-J. Zhong, X.-X. Xia, Hyper-production of large proteins of spider dragline silk MaSp2 by *Escherichia coli* via synthetic biology approach, Process Biochem. 51 (2016), 484-490.

[36] H. Dong, L. Nilsson, C.G. Kurland, Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction, J. Bacteriol. 177 (1995), 1497-1504.

[37] W.E. Bentley, N. Mirjalili, D.C. Andersen, R.H. Davis, D.S. Kompala, Plasmid-encoded protein: The principal factor in the “metabolic burden” associated with recombinant bacteria, Biotechnol. Bioeng. 35 (1990), 668-681.

[38] G. Georgiou, M.L. Shuler, D.B. Wilson, Release of periplasmic enzymes and other physiological effects of beta-lactamase overproduction in *Escherichia coli*, Biotechnol. Bioeng. 32 (1988), 741-748.

[39] P.S. Stewart, Diffusion in biofilms, J. Bacteriol. 185 (2003), 1485-1491.

[40] R. Heim, D.C. Prasher, R.Y. Tsien, Wavelength mutations and posttranslational autoxidation of green fluorescent protein, Proc. Natl. Acad. Sci. USA 91 (1994), 12501-12504.

[41] C. Prigent-Combaret, O. Vidal, C. Dorel, P. Lejeune, Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*, J. Bacteriol. 181 (1999), 5993-6002.

[42] M.A. Schembri, K. Kjaergaard, P. Klemm, Global gene expression in *Escherichia coli* biofilms, Mol. Microbiol. 48 (2003), 253-267.

[43] Y. Wang, J.Y. Shyy, S. Chien, Fluorescence proteins, live-cell imaging, and mechanobiology: seeing is believing, Annu. Rev. Biomed. Eng. 10 (2008), 1-38.

[44] D.M. Chudakov, M.V. Matz, S. Lukyanov, K.A. Lukyanov, Fluorescent proteins and their applications in imaging living cells and tissues, Physiol. Rev. 90 (2010), 1103-1163.

[45] H.A. Shuman, T.J. Silhavy, The art and design of genetic screens: *Escherichia coli*, Nat. Rev. Genet. 4 (2003), 419-431.

[46] C.L. Young, Z.T. Britton, A.S. Robinson, Recombinant protein expression and purification: a comprehensive review of affinity tags and microbial applications, Biotechnol. J. 7 (2012), 620-634.

[47] Y.Q. Tang, S.Y. Han, H. Zheng, L. Wu, M. Ueda, X.N. Wang, Y. Lin, Construction of cell surface-engineered yeasts displaying antigen to detect antibodies by immunofluorescence and yeast-ELISA, Appl. Microbiol. Biotechnol. 79 (2008), 1019-1026.

Table and Figure captions

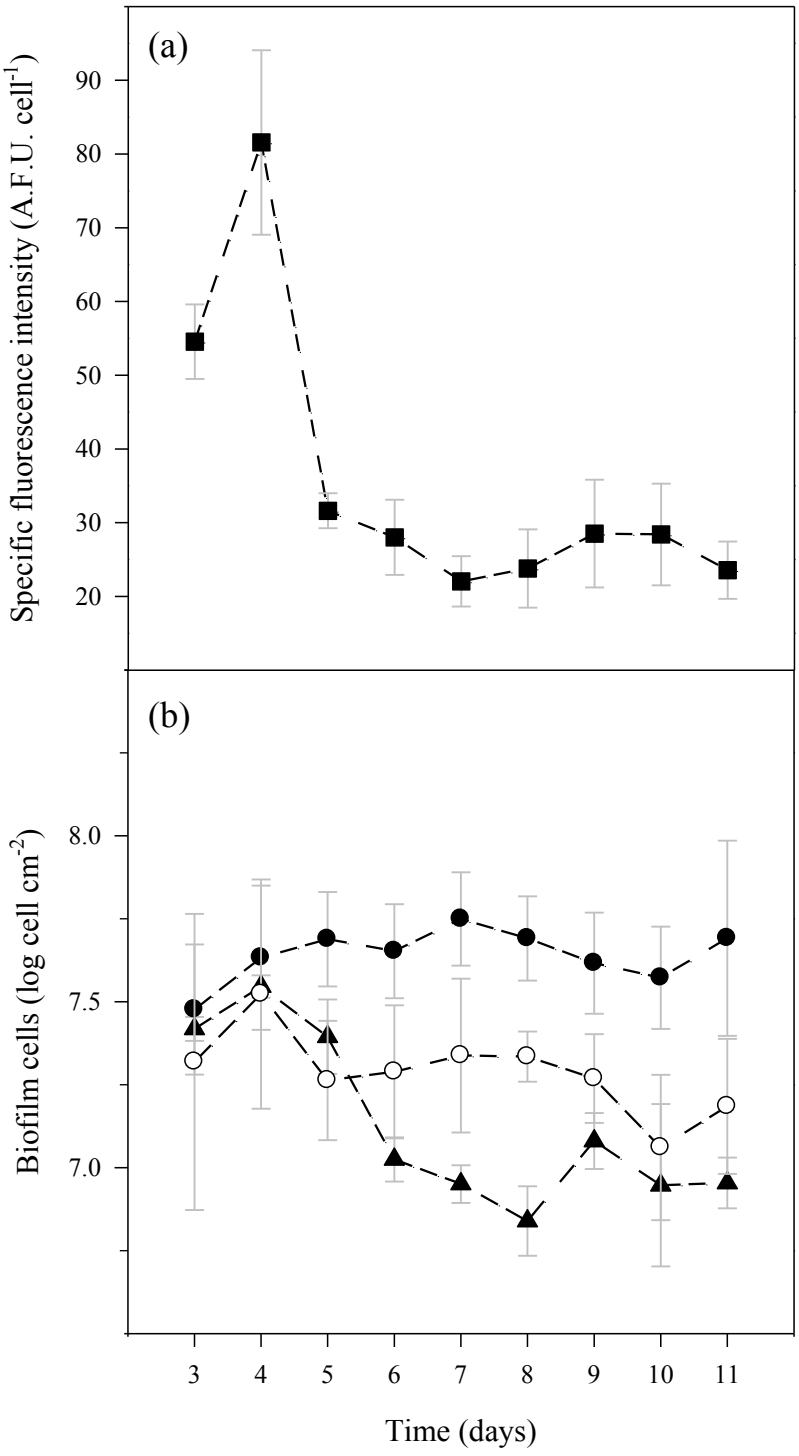
Fig. 1. Temporal evolution of (a) specific fluorescence intensity (-■-) and (b) total cell (-●-), viable cell (-○-) and eGFP-expressing (-▲-) cell number for biofilms formed by *E. coli* JM109(DE3) + pFM23. The means ± SDs for three independent experiments are illustrated.

Fig. 2. Illustrative epifluorescent micrographs of biofilm-detached cells after 3 ((a) and (d)), 7 ((b) and (e)) and 11 ((c) and (f)) days of operation of the flow cell system. Micrographs (a), (b) and (c) correspond to the representative colour-inverted images of the total *E. coli* cells stained with the Live/Dead® BacLight™ bacterial viability kit. Micrographs (d), (e) and (f) are the representative fields of *E. coli* cells expressing eGFP (bars = 10 μm).

Fig. 3. Temporal evolution of coefficients of variation for specific fluorescence intensity. Three distinct biofilm populations were identified: homogeneous (white), moderately heterogeneous (light grey) and strongly heterogeneous (dark grey).

Fig. 4. Correlation between the average of coefficient of variation for specific fluorescence intensity against time. The regression line is presented in black ($\boldsymbol{y = 1.28x + 2.36}$; $\boldsymbol{r = 0.92}$) and the 95% confidence band limits are presented in grey.

Fig. 5. Spatial heterogeneity of a 3-day-old biofilm formed by *E. coli* JM109(DE3) + pFM23: (a) section view of the CLSM images and (b) distribution of red and green fluorescence intensities along the vertical (*z*) biofilm position. The eGFP-expressing cells are labelled in green and the non-expressing cells are countermarked in red with Syto61. The dotted white line indicates the vertical section (bar = 50 μm).



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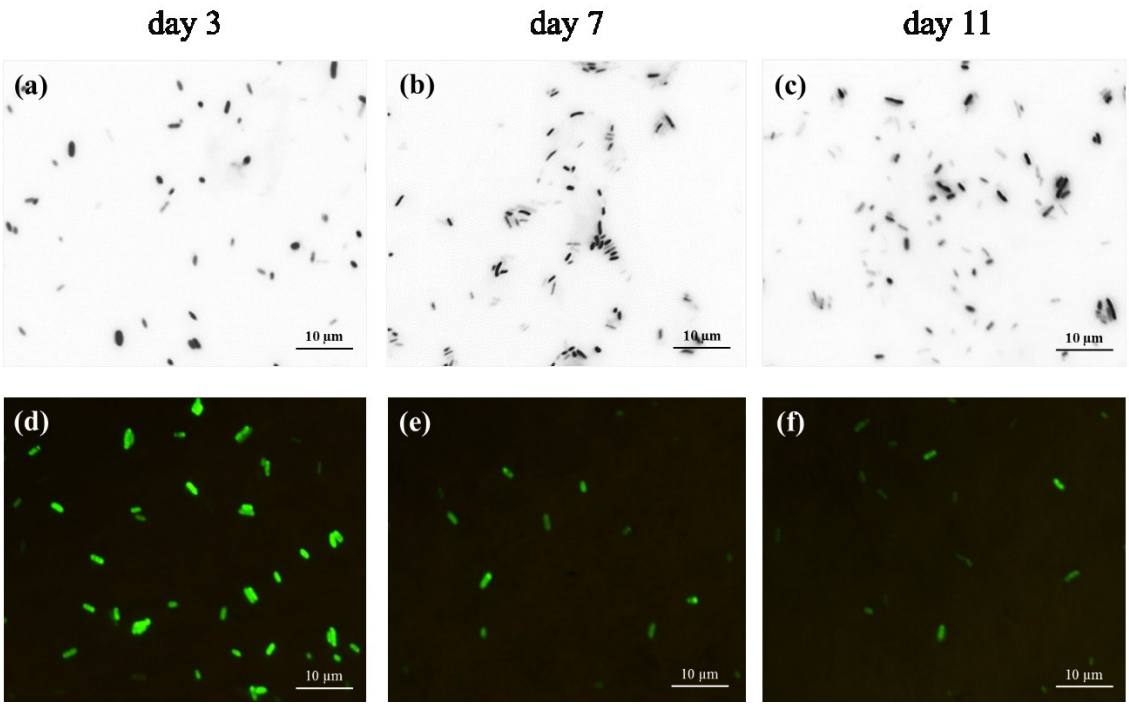

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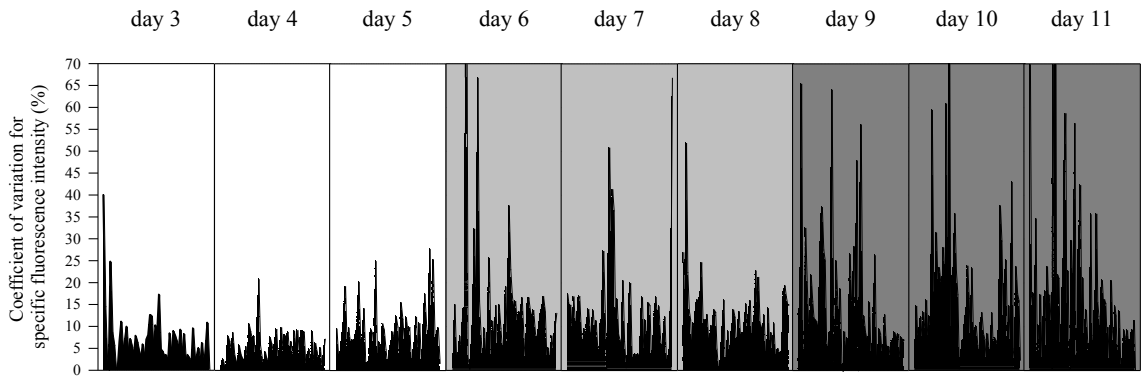

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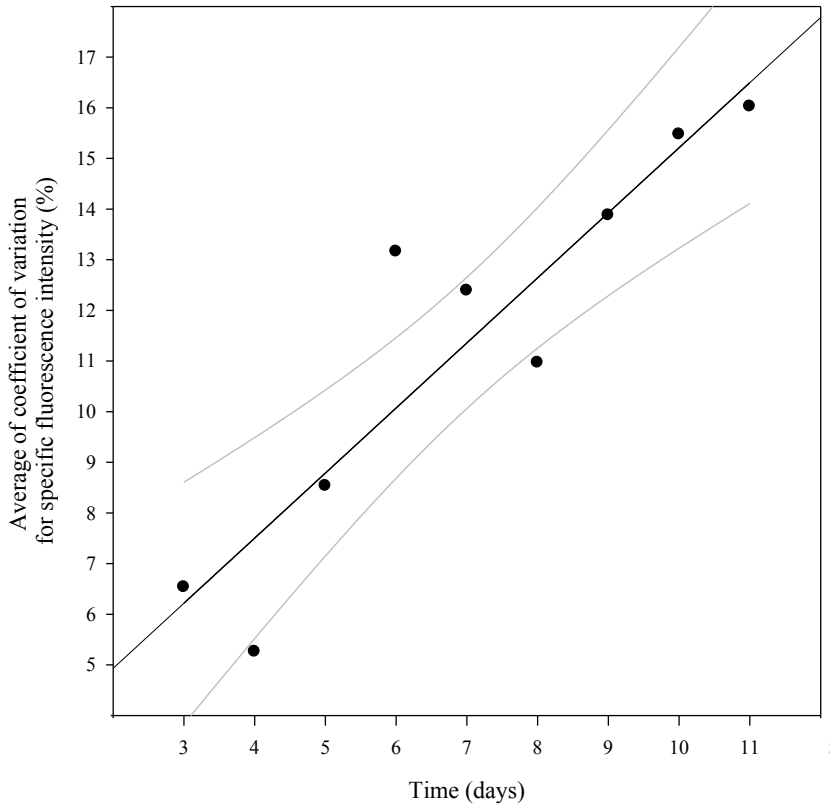

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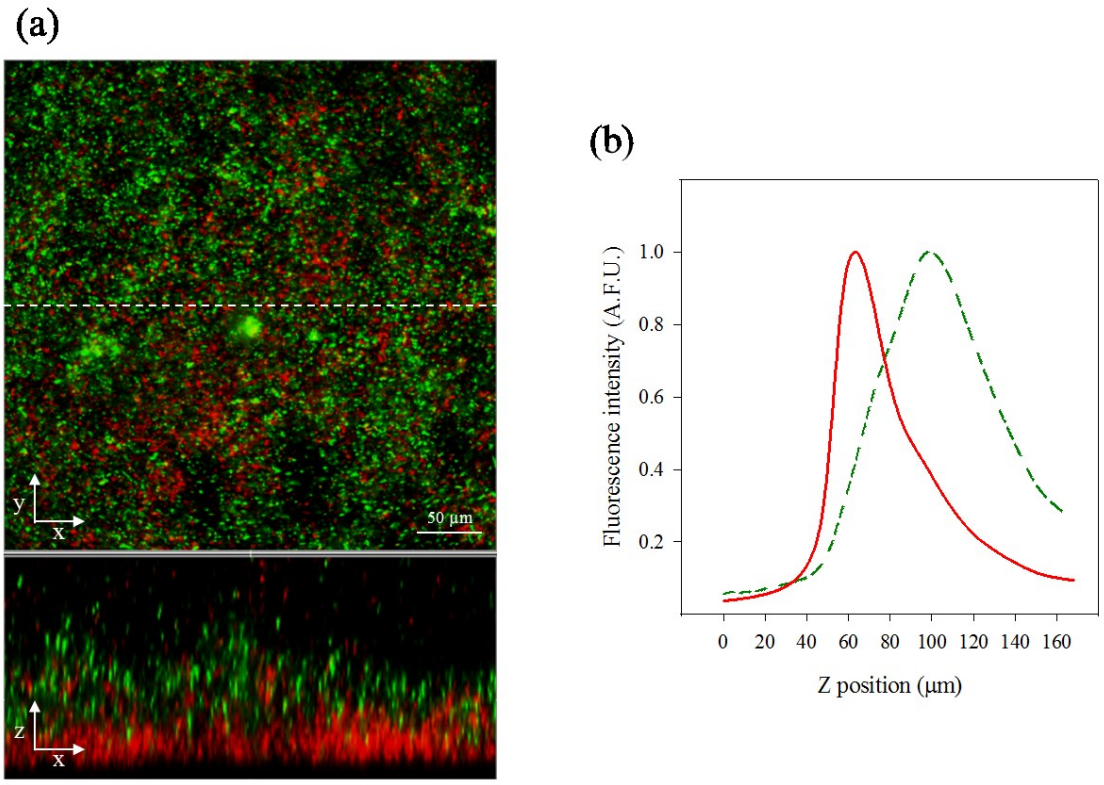

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