Evaluation of microbial globin promoters for oxygen-limited processes using *Escherichia coli*

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**Abstract**

Oxygen-responsive promoters can be useful for synthetic biology applications, however, information on their characteristics is still limited. Here, we characterized a group of heterologous microaerobic globin promoters in *Escherichia coli*. Globin promoters from *Bacillus subtilis*, *Campylobacter jejuni*, *Deinococcus radiodurans*, Streptomyces coelicolor, *Salmonella typhi* and *Vitreoscilla stercoraria* were used to express the FMN-binding fluorescent protein (FbFP), which is a non-oxygen dependent marker. FbFP fluorescence was monitored online in cultures at maximum oxygen transfer capacities (OTR max) of 7 and 11 mmol L⁻¹ h⁻¹. Different FbFP fluorescence intensities were observed and the OTR max affected the induction level and specific fluorescence emission rate (the product of the specific fluorescence intensity multiplied by the specific growth rate) of all promoters. The promoter from *S. typhi* displayed the highest fluorescence emission yields (the quotient of the fluorescence intensity divided by the scattered light intensity at every time-point) and rate, and together with the promoters from *D. radiodurans* and *S. coelicolor*, the highest induction ratios. These results show the potential of diverse heterologous globin promoters for oxygen-limited processes using *E. coli*.

**Keywords:** Microaerobic promoters, Oxygen-limited cultures, Globin promoters, FbFP expression, Microbioreactors

**Introduction**

Oxygen limitation can easily occur in high cell-density cultures due to technical and economic constraints that limit mass transfer in bioreactors. Oxygen limitation is commonly undesirable in cultures of *E. coli* because it causes strong unwanted metabolic deviations. However, operating the bioreactor at maximum oxygen transfer capacities (OTR max) would be advantageous from an economy standpoint. By modifying the metabolism of *E. coli*, it is possible to decrease the amount of byproducts formation and to improve the biomass yield and growth rate under microaerobic conditions [1]. Consequently, oxygen-limited bioprocesses could be an interesting option for the synthesis of valuable molecules, using self-inducible promoters that trigger transcription upon oxygen limitation. The development of such processes will require the availability of characterized promoters for the assembly of synthetic pathways. Oxygen-responsive promoters could also be applied as biosensors to detect oxygen-limited zones in bioreactors. We have previously characterized the performance of homologous oxygen sensitive promoters of *E. coli* and the promoter of the *Vitreoscilla stercoraria* hemoglobin (Pvgb) in oxygen-limited cultures [2]. From a group of 14 promoters evaluated, Pvgb showed interesting characteristics like good repression under aerobic conditions and the highest induction ratio. This suggests that heterologous globin promoters could be viable tools for driving oxygen responsive gene expression in *E. coli*. Koskenkorva and coworkers [3] searched globin promoters from *Bacillus subtilis* (Pbs), *Campylobacter jejuni* (Pcj), *Deinococcus radiodurans* (Pdr), Streptomyces coelicolor (Psc), and *Salmonella typhi* (Pst). The promoters were isolated and cloned in a plasmid to express chloramphenicol acetyl transferase (CAT) in *E. coli*. When cultured in shake flasks at low shaking frequency (150 rpm), maximum CAT activity was reported for all promoters after 2 h of culture, and decreased afterwards [3]. Despite the relevance of
such results, further characterization of the promoters under defined conditions is required. Namely, the cultures were performed in complex medium without pH and dissolved oxygen tension (DOT) monitoring. Furthermore, the OTR was not reported, and the dynamics of CAT expression in cultures not shown. Synthetic biology applications require standardized and well characterized parts. In this context, the effect of environmental conditions on the promoter activity is of prime relevance, particularly if bioprocess applications are sought. In the present contribution, the abovementioned promoters were synthesized and used to express the FMN binding fluorescent protein (FbFP). FbFP is an adequate reporter because of its fast activation independent from oxygen [4]. The assembly included the Shine-Dalgarno sequence and 8 bp spacer region as in our previous report [2], which allows a direct comparison of the results. Oxygen-limited cultures were performed in round well microtiter plates with optodes for pH and DOT monitoring using a chemically defined medium. Two filling volumes (1500 and 2400 μL per well) were used, which result in OTRmax values of ca. 11 and 7 mmol L⁻¹ h⁻¹, respectively [5]. Expression of FbFP under control of the constitutive promoter Pkat (which controls the expression of the aminoglycoside phosphotransferase gene kat), was used as a control to assess the effect of OTRmax in a constitutive expression system.

Results and discussion

Figure 1 shows the growth profiles of cultures expressing FbFP under control of Pkat. Cultures were oxygen-limited after 3 and 4 h of inoculation and lasted for 3.5 and 4 h at OTRmax ca. 7 and 11 mmol L⁻¹ h⁻¹, respectively (Fig. 1a). The pH decreased until glucose exhaustion (indicated by a sudden increase of DOT) and slightly increased thereafter, presumably due to the consumption of acid species like fermentative byproducts (Fig. 1a, b). Cell growth monitored by scattered light showed a change of trend when oxygen limitation started and ceased when DOT reached saturation (Fig. 1a, c). The FbFP fluorescence signal increased in parallel to scattered light. Both, final biomass and FbFP fluorescence were higher at OTRmax ca. 11 mmol L⁻¹ h⁻¹ than at OTRmax ca. 7 mmol L⁻¹ h⁻¹ (Fig. 1c, d). This can be attributed to the metabolic adaptations of *E. coli* when oxygen limits energy generation. The lower OTRmax caused a decrease of approximately 20% on the biomass and FbFP fluorescence attained (Fig. 1c, d).

The fluorescence emission yields (calculated by dividing the FbFP fluorescence intensity signal by the scattered light intensity signal at every time-point) relate the FbFP fluorescence intensity with the biomass concentration. As shown in Fig. 2, the fluorescence emission yields under both OTRmax conditions were relatively high during the aerobic phase, although displaying a strong variation. During the oxygen-limited phase, the fluorescence yields rapidly dropped to a relatively stable value near to 1.5 AU AU⁻¹. This suggests that the activity of the Pkat is affected by oxygen-limited conditions at the same extent than general biosynthetic capacity.

The growth profiles of the strains bearing the microbial globin promoters are shown in Fig. 3. Oxygen-limited cultures at two OTRmax (ca. 7 and 11 mmol L⁻¹ h⁻¹) were also performed to evaluate the sensitivity of the promoters to oxygen availability, which is very informative for bioreactor operation. A more restricted oxygen supply (resulting from a lower OTRmax) may mimic the effect of a higher concentration of a chemical inducer (for instance, IPTG in the case of Plac). However, under oxygen-limited conditions, energy generation is also limited by the capacity to regenerate NADH, which is also reflected in the capacity for biomass synthesis. In cultures at OTRmax ca. 7 mmol L⁻¹ h⁻¹, oxygen was depleted between 3 and 5 h after inoculation (Fig. 3a). Similar to culture profiles of Fig. 1, the pH decreased during the cultures until the raise of DOT signal, indicative of glucose exhaustion (Fig. 3c). The attained biomass was different for the strains bearing the different promoters and ranged from 22 (for Pvgb) to 28 (for PSc) AU (Fig. 3e). The FbFP fluorescence signals were very low during the first 4 h and increased importantly thereafter, coincident with the period of oxygen limitation (Fig. 3g). The highest FbFP fluorescence signal was recorded for PSt, which reached nearly 20 AU. Although this value is similar of that obtained using Pkat, the increase of fluorescence was observed only during the oxygen-limited period for PSt. The FbFP fluorescence readings for strains bearing Pbs, PDr, PSc and Pvgb were similar, while that of the culture using PCj was the lowest of all the studied promoters, attaining only 8 AU (Fig. 3g). In cultures at OTRmax ca. 11 mmol L⁻¹ h⁻¹, oxygen was depleted between 4 and 6 h after inoculation (Fig. 3b). The pH and DOT profiles were similar to those in cultures at OTRmax ca. 7 mmol L⁻¹ h⁻¹ (Fig. 3b, d). In contrast to cultures using Pkat, the biomass concentrations reached using the different globin promoters at OTRmax ca. 11 mmol L⁻¹ h⁻¹ were only slightly higher than those at OTRmax ca. 7 mmol L⁻¹ h⁻¹ for Pbs, PDr, PSc and Pvgb while slightly decreased for PSt and remained nearly the same for Pbs and PSc (Fig. 3e and f). In cultures at OTRmax ca. 11 mmol L⁻¹ h⁻¹ the FbFP fluorescence increased to a small extent for PCj, PSc, and PSt while remained unchanged for Pvgb and even decreased for Pbs and PDr.

Figure 4 shows the fluorescence emission yields through the cultures of the strains bearing the globin promoters. As can be seen for all the globin promoters, the fluorescence emission yields were low during the aerobic phase of the cultures. In this phase the fluorescence emission yields were disperse, which may be a result of a certain degree of induction during the
pre-culture development. Shortly after oxygen limitation, the fluorescence yields started to increase, indicating a fast induction of the \( fbfp \) gene (Fig. 4). A fast induction of FbFP expression was also observed under the control of promoters from fermentative pathways of \( E. coli \) [2]. Those promoters are activated by the protein FNR (fumarate nitrate reduction), which senses oxygen activating transcription through a redox reaction. It has been demonstrated that \( P_{vgb} \) [6] and \( P_{Bs} \) [7] are also activated by FNR. Koskenkorva and coworkers found FNR binding sites sequences in \( P_{Cj} \) and \( P_{St} \), but not in \( P_{Dr} \) and \( P_{Sc} \) [3]. The globins of \( C. jejuni \) and \( S. typhi \) are expressed in response to stress by nitric oxide, however, the role of FNR on the regulation of these promoters under oxygen-limited conditions is not completely defined [8, 9]. Nevertheless, from Fig. 4 it can be seen that all the globin promoters studied can efficiently trigger the expression of FbFP upon oxygen limitation in \( E. coli \). In cultures at \( OTR_{\text{max}} \) ca. 7 mmol L\(^{-1}\) h\(^{-1}\), the FbFP fluorescence remained relatively constant after oxygen limitation (Fig. 4 g). In contrast, in cultures at \( OTR_{\text{max}} \) ca. 11 mmol L\(^{-1}\) h\(^{-1}\), the FbFP fluorescence increased slightly for the different promoters after oxygen limitation. This indicates that some FbFP can be synthesized from re-assimilation of fermentative by-products (which are produced by \( E. coli \) under oxygen limitation) in cultures at \( OTR_{\text{max}} \) ca. 11 mmol L\(^{-1}\) h\(^{-1}\), but not at the lower \( OTR_{\text{max}} \). During the oxygen-limited period the fluorescence yields were similar for cultures at \( OTR_{\text{max}} \) of 7 or 11 mmol L\(^{-1}\) h\(^{-1}\) for all the promoters. The fluorescence yields for \( P_{St} \) and \( P_{vgb} \) were noticeably higher than for the rest of the globin promoters. These results differ from the previous report from Koskenkorva and coworkers [3], who found that the \( P_{Dr} \) displayed the highest activity. These differences could be related to genetic factors and culture conditions. First, RBS used in this work and the reported by Koskenkorva and coworkers [3] are different. Also, the use of different 5′ UTR sequences and/or reporter genes as compared with these authors could lead to differences in regulation or apparent promoter strength through unwanted interactions on different levels of expression [10, 11]. Concerning the culture conditions, the studies by Koskenkorva et al. [3] were performed using LB medium, and an \( E. coli \) K12 strain, which could produce different results. Moreover,
cultures were carried out in unbuffered medium [3], and therefore strong pH fluctuations are expected [12]. However, pH values were not informed by the authors. In the present study, the maximum fluorescence emission yields were reached during the phase of DOT raise. The maximum fluorescence emission yield was greater in cultures at OTR max ca. 11 mmol L$^{-1}$ h$^{-1}$ than in cultures at OTR max ca. 7 mmol L$^{-1}$ h$^{-1}$ for most promoters, except for P$_{St}$ and P$_{vgb}$. In all cases, the fluorescence yield were relatively stable after oxygen raise when the OTR max was ca. 7 mmol L$^{-1}$ h$^{-1}$, but rapidly decreased at OTR max ca. 11 mmol L$^{-1}$ h$^{-1}$. Again, P$_{St}$ and P$_{vgb}$ were the exceptions, as fluorescence yields decreased fast after the point of DOT raise (Fig. 4e and f).

The characterization of promoters should also consider factors like growth rate to provide information about the impact of the expression of the gene of interest on the general metabolic activity. The specific fluorescence emission rate involves the specific growth rate (not shown) during the time period of the calculation. Therefore, it is useful to give an insight of the production rate of a protein of interest under control of the promoter used. The specific fluorescence intensity was calculated over the aerobic and oxygen-limited phases of the cultures and depicted in Fig. 5a and b. The specific fluorescence intensity was very low for all promoters during the aerobic phase of the cultures and increased substantially under oxygen-limited conditions in close agreement with data from Fig. 4. In cultures at OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$, the highest specific fluorescence intensity was observed for P$_{St}$ (1.48 ± 0.02 AU AU$^{-1}$) and P$_{vgb}$ (0.92 ± 0.06 AU AU$^{-1}$) (Fig. 5a). In cultures at OTR$_{max}$ ca. 11 mmol L$^{-1}$ h$^{-1}$, most of the specific fluorescence intensity values were higher than those at OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$ (*p < 0.05 was evaluated and significant difference confirmed), except for P$_{vgb}$ that reached 0.64 ± 0.01 AU AU$^{-1}$ (Fig. 5b). Therefore, it can be concluded that stronger oxygen limitation resulted in stronger induction of P$_{vgb}$ and not for the other promoters. In cultures at OTR$_{max}$ ca. 11 mmol L$^{-1}$ h$^{-1}$, the highest specific fluorescence intensity was displayed again by P$_{St}$, followed by P$_{Cj}$, that reached values of 1.62 ± 0.10 and 0.69 ± 0.06 AU AU$^{-1}$, respectively (Fig. 5b). These values are in general greater than those of endogenous promoters of E. coli under similar culture conditions [2]. The specific fluorescence emission rate was similar for P$_{Bs}$ and P$_{Cj}$ during the aerobic and oxygen-limited phase of cultures at OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$ (Fig. 5c). This means that despite the increase of fluorescence intensity observed upon oxygen depletion for these promoters, the decline of growth rate was more pronounced, resulting in a nearly unchanged fluorescence emission rate. For all the other promoters, the specific fluorescence emission rates increased during the oxygen-limited phase, compared to the aerobic phase of the culture at OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$ (Fig. 5c).

The highest specific fluorescence emission rate under oxygen-limited conditions was observed for P$_{St}$ (Fig. 5c). In cultures at OTR$_{max}$ ca. 11 mmol L$^{-1}$ h$^{-1}$ the specific fluorescence emission rate increased during the oxygen-limited conditions.
phase, compared to the aerobic phase for the different promoters, except for $P_{vgb}$ (Fig. 5d). The result for $P_{vgb}$ is coincident with a previous study under similar conditions [2]. The specific fluorescence emission rates under oxygen-limited conditions were greater at OTR$_{max}$ ca. 11 mmol L$^{-1}$ h$^{-1}$, compared to those obtained at OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$. This is most probably a result of the limited resources for energy generation and biomass synthesis under oxygen-limitation.

Figure 6 depicts the induction ratio. This parameter represents the change of specific fluorescence intensity under uninduced (aerobic) and induced (oxygen-limited) conditions in the cultures at different OTR$_{max}$. The induction ratio was greater at OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$
for $P_{Dr}$ and $P_{vgb}$, while for the other promoters no significant differences were found using a T-test ($p < 0.05$). While $P_{Sc}$ produced the greatest fluorescence intensity and fluorescence emission rate, expression under control of $P_{Sc}$ was better repressed under aerobic conditions and yielded the highest induction ratio. The induction ratio of all the globin promoters was greater than the reported for endogenous promoters [2]. Although the used promoters, except $P_{Sc}$ and $P_{Dr}$, have putative regions for regulation by CRP, ArcA and FNR, the positions of these transcriptional elements are different for each promoter [3] and from the typical positions in *E. coli* [13]. It is possible then that the exact architecture and binding sequences of the heterologous promoters drive a more efficient induction under oxygen-limited conditions than the homologous promoters reported elsewhere [2].

The data set presented here provides useful information for the selection of oxygen sensitive promoters for particular designs. Severe oxygen limitation (OTR$_{max}$ ca. 7 mmol L$^{-1}$ h$^{-1}$) seems to negatively affect the activity of most of the globin promoters studied. Nevertheless, cell engineering strategies aimed at improving the metabolic performance and energy generation by aerobic respiration of *E. coli* under oxygen-limited conditions can increase the specific fluorescence emission rate [2]. Altogether, the information shown contributes to expand the toolbox for synthetic biology applications under bioprocessing conditions. For example, it opens the possibility to explore further combinations of these promoters with other reporter genes, 5’UTR and RBS sequences [10, 11].

**Methods**

**Strains**

*Escherichia coli* strain BL21 (DE3) was used as expression host. *E. coli* BL21 was transformed with each plasmid and conserved at $-80$ °C in a solution of 40% v/v glycerol.

**Parts synthesis and assembly**

The globin promoters used correspond to the reported by Koskenkorva and co-workers [3]. The sequences were obtained from the NCBI database and are detailed, together with their accession number, in the Additional file 1. A ribosome binding site (RBS) (Shine-Dalgarno sequence) and a spacer region of 8 bases were added previous to the start codon. FbFP sequence was taken from Evocatal (Düsseldorf, Germany, Cat. No.: 2.1.030) and the *rrnb T1* terminator was added downstream. All the sequences were flanked by a HindIII restriction sequence and cloned in the same orientation (5’-3’). The complete sequences were synthesized and cloned in pUC57kan by GenScript (Piscataway, NJ, USA).
Culture media
Precultures were grown in terrific broth (TB) consisting of 12 g L\(^{-1}\) tryptone, 24 g L\(^{-1}\) yeast extract, 12.54 g L\(^{-1}\), K\(_2\)HPO\(_4\), 2.31 g L\(^{-1}\), KH\(_2\)PO\(_4\), and 5 g L\(^{-1}\) glycerol. The main cultures were carried out using a mineral medium supplemented with 3-(N-morpholino)-propanesulfonic acid (MOPS) at a final concentration of 0.2 M, described elsewhere [2] and the pH was adjusted to 7.4 prior to sterilization. Glucose was added at final concentration of 5 g L\(^{-1}\). Kanamycin sulfate was used in all the cultures at a concentration of 50 \(\mu\)g L\(^{-1}\).

Culture conditions
For pre-culture development, 100 \(\mu\)L of cryopreserved cells were used to inoculate 10 mL of TB and grown at 30 °C in 250 mL Erlenmeyer flasks shaken at a frequency of 300 rpm with a shaking diameter of 50 mm for 8 h. 1 mL of this culture was transferred to 250 mL Erlenmeyer flasks containing 50 mL of the mineral medium. The cells grew at 37 °C and shaking frequency of 300 rpm for 6–8 h. This time corresponds to the exponential growth phase, and the absorbance of the broth (measured at 600 nm) was around 2.0. This culture was used to inoculate the microbioreactors at an initial absorbance of 0.1 units. Microbioreactor cultures were performed using the BioLector system (m2p Labs, Beasweiler, Germany), which allows online measurement of cell growth, DOT, pH and fluorescence as indicator of FbFP expression using 48 round wells plates (MTP-R48-BOH, Lot 1402, m2p Labs, Beasweiler, Germany). Plates were sealed with a hydrophobic porous rayon sterile sealing film (AeraSeal, Excel Scientific, CA, USA). Cultures were performed at 37 °C, 85% humidity, shaking diameter of 3 mm, and shaking frequency of 700 rpm. Depending on the experiment, the culture volume per well was 1500 or 2400 \(\mu\)L. Biomass was monitored by scattered light (\(\lambda_{ex} = 620\) nm; gain: 20). Fluorescence was used to monitor DOT (\(\lambda_{ex} = 520\) nm; \(\lambda_{em} = 600\) nm; gain: 83), pH (\(\lambda_{ex} = 485\) nm; \(\lambda_{em} = 530\) nm; gain: 45) and FbFP (\(\lambda_{ex} = 450\) nm; \(\lambda_{em} = 492\) nm; gain: 90). The OTR\(_{max}\) values were taken from Funke et al. 2009 [5]. All the experiments included three technical replicates.

Data analysis
The initial data of scattered light and fluorescence intensity were subtracted from the measured data. Parameters for promoter characterization were determined during
the aerobic or oxygen-limited phases. Specific fluorescence intensity was determined as the slope in the plot of fluorescence intensity ($F-F_0$) versus scattered light intensity ($I-I_0$) data points. The specific fluorescence emission rate was calculated as the product of $\mu$ multiplied by the specific fluorescence intensity. Fluorescence emission yields were calculated dividing the $F_{bFP}$ fluorescence intensity by the scattered light intensity of each time-point. For calculating the parameters under aerobic conditions, data corresponding to 2–4 h of culture were used for both $O_{TR_{max}}$ conditions, except for $P_{vgb}$, for which data from 1 to 2.5 h ($O_{TR_{max}}$ ca. 7 mmol L$^{-1}$ h$^{-1}$) and 1–3.5 h ($O_{TR_{max}}$ ca. 11 mmol L$^{-1}$ h$^{-1}$) were used. For oxygen-limited conditions at $O_{TR_{max}}$ ca. 7 mmol L$^{-1}$ h$^{-1}$, the data from 4 to 7.5 ($P_{kat}$), 4.9–8.7 ($P_{Bs}$), 4–8.5 ($P_{Cj}$), 4.2–8.9 ($P_{Dr}$ and $P_{Sc}$), 4.9–9.4 ($P_{St}$) and 2.6–8.7 ($P_{vgb}$) h of culture were used. For calculating the parameters in cultures under oxygen-limited conditions at $O_{TR_{max}}$ ca. 11 mmol L$^{-1}$ h$^{-1}$, the data from 4 to 7.5 ($P_{kat}$), 4.9–8.7 ($P_{Bs}$), 4.5–8.2 ($P_{Cj}$), 5.2–8.9 ($P_{Dr}$ and $P_{Sc}$), 6.4–9.8 ($P_{St}$) and 3.5–7.9 ($P_{vgb}$) h of culture were used.

Nomenclature

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ArcA | Component A of the Aerobic respiratory control protein (the response regulator component) |
| CRP | Cyclic AMP receptor protein |
| $d_0$ | Shaking diameter (mm) |
| DOT | Dissolved oxygen tension (% air saturation) |
| $F_{bFP}$ | FMN binding fluorescent protein |
| FNR | Fumarate and nitrate reductase (transcriptional activator) |
| $n$ | Shaking frequency (rpm) |
| $q_F$ | Specific fluorescence intensity rate (AU AU$^{-1}$ h$^{-1}$) |
| $O_{TR_{max}}$ | Oxygen transfer rate (mmol L$^{-1}$ h$^{-1}$) |
| $V_L$ | Volume of the liquid phase (μL) |

Symbols

| Symbol | Description |
|--------|-------------|
| $\lambda_{em}$ | Emission wavelength [nm] |
| $\lambda_{ex}$ | Excitation wavelength [nm] |
| $\mu$ | Specific growth rate (h$^{-1}$) |

Additional file

Additional file 1: Complete sequences cloned in the plasmid pUC57kan used in this study. (DOCX 17 kb)

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Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article and its additional file.

Supporting information

Promoters sequences used to control the expression of $F_{bFP}$.

Fig. 6 Induction ratio of the globin promoters characterized. The induction ratio was calculated dividing the value of the specific fluorescence intensity during the aerobic phase by the corresponding value during the oxygen-limited phase. White bars correspond to cultures at $O_{TR_{max}}$ ca. 7 mmol L$^{-1}$ h$^{-1}$ and black bars to cultures at $O_{TR_{max}}$ ca. 11 mmol L$^{-1}$ h$^{-1}$. Values are average of three cultures. Vertical lines indicate the standard deviation ($n = 3$) of average values.
Authors’ contributions
ARL conceived the project, performed the cultures and data analyses. KEJ and JCS contributed to the design of the expression systems. LR and JB contributed in the design of the experiments and general data interpretation. All the authors participated in preparing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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