The new pLAI (lux regulon based auto-inducible) expression system for recombinant protein production in Escherichia coli

Salvatore Nocadello* and Erwin Frans Swennen

Abstract

Background: After many years of intensive research, it is generally assumed that no universal expression system can exist for high-level production of a given recombinant protein. Among the different expression systems, the inducible systems are the most popular for their tight regulation. However, induction is in many cases less favorable due to the high cost and/or toxicity of inducers, incompatibilities with industrial scale-up or detrimental growth conditions. Expression systems using autoinduction (or self-induction) prove to be extremely versatile allowing growth and induction of recombinant proteins without the need to monitor cell density or add inducer. Unfortunately, almost all the actual auto inducible expression systems need endogenous or induced metabolic changes during the growth to trigger induction, both frequently linked to detrimental condition to cell growth. In this context, we use a simple modular approach for a cell density-based genetic regulation in order to assemble an autoinducible recombinant protein expression system in E. coli.

Result: The newly designed pLAI expression system places the expression of recombinant proteins in Escherichia coli under control of the regulatory genes of the lux regulon of Vibrio fischeri’s Quorum Sensing (QS) system. The pLAI system allows tight regulation of the recombinant gene allowing a negligible basal expression and expression only at high cell density. Sequence optimization of regulative genes of QS of V. fischeri for expression in E. coli upgraded the system to high level expression. Moreover, partition of regulative genes between the plasmid and the host genome and introduction of a molecular safety lock permitted tighter control of gene expression.

Conclusion: Coupling gene expression to cell density using cell-to-cell communication provides a promising approach for recombinant protein production. The system allows the control of expression of the target recombinant gene independently from external inducers or drastic changes in metabolic conditions and enabling tight regulation of expression.

Keywords: autoinduction, recombinant protein production, Quorum Sensing

Background

After many years of intensive research on expression of heterologous proteins, some empirical “rules” guiding the design of expression systems have emerged. Among these, tight regulation of promoter activity allows a fast initial period of cell growth to high density. Once an optimal cell density is obtained, protein expression can be triggered through inducible activation of the promoter. The promoters P_{lac}, P_{trp}, P_{tac}, λP_{L}, P_{T7}, P_{BAD}, P_{lacUV5} and P_{T5} are commonly utilized in this approach [1-3].

However, despite the availability of excellent expression systems for high-level production of recombinant proteins, the approaches using inducible promoters require monitoring of the metabolic state and cell density. In addition, balancing the cell growth rate with the rate of heterologous protein production is required to maximize the overall levels of protein production [4,5]. The different inducible expression systems offer few possibilities to fine-tune gene expression and a heterogeneous uptake of the inducer by the cells of the culture is very common [6-10]. Finally, the high cost and potential toxicity of inducers, such as isopropyl-b-D-thiogalactoside (IPTG),
limit their use for industrial scale protein expression and for production of therapeutic proteins [11-20].

The use of auto-inducing (or self-inducing) expression systems for recombinant protein production eliminates the necessity to monitor cell growth and to actively induce the expression of a target gene at the appropriate moment and to the desired level. Unfortunately, almost all auto-inducible promoters respond to the metabolic state of population or to the imposition of specific culture conditions. Furthermore, several reported auto-induction mechanisms are regulated by growth rate and a number of intracellular metabolic signals as well as specific growth conditions, such as catabolite repression, nutrient availability, dissolved oxygen tension, pH and osmolarity. These auto-induction approaches demand reactions that could be detrimental or unnecessary for production purposes [21-25].

Among known regulatory systems, Quorum Sensing (QS) offers a unique opportunity to link the expression of a recombinant gene to population density and enable auto-induction under fine regulated genetic control. Recently, Tsao et al. [26] re-engineered the native QS regulon of Escherichia coli to initiate and drive autonomously recombinant protein expression in response to prevailing metabolic state of the bacteria population. The circuit was designed to enabled modulated expression of the target gene under the control of P~lux~ promoter and the incorporated of a T7Rpol under the control of QS in a two-plasmid based expression system.

In the present study, we use a heterologous QS genetic circuit for recombinant protein expression in E. coli in order to provide a compact genetically engineered gene regulatory network that responds to cell density and that is minimally affected by the metabolic state of the host. The Vibrio fischeri’s genetic circuit for QS has been shown previously to work both in prokaryotic and eukaryotic cells in various innovative synthetic-biology applications [27-30]. We employ a simple modular design strategy to create a recombinant protein expression system in E. coli strains where a genetic ‘toggle switch’ is interfaced with the transgenic QS signaling pathway from V. fischeri. The LuxI protein, from the well-characterized LuxI/LuxR system, synthesizes a small and specific signal molecule N-3-(oxohexanoyl)-l-homoserine lactone (the auto-inducer, AI) [31,32]. LuxI produces the AI from a common precursor of the host’s metabolism, S-adenosylmethionine, and an acylated acyl carrier protein from the fatty acid biosynthesis pathway [33]. As the cell density increases AI accumulates in the medium and inside the cells. At a critical concentration, the chemical equilibrium shifts favoring formation of a complex with the transcriptional factor LuxR. The AI-LuxR complex acts on a 20-bp palindromic sequence (the LUX BOX) and directs the over-expression of the P~lux~ Promoter and the operon containing the gene. The diffusible nature of AI is the regulative key of the genetic circuit. It enables cell density-dependent induction of gene expression that is homogeneous in all cells of the culture [32]. Moreover, the genetic organization of V. fischeri’s Lux regulon was conserved in such a way that a positive feedback loop creates a very sensitive switch to turn on the expression without the necessity to amplify the signal with other complex genetic circuits [31].

Results and discussion

Construction and characteristics of expression vectors

The pLAI system is based on the luxI promoter, P~luxI~, the transcriptional activator gene, luxR, the LUX-BOX, and the luxI gene (Figure 1). However, additional features were included in the design of the pLAI expression system in order to build a set of expression vectors for a wider panel of options. His-tag, TEV protease cleavage site, MCSs, stop codons and rrrnb T1 transcriptional terminator where considered and arranged on different vectors (Figure 1B). We designed a MCS compatible with other commercial vectors, simplifying cloning operations. In fact, MCS1 has compatible restriction sites with some MCS of vectors of the pET system (pET21 series). Moreover, the vectors contain a pBR322 origin of replication (ori) or a pUC ori and an antibiotic resistance gene (ampicillin (Ap') or kanamycin (Km')). The modular approach allows the incorporation of all regulatory elements in one plasmid (pMKSal, pLAIET32, pLAIR32 or pLAIR42) or the partition of regulatory genes between the plasmid (pMKSal-LuxI) and the host genome (MM294.1::luxI). Furthermore, codon-optimized luxI and luxR and the IPTG inducible promoter P~T7~ were included in the pMKSal series. Promoter P~T7~ in divergent arrangement with respect to luxR was used in order to test the repression of QS by using inducible transcriptional interference, a molecular switch controlling repression of QS at high cell density.

Optimization of the vector pMKSal

One of the most important properties of an expression system is the high rate of recombinant gene transcription that can be achieved. Induction of the P~lux~ promoter increases exponentially the strength of expression to approximately 10^7-fold over the basal level [19]. These characteristics allow the protein to accumulate to very high concentration after induction, while deleterious effects of production of toxic gene products leading to growth inhibition should be minimized by maintaining the level of basal expression extremely low during the growth phase.

It is interesting to note that we found many rare codons in luxI and luxR for their translation in E. coli.
In fact, ORFs of luxI and luxR have 16 and 11 codons, respectively, with an usage frequency lower than 30%. In order to allow efficient translation according to the codon usage of the host organism, the codon usage and the GC content of the gene sequences for AI-synthase luxI and the AI-dependent transcriptional factor luxR, were changed. The optimized luxI and luxR sequences have several codon modifications and are 74.742% and 74.235% identical to the original sequences, respectively. The GC% of luxI and luxR changed from 32% and 30%, respectively, to 46%. Afterwards, the use of pBR322 origin of replication for a medium plasmid copy number and a different antibiotic resistance cassette was evaluated in combination with the use of optimized luxI and luxR sequences in the pMKSal.

The optimization of pMKSal vector increases the expression of the reporter gene when compared with non optimized pGL506 vector (Figure 2). In fact, the fluorescence intensity of GFP increased to 250% in fully-induced conditions. The vector optimization also affected the threshold of response to cell density. In fact, the pMKSal-gfp harboring cells showed induction of expression at lower cell density than the vector containing the non-optimized control genes. At low cell density, the fluorescence value is not different from the negative control for both pMKsal-gfp and pGL506.

The optimization of pMKSal vector demonstrated to improve the expression level. The rare codons in the V. fischeri’s regulatory genes could affect their expression in E. coli and consequently the availability of LuxR and LuxI reducing the formation of the LuxR-AI complex. Furthermore, the sequence of luxI is particularly important considering the polycistronic expression of the recombinant gene and the mRNA stability that is associated with codon frequency [34]. Codon optimization should affect also the translation of luxI mRNA allowing...
the critical concentration of AI to be reached at lower cell density and triggering induction (Figure 2). Therefore, the optimization of pMKSal vector strongly affects the dynamic response of induction to cell density.

**Reduction of gene dosage of luxI**

Inducible expression systems fulfill a fundamental requirement regarding recombinant protein production compared to constitutive promoter-based systems with respect to their ability to uncouple biomass production from the expression of the target gene [35]. The rate of synthesis of AI determines the cell density at which the AI reaches the critical concentration to induce the expression. In order to obtain induction at higher cell density, we argue to reduce the rate of synthesis of AI, by reducing the rate of expression of AI synthetase. Therefore, the gene dosage of luxI was reduced by integrating non-optimized luxI gene into the genome of *E. coli* MM294.1 (see Materials and Methods) (see Additional file 1, Figure S1). The luxI gene was truncated in the plasmid pMKSal by using two sites for the KpnI restriction endonuclease that eliminates approximately 70% of the ORF to generate pMKSal-ΔluxI (see Additional file 1, Figure S2). Cell density induction, dynamic expression and growth rate of the decoupled luxI/luxR auto-inducible system (pMKSal-ΔluxI-gfp/MM294.1::luxI) was compared with plasmids carrying luxI-luxR optimized sequences (pMKSal-gfp) (Figure 3). As expected, the reduction of gene dosage of luxI in the pMKSal-ΔluxI-gfp/MM294.1 combination resulted in induction of the gfp reporter gene at higher cell density. Although the specific fluorescence in fully induced conditions of the pMKSal-ΔluxI-gfp/MM294.1 combination was reduced by about 20% with respect to pMKSal-gfp/MM294.1, induction at higher cell density resulted in a lower impact on the growth rate. A higher final cell density was obtained increasing the volumetric productivity (amount of product produced per liter) of approximately 10% measured by fluorescence. Therefore, by reducing the gene dosage of luxI to 1 copy per genome the expression system is induced after reaching a higher cell density but with a stronger feedback effect than the other constructs used for comparison in this study. The data suggest that the uncoupling of the luxI-gene dosage from the plasmid-copy-number should lead to a tighter control of basal expression at low cell density. This strategy could be considered for production of toxic proteins.

**Repression of the auto-inducible system**

A useful property of a tightly regulated expression system is the control of the un-induced state at high cell density. This feature is exploited during pre-culturing for the normalization of different growths using the “grown to saturation” strategy [25], or during scale-up in industrial processes.
In order to provide the genetic regulatory circuit with a system for repression a novel approach was developed. The molecular mechanism of repression is based on transcriptional interference as the suppressive influence of one transcriptional process, directly and in cis on a second transcriptional process [36,37].

To test the efficiency of repression of QS activation by the transcriptional interference mechanism, an inducible promoter, \( P_{T7} \), was introduced in divergent arrangement with respect to \( luxR \) in the pMKSal plasmid (Figure 1). \( P_{T7} \) promoter is induced on pMKSal plasmid only in the BL21(DE3) strain by IPTG. Furthermore, LuxR is required for cell density-dependent induction [38].

The fluorescence of pMKSal-gfp/BL21(DE3) without and with IPTG at different concentrations was compared in Figure 4. The presence of 1 mM of IPTG in the growth medium allowed complete repression of the lux regulatory circuit and the specific fluorescence was at comparable levels to the negative control (pMKSal/MM294.1).

Insertion of the transcriptional interference mechanism provides a useful approach to repress the expression of target genes. This property may be of advantage in industrial fermentation during the scale up process. The IPTG inducible promoter \( P_{T7} \) was used to test the repression of QS by using inducible transcriptional interference. However, the use of other inducible promoters could be explored.

**Conclusion**

Since Grossman et al. [39] encountered unintended induction in the T7 based expression system auto-induction of expression for recombinant protein production is generally referred to as a phenomenon intimately related to the medium composition. It allows induction and production of substantial amounts of target protein in absence of an added inducer. Consequently, many types of auto-inducible media were developed and auto-induction has become a standard procedure in many laboratories for testing expression and solubility of many proteins and for producing target proteins in large amounts for purification [25]. In fact, auto-induction is more convenient than inducer-dependent induction. The expression strain is inoculated into well-defined auto-inducing medium and grown to saturation without the need to monitor culture growth or to add an inducer at a specific point in time of the process. Any promoter that is induced by a change in metabolic state of the growth culture, such as the transition to depletion of a specific nutrient, catabolite repression, stationary growth, pH, oxygen levels or osmolarity, can be applied in the auto-inductive approach [22]. Nonetheless, most of these systems are strictly related to a particular medium definition and/or growth conditions, often causing stress upon the cells during growth and during the recombinant protein production phase.

This work describes a different approach for the auto-induction of recombinant protein expression. QS
provides a strictly regulated genetic control enabling recombinant gene expression to be linked to population density. While Tsao et al. [27] developed a autoinducible system by rewiring native QS of *E. coli*, in our approach, we were looking for a simple molecular switch that was i) directly linked to cell-density, ii) independent from any endogenous signals and from reorganization of host's metabolic state. We demonstrated that the regulation of *V. fischeri*’s QS system, optimized in the pLAI expression system, is a very useful mechanism to control gene expression for protein production. Since *E. coli* is the prokaryotic expression host of choice for the production of many recombinant proteins, we adapted the simplified cell-density dependent genetic switch of *V. fischeri*’s QS to *E. coli* strains.

The amphipathic, diffusible character of AI simplifies the genetic regulatory circuit avoiding the necessity of a transporter for the inducer. In several available *E. coli* expression systems only all-or-none gene expression is possible because expression of the gene encoding the transporter for the inducer is controlled by the inducer itself [40-43]. In these expression systems, expression is not adjustable in individual cells and protein expression is not homogeneous. In contrast, the pLAI expression system ensures homogeneous induction due to the high permeability of the cells for AI [32].

The system does not need specific auto-inducible medium and/or growth conditions to guarantee auto-induction. Rather, the system responds directly to cell density during growth. However, growth conditions exist that allow modification of the cell density perception [45] and also the protein expression profile. For example, it is reported that the half-life of AI affects cell-to-cell communication and the detection of cell density [38]. Indeed, the AI degradation rate is accelerated by increasing the pH of the medium [19]. Furthermore, the regulation of the LuxI/LuxR system of *V. fischeri* is also controlled by catabolite repression [44]. Our preliminary data suggest that pH and glucose concentration are useful fermentative variables that could be used to adjust the cell-density dependent induction or the dynamic expression pattern to respond to specific production requirements (see Additional file 1, Figures S3, S4, S5). However, more experimental data are needed.

In conclusion, we have generated an effective, autonomous system of auto-induction which does not rely on the addition of an inducer, the use of specific culture conditions or imposition of specific stress factors. Furthermore, it enables tight regulation of gene expression, homogeneous induction and high maximal expression. Different genetic approaches provided a panel of vectors that make the pLAI system very flexible for different protein production requirements.

Auto-induction is convenient, efficient and economical for production of proteins for research and industrial purposes since cultures for auto-induction are simply inoculated and grown to saturation. While direct application of this expression system in industrial scale is ongoing, we welcome these cell density-based autoinducible expression systems for high throughput recombinant protein expression and lab-scale production processes.

**Methods**

**Media and bacterial strains**

Cultures were grown in Luria-Bertani (LB) broth at 37°C or as indicated in the figure legends. Complex medium (CM) contains yeast extract (30 g/l), KH₂PO₄ 5.4 g/l, K₂HPO₄ 15.6 g/l, MgSO₄ 250 mg/l, glycerol 15 g/l, pH 7.4. YE3X medium contains yeast extract (45 g/l), KH₂PO₄ 4 g/l, K₂HPO₄ 16 g/l, glycerol 15 g/l, pH 7.2. Antibiotics were used as follows: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml and erythromycin 50 μg/ml. Media were supplemented with glucose or IPTG as indicated in the figure legends (the materials were obtained from Sigma, St. Louis, Mo.). Cell growth was monitored as the optical density at a wavelength of 590 nm (OD₅₉₀).

The bacterial strains used in the present study are listed in Table 1. All DNA manipulations were performed in *E. coli* DH5α by using established protocols [23].

Strain *E. coli* MM294.1::luxI was constructed applying a modified PCR-mediated gene recombination method [45]. Briefly, luxI gene, including its promoter, was amplified by PCR using the primers LxIAscIF-TTTTGGCCGCCAATTATTTTCCATATAATAC TTAGTA and LxIAscIR-TTTTGGCCGCCCTAAAAC CGGTAATAGTTGACA and chromosomal DNA of *V. fischeri* ATCC7744. The PCR product was digested with Ascl. Concomitantly, for pGLEM constructions, metE gene was amplified from genomic DNA of *E. coli* MM294.1 with the primers metEScaIL-GAAAAAG TACTGCTTTGTAGCGTTTTTCCGTTG and metEScaIRGAAAAATCTGGAAGAAGTCGCTGTAAATG and cloned in pGLOW (Invitrogen). The restriction site for EcoRV in metE was used to produce blunt-ends to be ligated with a blunt-end fragment containing the ery gene, extracted from pBS-Ery with *SmaI* [46]. The restriction site for Ascl in ΔmetE, proximal to the ery gene, was used to insert the digested PCR product containing non codon-optimized luxI, generating pGLEM-luxI. The entire linear fragment containing ΔmetE-ery-luxIΔmetE was PCR amplified from pGLEM-luxI, using MetEL-GAAAAAGTACTGCTTTGTAGCGTTTTTCCGTTG and MetER-GAAAAATCTGGAAGAAGTCGCTGTAAATG and the ampiclon was used to transform *E. coli*
MM294.1 carrying the pKobeg plasmid and erythromycin-resistant candidate integrants were analyzed by PCR (using LuxI4Fr-TCAAATGTCAATCTATTACCG and LuxI4Rv-TCCTTACCTATTGTTTGTCG) and Southern blot [45].

Briefly, for Southern blot, genomic DNA was prepared from an overnight liquid culture of *E. coli* MM294.1 and its isogenic *luxI* mutant using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Five μg of genomic DNA of each mutant was digested overnight with *XmaI* and *AatII* restriction enzymes at 37°C and loaded on a 0.7% agarose gel with appropriate DNA size markers. A 513-bp DNA probe, annealing within the *luxI* gene, was prepared by PCR from pGLEM-*luxI* using the primers LuxI4Fr and LuxI4Rv. Southern blot was performed with the ECL Direct Nucleic Acid Labeling and Detection Systems kit (GE Healthcare) as described by the manufacturer.

**Construction of pLAI vectors**

Constructed vectors differ in multicloning sites (MCS), the antibiotic resistant gene and origin of replication (ori).

The **luxR-luxI** containing region of the *lux* regulon was amplified from genomic DNA of *V. fischeri* ATCC7744 with LuxIRFw-AAGCTTTACTTACGTACTAATTTTA and LuxIRRv-TCATTATTTCCCCATAATACCTTAGT. The PCR product was inserted in topoisomerase recombination sites of pGLOW (Invitrogen) to produce pGL506. *luxR* and *luxI* genes were codon optimized as previously described by Richardson et al. [47]. Two enzymatic restriction sites, for *KpnI* and *XbaI*, were introduced in *luxI*. A DNA fragment containing the T7 promoter, the optimized *luxR* gene, the *luxR-luxI* intergenic region, the optimized *luxI* gene, a multiple cloning site (MCS1) and the transcription terminator was designed and synthesized (GeneArt). This fragment was inserted into a pMK vector using *AscI* and *PacI* cloning sites, producing pMKSal [48]. The *gfp* gene was amplified from pGLOW (Invitrogen) using GFP Eco-GAATTCAAGCTTAAAGAGAAGAAGACT and GFP NotI-GCGGCCGCTTATTTGTAGCTCATTCCA and inserted in the MCS1 of pMKSal using EcoRI and NotI. The final plasmid is pMKSal-gfp.

**Table 1 Strains or plasmids used in this work**

| Strains or plasmids | Descriptions | References or sources |
|---------------------|--------------|-----------------------|
| Strains | | |
| *V. fischeri* | ATCC7744 | ATCC7744 |
| E. coli DH5α | F' Δ(lacZΔM15 ΔlacZYA-argF)U169 deoR recA1 endA1 hsdR17(r-k, m+k) phoA supE44 thi-1 gyrA96 relA1 | Invitrogen |
| E. coli TOP10 | F' mcrA Δ(met-1::MCS-ermB) Δ(lacQ-151) 7697 galU galK rpsL (Strr) | Invitrogen |
| E. coli MM294.1 | F' supE44 hsdR17 endA1 thi-1 | Novartis Collection |
| E. coli MM294.1::luxI | F' supE44 hsdR17 endA1 thi-1Δmet-1::eryluxI | This study |
| E. coli BL21(DE3) | F' ompT gal dcm lon hsdSB(rB- mB-) λDE3 (lacI lacUV5-T7 geneI ind1 sam7 nin5) | Novagen |
| Plasmids | | |
| pBS-Ery | pBR322 ori; Ap'; carrying ery (Er') | 51 |
| pET21b | IPTG inducible; pBR322 ori; Ap'; | Novagen |
| pET21b-gfp | pET21b carrying gfp; Ap'; | This study |
| pGlow | TOPO-TA vector, promoterless gfp; pUC ori; Ap'; | Invitrogen |
| pGL506 | pGlow carrying luxR-luxI amplicon in TA cloning site; pUC ori; Ap'; | This study |
| pGLEM | pGlow carrying Δmet-E-ery in TA cloning site; pUC ori; Ap'; | Novartis Collection |
| pGLEM-luxI | pGLEM carrying luxI; pUC ori; Ap'; | This study |
| pKobeg | Arabinose inducible expression for gam, bet, exa, R101 ori; Cm'; | 7 |
| pMK | pBR322 ori; Km'; | 1 |
| pMKSal | MCS1; pBR322 ori; Km'; | This study |
| pMKSal-GFP | pMKSal carrying gfp; Km'; | This study |
| pMKSal-luxI | pMKSal with truncated luxI; Km'; | This study |
| pMKSal-luxI-GFP | pMKSal-luxI carrying gfp; Km'; | This study |
| pLAIM2 | MCS2; pUC ori; Ap' and Km'; | This study |
| pLAIM4 | MCS3; pUC ori; Ap' and Km'; | This study |
| pLAIMET2 | MCS2; pBR322 ori; Ap'; | This study |

*Ap', ampicillin resistance; Cm', chloramphenicol resistance; Km', kanamycin resistance; Er', Erythromycin resistance; ori, replication origin; IPTG, Isopropyl-β-D-thiogalactosidase.*

MM294.1 carrying the pKobeg plasmid and erythromycin-resistant candidate integrants were analyzed by PCR (using LuxI4Fr-TCAAATGTCAATCTATTACCG and LuxI4Rv-TCCTTACCTATTGTTTGTCG) and Southern blot [45].

Briefly, for Southern blot, genomic DNA was prepared from an overnight liquid culture of *E. coli* MM294.1 and its isogenic *luxI* mutant using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Five μg of genomic DNA of each mutant was digested overnight with *XmaI* and *AatII* restriction enzymes at 37°C and loaded on a 0.7% agarose gel with appropriate DNA size markers. A 513-bp DNA probe, annealing within the *luxI* gene, was prepared by PCR from pGLEM-luxI using the primers LuxI4Fr and LuxI4Rv. Southern blot was performed with the ECL Direct Nucleic Acid Labeling and Detection Systems kit (GE Healthcare) as described by the manufacturer.
pMKSal-ΔluxI is a pMKSal derivative plasmid obtained by its digestion with KpnI and self-circularization in order to generate a non functional LuxI. For pLAIET32, pLAIR32 and pLAIR42 vector constructions, 2 DNA fragments, were assembled by using long primer and assembly-PCR, as previously reported [49]. Both sequences containing MluI-AatII-MCS2-Ndel or MluI-AatII-MCS3-Ndel, were cloned in pCR1 (Invitrogen). The luxR-luxI containing fragment, amplified from pGL506 plasmid with LF/MluI-TTTTACCGGTACCTTGTTAACCTAATTATTTGAACGTCTTCATTATTTCCCCTATAATATA and LR/NdeII sequences containing T-TTTTACGCGTGAGAAGCAGGCCATTATCGC and T-TTTT

When the OD590 reached 0.5, the cells were grown at different temperatures, as indicated in figure legends. When the OD590 reached 0.5, the cells harboring the IPTG inducible promoter were induced with 1 mM of IPTG or as indicated in figure legends. GFP was used to provide an indirect, quantitative measurement of the transcriptional properties of the cloned gene [50,51].

GFP fluorescence in batch cultures of E. coli containing the reporter plasmids expressing gfp was measured by Tecan Infinite M200 plate reader (Tecan) using an excitation wavelength of 405 nm and an emission wavelength of 535 nm. GFP fluorescence was normalized for cell density (GFP fluorescence per OD590 unit).

Protein extracts, SDS-polyacrylamide gel electrophoresis, and Western blotting
All samples for SDS-page or Western blotting were normalized by cell density. Cell cultures were centrifugated (13000 rpm for 5 min) and the pellets were resuspended in 1 ml of buffer B-PER (Thermo scientific), 10 μl of lysozyme (100 mg/ml, Invitrogen) and 20 μl of DNase (1000 kunits/ml, Invitrogen). The samples were incubated at room temperature for 30 min. To 120 μl of sample, 40 μl of Loading Sample Buffer 4 × (0.4 M DTT, 8% SDS, 200 mM Tris-HCl pH 6.8, 0.1% bromo-phenol blue, 40% glycerol) was added. The samples were boiled for 10 min at 95°C. For each sample, protein extracts were loaded on SDS-12% polyacrylamide gels, and polyacrylamide gel electrophoresis was performed at 180 mA for 1 h. Immunoblotting was performed with a commercial anti-GFP antibody (Invitrogen) as first primary antibody, as previously described [23].

Additional material

Additional file 1: Figure S1. Molecular characterization of luxI integration into the E. coli genome. A) PCR analysis of MM294.1::luxI. Chromosomal DNA of MM294.1::luxI (line 1) and chromosomal DNA of MM294.1::luxI (line 2) and chromosomal DNA of MM294.1::luxI (line 3) were used as a template for PCRs with primers LuxF/luxR, specifically annealing to luxI. A molecular weight ladder is also shown, on the right. B) Southern blot analysis of wild type and MM294.1::luxI mutant strain. Plasmid pGLEM-luxI (line 3; positive control), Chromosomal DNA from MM294.1::luxI mutant (line 4) and wild type MM294.1 (line 5) were digested with XmaI/AatII restriction enzymes. Fragments were separated on an agarose gel and transferred to nitrocellulose membrane for Southern blot analysis using a 513-bp PCR (primers LuxF/luxR) that fully probed within the luxI gene. As a control of the Southern blot efficiency, a 513-bp PCR (primers LuxF/luxR) from plasmid pGL506 (line 1) and chromosomal DNA of MM294.1::luxI (line 2) were loaded onto the agarose gel. C) Schematic representation of luxI integration into the genome. Figure S2. Schematic decoding of coupled luxI/luxR auto-inducible system. LuxI is produced from the genome, reducing the luxI gene dosage to 1/genome, while luxR and the target gene dosages depend on plasmid copy number of pMKSal-ΔluxI. Figure S3. pH effect on the pattern of expression. Comparison of pMKSal/gfp/MM294.1 expression at different cell densities at two different pH set points (6.2 ± 0.1 and 7.2 ± 0.1) controlled during bacterial growth in a bioreactor. Batch processes were carried out in a 7-liter bioreactor (Applikon) under the following conditions: 5L of YE3X containing 15 g/l of glycerol, 25°C, 0.5 VVM (air Volume per Volume of culture medium per Minute) of airflow and a 300rpm stirrer speed. The glucose was added to the medium where indicated. An Applikon programmable logic controller (ADI1030) was used for maintaining temperature at 25°C and pH set-point. The pH of the medium was maintained with 2 M H3PO4 and 4 M NaOH. The concentration of dissolved oxygen was maintained at 65% by controlling the stirrer speed up to a maximum speed of 800 rpm and O2 flow by electronic valve. The sampling was done for SDS-PAGE, Western blotting and fluorescence assays as reported above. During the growth at 25°C samples were collected at the indicated cell density. The fluorescence level was determined for 3 replicates of each sample. Controls pMKSal/MM294.1 (negative) and pET21::gfp/BL21(DE3) (positive) were grown in the same conditions. pET21::gfp/BL21(DE3) was induced at 30°C with 1 mM of IPTG and collected after 3 hours from the induction. Controls pMKSal/MM294.1 and pET21::gfp/BL21(DE3) were collected at 134 OD590 and 12.5 OD600, respectively. Error bars show the standard deviation of the experiment. Figure S4. Glucose effect on the pattern of expression. Comparison of culture-average fluorescence (fluorescence per OD590 unit) of E. coli harboring pMKSal-gfp grown in LB (squares) or YESX (triangles) without (open symbols) or with the addition of 2.5 g per liter of glucose (solid symbols). Cells grown overnight at 37°C in LB medium containing the appropriate antibiotic, were used to inoculate LB and YESX media with antibiotic and with or without glucose, in 96-well plates at 27°C with shaking in a Tecan Infinite M200, monitoring the optical density and the fluorescence. 5 replicates of each condition were incubated. Figure S5 Western blot analysis of GFP expression during the batch process under conditions of catabolite repression. E. coli cells harboring pGL506 grown overnight at 37°C in shaking a flask with YESX with the appropriate antibiotic were used to inoculate 7 L Applikon.
bioreactor containing 5 L of YESX medium with glucose (1 g/L), 15 g/L of glycine. The fermentation was performed at 25°C, with 0.5 VVM (air volume per volume of culture medium per minute) of airflow and a 300 rpm stirrer speed. An Applikon programmable logic controller (AD1030) was used for maintaining temperature at 25°C and pH set-point. The pH of the medium was maintained with 2 M H3PO4 and 4 M NaOH. The concentration of dissolved oxygen was maintained at 65% by controlling the stirrer speed up to a maximum speed of 800 rpm and O2 flow by electronic valve. Samples were collected at the indicated cell densities. All proteins were isolated at various times at the cell density reported in each well. The proteins were separated on an SDS 10% polyacrylamide gel and blotted onto a nitrocellulose membrane to be incubated with an anti-GFP polyclonal antibody (Invitrogen). All samples were normalized based on the biomass. As a positive control, 0.2 μg of purified GFP (Invitrogen) was used.

List of abbreviations

QS: Quorum Sensing; AI: autoinducer.

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Authors’ contributions

SN carried out the studies and drafted the manuscript. ES participated in the design of the study and coordination and revised the manuscript. All authors read and approved the final manuscript.

Authors’ information

SN, PhD, current position: post-doc at Novartis Vaccines and Diagnostics, ES, PhD, current position: lab head at Novartis Vaccines and Diagnostics.

Competing interests

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