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

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## 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 a 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  $P_{lac}$ ,  $P_{trp}$ ,  $P_{tac}$ ,  $\lambda P_L$ ,  $P_{T7}$ ,  $P_{BAD}$ ,  $P_{lacUV5}$  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),

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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 enable modulated expression of the target gene under the control of  $P_{T7}$  promoter and the incorporation 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

$P_{luxI}$  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

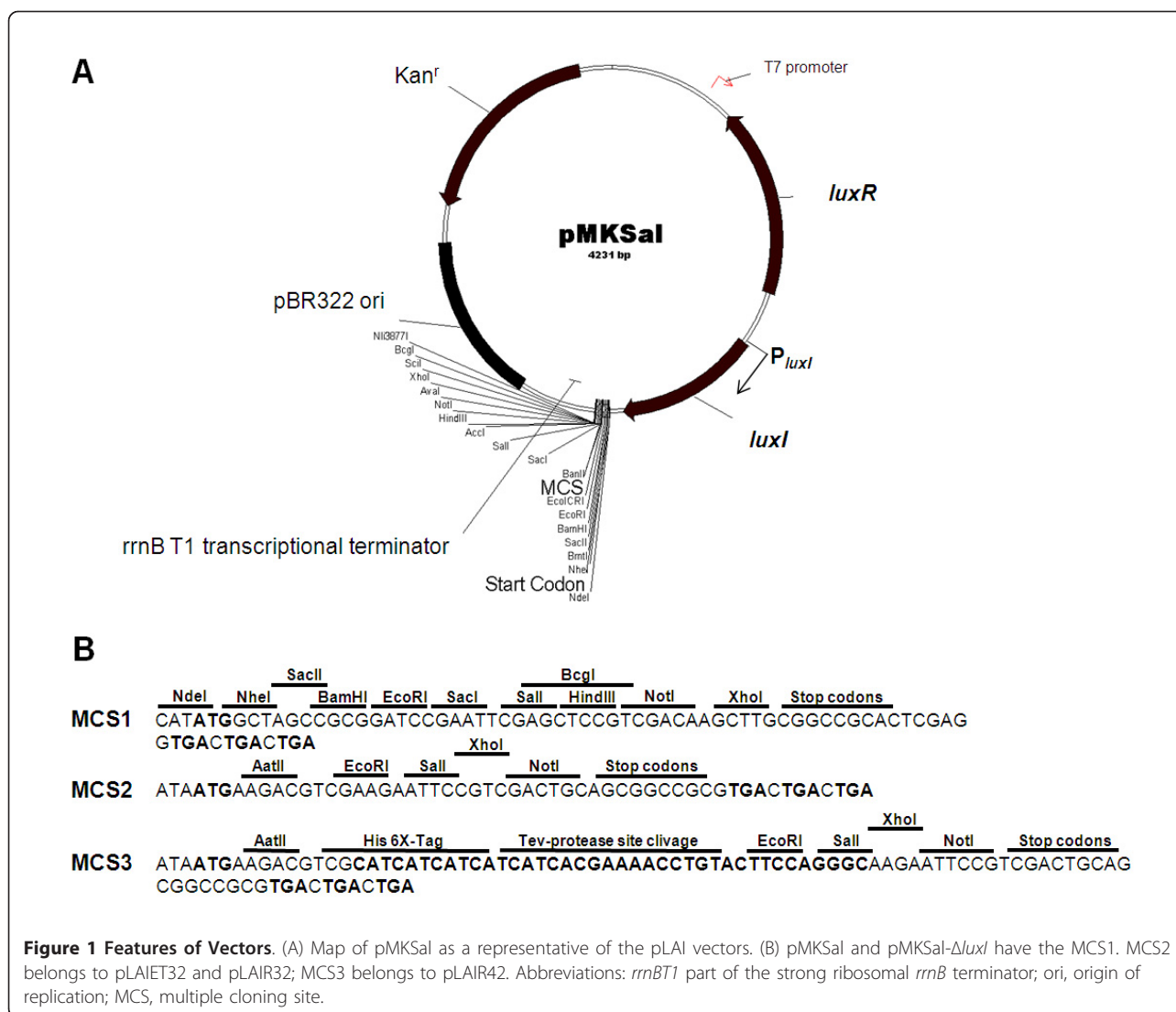
### Construction and characteristics of expression vectors

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 *rrnB T1* transcriptional terminator were 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 ( $Ap^r$ ) or kanamycin ( $Km^r$ )). The modular approach allows the incorporation of all regulatory elements in one plasmid (pMKSaI, pLAIET32, pLAIR32 or pLAIR42) or the partition of regulative genes between the plasmid (pMKSaI- $\Delta luxI$ ) and the host genome (MM294.1::*luxI*). Furthermore, codon-optimized *luxI* and *luxR* and the IPTG inducible promoter  $P_{T7}$  were included in the pMKSaI 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 pMKSaI

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  $P_{luxI}$  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*.

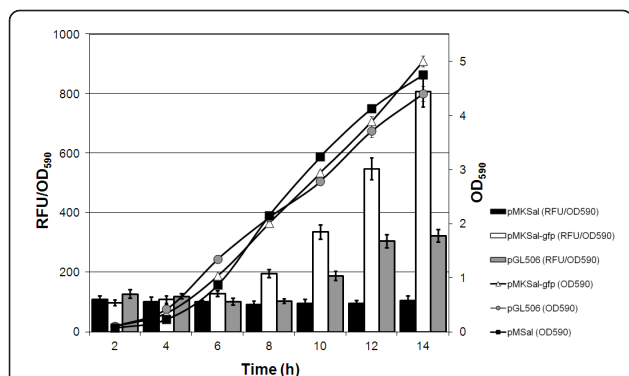


In fact, ORFs of *luxI* and *luxR* have 16 and 11 codons, respectively, with an usage frequency lower than 30%. In order to allow efficient translation according to the codon usage of the host organism, the codon usage and the GC content of the gene sequences for AI-synthase *luxI* and the AI-dependent 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 pMKSaI.

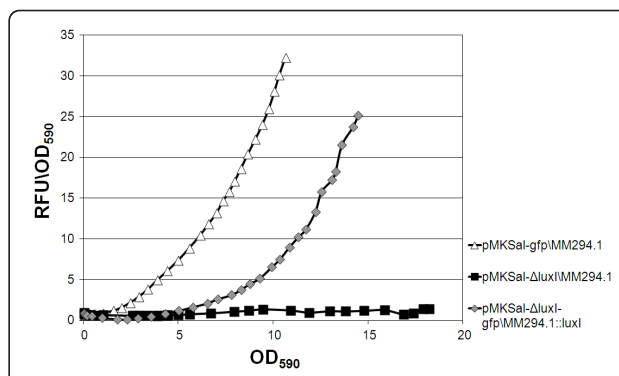
The optimization of pMKSaI vector increases the expression of the reporter gene when compared with non optimized pGL506 vector (Figure 2). In fact, the

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 pMKSaI-*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 pMKSaI-*gfp* and pGL506.

The optimization of pMKSaI 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



**Figure 2 Optimization of pMKSsal vector.** Comparison of growth curve and culture-average fluorescence (Relative Fluorescence Unit per OD<sub>590</sub> unit) of *E. coli* harboring pMKSsal-gfp (*luxI* and *luxR* optimized; open triangles and white bar blots for OD<sub>590</sub> and fluorescence, respectively), pGL506 (*luxI* and *luxR* non-optimized; grey circles and grey histogram for OD<sub>590</sub> and fluorescence, respectively) and pMKSsal (negative control, *luxI* and *luxR* optimized; black squares and black histogram for OD<sub>590</sub> and fluorescence, respectively). Cell cultures were grown overnight in LB at 37°C and sub-cultured in CM medium with the appropriate antibiotic, to a final density of 0.15 OD<sub>590</sub>. 5 replicates of each clone were incubated in 96-well plates at 30°C with shaking, monitoring the optical density and the fluorescence in a Tecan Infinite M200. Error bars show the standard deviation of the experiment.



**Figure 3 Dynamic expression of the decoupled *luxI/luxR* auto-inducible system.** Comparison of culture-average fluorescence (Relative Fluorescence Unit per OD<sub>590</sub> unit) and cell density associated with specific fluorescence of the decoupled *luxI/luxR* auto-inducible system (pMKSsal-Δ*luxI*-gfp/MM294.1::*luxI*; grey diamonds), coupled *luxI/luxR* optimized-sequences auto-inducible system (pMKSsal-gfp/MM294.1; open triangles), and control (pMKSsal/MM294.1; solid squares). Cells carrying the plasmid, grown overnight in LB at 37°C, were sub-cultured in CM medium with the appropriate antibiotic, to a final density of 0.15 OD<sub>590</sub>. 5 replicates of each clone were incubated in 96-well plates at 27°C with shaking, in a Tecan Infinite M200, monitoring the optical density and the fluorescence. The temperature was decreased at 27°C in order to reach high cell density in rich medium with a low aerated condition.

the critical concentration of AI to be reached at lower cell density and triggering induction (Figure 2). Therefore, the optimization of pMKSsal 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 pMKSsal by using two sites for the *KpnI* restriction endonuclease that eliminates approximately 70% of the ORF to generate pMKSsal-Δ*luxI* (see Additional file 1, Figure S2). Cell density induction, dynamic expression and growth rate of the decoupled *luxI/luxR* auto-inducible system (pMKSsal-Δ*luxI*-gfp/MM294.1::*luxI*) was compared with plasmids carrying *luxI-luxR* optimized sequences (pMKSsal-gfp) (Figure 3). As

expected, the reduction of gene dosage of *luxI* in the pMKSsal-Δ*luxI*-gfp/MM294.1::*luxI* combination resulted in induction of the *gfp* reporter gene at higher cell density. Although the specific fluorescence in fully induced conditions of the pMKSsal-Δ*luxI*-gfp/MM294.1::*luxI* combination is reduced by about 20% with respect to pMKSsal-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 pMKSaI plasmid (Figure 1).  $P_{T7}$  promoter is induced on pMKSaI plasmid only in the BL21(DE3) strain by IPTG. Furthermore, LuxR is required for cell density-dependent induction [38].

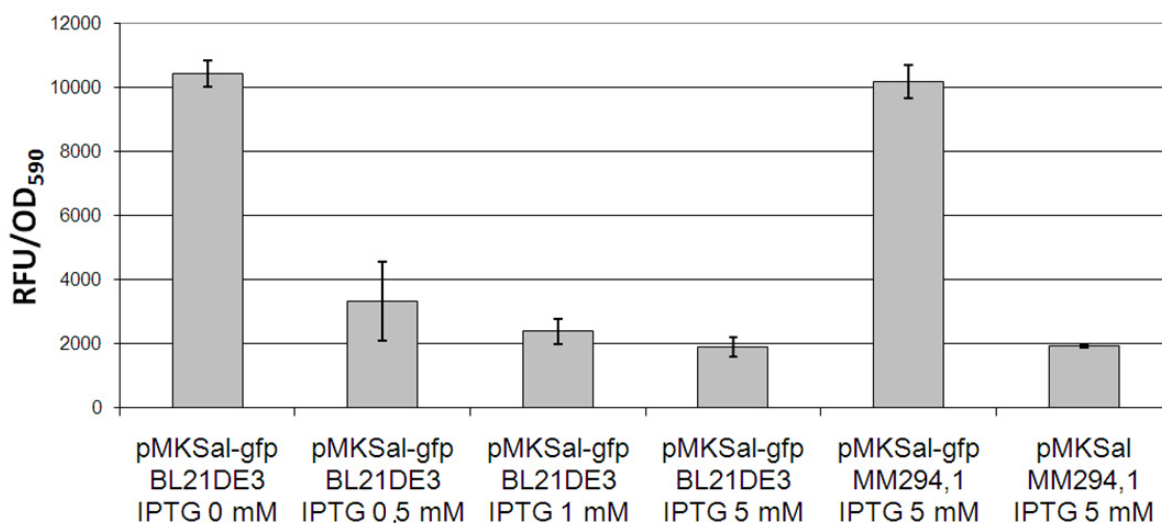
The fluorescence of pMKSaI-*gfp*/BL21(DE3) without and with IPTG at different concentrations was compared in Figure 4. The presence of 1 mM of IPTG in the growth medium allowed complete repression of the *lux* regulatory circuit and the specific fluorescence was at comparable levels to the negative control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



**Figure 4 Repression of the auto-inducible system.** Comparison of efficiency of repression of the auto-inducible system by transcriptional interference on *luxR*. pMKSaI-*gfp*/MM294.1, pMKSaI-*gfp*/BL21DE3 and pMKSaI/MM294.1 were grown in LB at 37°C with shaking. For each plasmid/strain combination, 3 replicates were incubated for each of the 4 concentrations of IPTG: 0, 0.5, 1 and 5 mM. After 16 h of growth, GFP expression was determined. pMKSaI-*gfp*/MM294.1 and pMKSaI/MM294.1 combinations were used as positive and negative controls. Error bars show the standard deviation of experiments. RFU/OD<sub>590</sub>, Relative Fluorescence Unit per OD<sub>590</sub> unit.

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 and thus 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), KH<sub>2</sub>PO<sub>4</sub> 5,4 g/l, K<sub>2</sub>HPO<sub>4</sub> 15,6 g/l, MgSO<sub>4</sub> 250 mg/l, glycerol 15 g/l, pH 7.4. YE3X medium contains yeast extract (45 g/l), KH<sub>2</sub>PO<sub>4</sub> 4 g/l, K<sub>2</sub>HPO<sub>4</sub> 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 (OD<sub>590</sub>).

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-TTTTGGCGCGCCATTATTTCCCCTATAATATAC TTAGTA and LxIAscIR-TTTTGGCGCGCCTAAAA CGGTAATAGATTGACA and chromosomal DNA of *V. fischeri* ATCC7744. The PCR product was digested with *AscI*. Concomitantly, for pGLEM constructions, *metE* gene was amplified from genomic DNA of *E. coli* MM294.1 with the primers metEScaIL-GAAAAGTACTGCTTGTAGCGTTTTTCAGGTG and metEScaIR-GAAAAGTACTGGGAAGAAGTCGCTGTAATG 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 *AscI* in  $\Delta$ *metE*, 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  $\Delta$ *metE*-*ery*-*luxI*- $\Delta$ *metE* was PCR amplified from pGLEM-*luxI*, using MetEL-GAAAAGTACTGCTTGTAGCGTTTTTCAGGTG and MetER-GAAAAGTACTGGGAAGAAGTCGCTGTAATG. The amplicon was used to transform *E. coli*

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

Strains or plasmids	Descriptions <sup>a</sup>	References or sources
<b>Strains</b>		
<i>V. fischeri</i>	ATCC7744	ATCC7744
<i>E. coli</i> DH5 $\alpha$	F' $\Phi$ 80lacZ $\Delta$ M15 $\Delta$ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r-k, m+k) phoA supE44 thi-1 gyrA96 relA1 $\lambda$	Invitrogen
<i>E. coli</i> TOP10	F' mcrA $\Delta$ (mrr-hsdRMS-mcrBC) $\Phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 recA1 araD139 $\Delta$ (ara-leu) 7697 galU galk rpsL (Str <sup>r</sup> ) endA1 nupG	Invitrogen
<i>E. coli</i> MM294.1	F' supE44 hsdR17 endA1 thi-1 $\lambda$	Novartis Collection
<i>E. coli</i> MM294.1::luxI	F' supE44 hsdR17 endA1 thi-1 $\lambda$ : metE::ery\luxI.	This study
<i>E. coli</i> BL21(DE3)	F' ompT gal dcm lon hsdSB(rB- mB-) $\lambda$ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
<b>Plasmids</b>		
pBS-Ery	pBR322 ori; Ap <sup>r</sup> ; carrying ery (Er <sup>r</sup> );	51
pET21b	IPTG inducible; pBR322 ori; Ap <sup>r</sup> ;	Novagen
pET21b-gfp	pET21b carrying gfp; Ap <sup>r</sup> ;	This study
pGlow	TOPO-TA vector; promoterless gfp; pUC ori; Ap <sup>r</sup> ;	Invitrogen
pGL506	pGlow carrying luxR-luxI amplicon in TA cloning site; pUC ori; Ap <sup>r</sup> ;	This study
pGLEM	pGlow carrying $\Delta$ metE-ery in TA cloning site; pUC ori; Ap <sup>r</sup> ;	Novartis Collection
pGLEM-luxI	pGLEM carrying luxI; pUC ori; Ap <sup>r</sup> ;	This study
pKobeg	Arabinose inducible expression for gam, bet, exo; R101 ori; Cm <sup>r</sup> ;	7
pMK	pBR322 ori; Km <sup>r</sup> ;	1
pMKSsal	MCS1; pBR322 ori; Km <sup>r</sup> ;	This study
pMKSsal-GFP	pMKSsal carrying gfp; Km <sup>r</sup> ;	This study
pMKSsal- $\Delta$ luxI	pMKSsal with truncated luxI; Km <sup>r</sup> ;	This study
pMKSsal- $\Delta$ luxI-GFP	pMKSsal- $\Delta$ luxI carrying gfp; Km <sup>r</sup> ;	This study
pLAIR32	MCS2; pUC ori; Ap <sup>r</sup> and Km <sup>r</sup> ;	This study
pLAIR42	MCS3; pUC ori; Ap <sup>r</sup> and Km <sup>r</sup> ;	This study
pLAJET32	MCS2; pBR322 ori; Ap <sup>r</sup> ;	This study

<sup>a</sup> Ap<sup>r</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Km<sup>r</sup>, kanamycin resistance; Er<sup>r</sup>, Erythromycin resistance; ori, replication origin; IPTG, Isopropyl- $\beta$ -D-thiogalactoside.

MM294.1 carrying the pKobeg plasmid and erythromycin-resistant candidate integrants were analyzed by PCR (using LuxI4Fr-TCAAATGTCAATCTATTACCG and LuxI4Rv-TCCTTACCTATTGTTTGTGCG) 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  $\mu$ g 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-AAGCTTTACTTACGT ACTTAACTTTTA and LuxIRRv-TCATTATTTCCCC TATAATATACTTAGT. 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 *Ascl* and *PacI* cloning sites, producing pMKSsal [48]. The *gfp* gene was amplified from pGLOW (Invitrogen) using GFPEco-GAATT-CAATGGCTAGCAAAGGAGAAGAAGT and GFP NotI-GCGGCCGCTTATTTGTAGAGCTCATCCA and inserted in the MCS1 of pMKSsal using *EcoRI* and *NotI*. The final plasmid is pMKSsal-*gfp*.

pMKSsal-*ΔluxI* is a pMKSsal 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-*NdeI* or *MluI*-*AatII*-MCS3-*NdeI*, were cloned in pCRII (Invitrogen). The *luxR-luxI* containing fragment, amplified from pGL506 plasmid with LFMluIT-TTTTACGCGTTACT-TACGTACTTAACTTTTA and LRAatIIT-TTTT GACGTCCTTCATTATTTCCCCTATAATATA, was inserted in sequences containing MCS2 or MCS3 on pCRII by using *MluI/AatII*, to generate pLAIR32 and pLAIR42. The sequence containing *luxR-luxI*-MCS2 was extracted from pLAIR32 by *MluI/NdeI* digestion. The PCR-product of pET21 (Novagen), pETORIAMP*MluI*Fr-TTTTACGCGTGAGAAGCAGGCCATTATCGC and pETORIAMP*NdeI*Rv-TTTTCATATGATTT-CAGGTGGCACTTTTCG, containing pBR322 *ori* and the *bla* gene, was digested with *MluI/NdeI* and was ligated with the *luxR-luxI*-MCS2 DNA fragment. The final plasmid is pLAIET32. The enzymes were obtained from New England Biolabs.

#### Transcriptional fusion studies of pGL506, pMKSsal, pET21 promoters linked to the *gfp* reporter gene

A seed culture was made by inoculating cells into LB medium containing the appropriate antibiotic and growing the cells overnight at 37°C. Each seed culture was inoculated in fresh LB, YE3X or CM medium with antibiotics, to obtain a final optical density of 0.15 (OD<sub>590</sub>). The cells were grown at different temperatures, as indicated in figure legends. When the OD<sub>590</sub> 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 OD<sub>590</sub> 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% bromophenol 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 from wild type MM294.1 (line 1), plasmid pGL506 (line 2) and chromosomal DNA of MM294.1::luxI (line 3) were used as a template for PCRs with primers LuxI4Fr/LuxI4Rv, 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 LuxI4Fr/LuxI4Rv) that fully probed within the *luxI* gene. As a control of the Southern blot efficiency, a 513-bp PCR (primers LuxI4Fr/LuxI4Rv) 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 representation of decoupled 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 pMKSsal-*ΔluxI*. **Figure S3. pH effect on the pattern of expression.** Comparison of pMKSsal-*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 300-rpm 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 H<sub>3</sub>PO<sub>4</sub> 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 O<sub>2</sub> 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 pMKSsal/MM294.1 (negative) and pET21-*gfp*/BL21(DE3) (positive) were grown in the same conditions. pET21-*gfp*/BL21(DE3) was induced at 3OD<sub>590</sub> with 1 mM of IPTG and collected after 3 hours from the induction. Controls pMKSsal/MM294.1 and pET21-*gfp*/BL21(DE3) were collected at 13.4 OD<sub>590</sub> and 12.5 OD<sub>590</sub> 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 OD<sub>590</sub> unit) of *E. coli* harboring pMKSsal-*gfp* grown in LB (squares) or YE3X (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 YE3X 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 YE3X with the appropriate antibiotic were used to inoculate 7 L Applikon



bioreactor containing 5 L of YE3X medium with glucose (1 g/L), 15 g/l of glycerol. The process was performed at 25°C, with 0.5 WM (air Volume per Volume of culture medium per Minute) of airflow and a 300-rpm stirrer speed. 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 H<sub>3</sub>PO<sub>4</sub> 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 O<sub>2</sub> flow by electronic valve. Samples were collected at the indicated cell densities. Total 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.

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#### References

1. Baneyx F: Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotechnol* 1999, **10**:411-421.
2. Choi YJ, Morel L, Le François T, Bourque D, Bourget L, Groleau D, Massie B, Miguez CB: Novel, versatile, and tightly regulated expression system for *Escherichia coli* strains. *Appl Environ Microbiol* 2010, **76**:5058-5066.
3. Hanahan D: Techniques for transformation of *Escherichia coli*. DNA cloning: a practical approach. Edited by: Glover DM 1985, 109-135.
4. Guda C, Zhang X, McPherson DT, Xu J, Cherry JH, Urry DW, Daniell H: Hyper expression of an environmentally friendly synthetic polymer gene. *Biotechnology Letters* 1995, **17**:745-750.
5. Masui Y, Mizuno T, Noye M: Novel high-level expression cloning vehicles: 10<sup>4</sup>-fold amplification of *Escherichia coli* minor protein. *Bio-Technology* 1984, **2**:81-85.
6. Giacalone MJ: Toxic protein expression in *Escherichia coli* using a rhamnose-based tightly regulated and tunable promoter system. *Biotechniques* 2006, **40**:596-596.
7. Kurland CG, Dong HJ: Bacterial growth inhibition by overproduction of protein. *Molecular Microbiology* 1996, **21**:1-4.
8. Patkar A, Vijayasankaran N, Urry DW, Srien F: Flow cytometry as a useful tool for process development: rapid evaluation of expression systems. *Journal of Biotechnology* 2002, **93**:217-229.
9. Schein CH, Noteborn MHM: Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. *Bio-Technology* 1988, **6**:291-294.
10. Sevastyanovich YR, Alfasi SN, Cole JA: Sense and nonsense from a systems biology approach to microbial recombinant protein production. *Biotechnology and Applied Biochemistry* 2010, **55**:9-28.
11. Andrews B, Adari H, Hanning G, Lahue E, Gosselin M, Martin S, Ahmed A, Ford PJ, Hayman EG, Makrides SC: A tightly regulated high level expression vector that utilizes a thermosensitive lac repressor: production of the human T cell receptor V beta 5.3 in *Escherichia coli*. *Gene* 1996, **182**:101.
12. Bhandari P, Gowrishankar J: An *Escherichia coli* host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. *J Bacterio* 1997, **179**:4403-4406.
13. Darby RAJ, Hine AV: LacI-mediated sequence-specific affinity purification of plasmid DNA for therapeutic applications. *Faseb Journal* 2005, **19**:801.
14. Figge J, Wright C, Collins CJ, Roberts TM, Livingston DM: Stringent regulation of stably integrated chloramphenicol acetyl transferase genes by *Escherichia coli* Lac repressor in monkey cells. *Cell* 1988, **52**:713-722.
15. Jana S, Deb JK: Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Applied Microbiology and Biotechnology* 2005, **67**:289-298.
16. Knodler LA, Sekyere EO, Stewart TS, Schofield PJ, Edwards MR: Cloning and expression of a prokaryotic enzyme, arginine deiminase, from a primitive eukaryote *Giardia intestinalis*. *Journal of Biological Chemistry* 1998, **273**:4470-4477.
17. Kosinski MJ, Rinas U, Bailey JE: Isopropyl-β-D-thiogalactopyranoside influences the metabolism of *Escherichia coli*. *Applied Microbiology and Biotechnology* 1992, **36**:782-784.
18. Pal Y, Gupta JC, Mukherjee KJ: Optimizing recombinant protein expression in the T7 system under the control of the *proUp* promoter. *Biotechnology Letters* 2001, **23**:41-46.
19. Thomas MD, van Tilburg A: Overexpression of foreign proteins using the *Vibrio fischeri lux* control system. *Bioluminescence and Chemiluminescence, Pt C* 2000, **305**:315-329.
20. Schumann W: Production of recombinant proteins in *Bacillus subtilis*. *Advances in Applied Microbiology, Vol 62* 2007, **62**:137-189.
21. Lee SK, Keasling JD: A propionate-inducible expression system for enteric bacteria. *Applied and Environmental Microbiology* 2005, **71**:6856-6862.
22. Miksch G, Bettenworth F, Friehs K, Flaschel E, Saalbach A, Twellmann T, Nattkemper TW: Libraries of synthetic stationary-phase and stress promoters as a tool for fine-tuning of expression of recombinant proteins in *Escherichia coli*. *Journal of Biotechnology* 2005, **120**:25-37.
23. Sambrook J: *Molecular cloning: a laboratory manual*. CSHL Press; 2001.
24. Srinivasan S, Barnard GC, Gerngross TU: A novel high-cell-density protein expression system based on *Ralstonia eutropha*. *Applied and Environmental Microbiology* 2002, **68**:5925-5932.
25. Studier FW: Protein production by auto-induction in high-density shaking cultures. *Prot Expr Purif* 2005, **41**:207-234.
26. Anderson JC, Clarke EJ, Arkin AP, Voigt CA: Environmentally controlled invasion of cancer cells by engineered bacteria. *Journal of Molecular Biology* 2006, **355**:619-627.
27. Tsao CY, Hooshangi S, Wu HC, Valdes JJ, Bentley WE: Autonomous induction of recombinant proteins by minimally rewiring native quorum sensing regulon of *E. coli*. *Metab Eng* 2010, **12**:291-297.
28. Basu S, Mehreja R, Thiberge S, Chen MT, Weiss R: Spatiotemporal control of gene expression with pulse-generating networks. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**:6355-6360.
29. Sayut DJ, Niu Y, Sun LH: Construction and engineering of positive feedback loops. *ACS Chemical Biology* 2006, **1**:692-696.
30. You LC, Cox RS, Weiss R, Arnold FH: Programmed population control by cell-cell communication and regulated killing. *Nature* 2004, **428**:868-871.
31. Fuqua C, Greenberg EP: Listening in on bacteria: Acyl-homoserine lactone signalling. *Nature Reviews Molecular Cell Biology* 2002, **3**:685-695.
32. Whitehead NA, Barnard AML, Slater H, Simpson NJL, Salmond GPC: Quorum-sensing in gram-negative bacteria. *Fems Microbiology Reviews* 2001, **25**:365-404.
33. Miller MB, Bassler BL: Quorum sensing in bacteria. *Annual Review of Microbiology* 2001, **55**:165-199.
34. Deana A, Ehrlich R, Reiss C: Synonymous codon selection controls in vivo turnover and amount of mRNA in *Escherichia coli bla* and *ompA* genes. *Journal of Bacteriology* 1996, **178**:2718-2720.
35. Rowe DCD, Summers DK: The quiescent-cell expression system for protein synthesis in *Escherichia coli*. *Applied and Environmental Microbiology* 1999, **65**:2710-2715.

36. Elledge SJ, Davis RW: **Position and density effects on repression by stationary and mobile DNA-binding proteins.** *Genes & Development* 1989, **3**:185-197.
37. Eszterhas SK, Bouhassira EE, Martin DIK, Fiering S: **Transcriptional interference by independently regulated genes occurs in any relative arrangement of the genes and is influenced by chromosomal integration position.** *Molecular and Cellular Biology* 2002, **22**:469-479.
38. Stevens AM, Greenberg EP: **Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes.** *Journal of Bacteriology* 1997, **179**:557-562.
39. Grossman TH, Kawasaki ES, Punreddy SR, Osburne MS: **Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability.** *Gene* 1998, **209**:95-103.
40. Joseph S, William RD: **Molecular cloning: a laboratory manual.** CSHL Press edition; 20011.
41. Khlebnikov A, Datsenko KA, Skaug T, Wanner BL, Keasling JD: **Homogeneous expression of the P-BAD promoter in *Escherichia coli* by constitutive expression of the low-affinity high-capacity AraE transporter.** *Microbiology-Sgm* 2001, **147**:3241-3247.
42. Khlebnikov A, Keasling JD: **Effect of *lacY* expression on homogeneity of induction from the P-tac and P-trc promoters by natural and synthetic inducers.** *Biotechnology Progress* 2002, **18**:672-674.
43. Novick A, Weiner M: **Enzyme induction as an all-or-none phenomenon.** *PNAS* 1957, **43**:553-566.
44. Dunlap PV, Greenberg EP: **Control of *Vibrio fischeri* luminescence gene expression in *Escherichia coli* by cyclic-AMP and cyclic-AMP receptor protein.** *Journal of Bacteriology* 1985, **164**:45-50.
45. Chaverroche MK, Ghigo JM, d'Enfert C: **A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*.** *Nucleic Acids Res* 2000, **28**:97.
46. Ye R, Rehemtulla SN, Wong SL: **Glucitol induction in *Bacillus subtilis* is mediated by a regulatory factor, GutR.** *J Bacteriol* 1994, **176**:3321-3327.
47. Richardson SM, Wheelan SJ, Yarrington RM, Boeke JD: **GeneDesign: rapid, automated design of multikilobase synthetic genes.** *Genome Res* 2006, **16**:550-556.
48. Ames RS, Tornetta MA, Jones CS, Tsui P: **Isolation of neutralizing anti-C5a monoclonal antibodies from a filamentous phage monovalent Fab display library.** *J Immunol* 1994, **153**:910.
49. Rydzanicz R, Zhao XS, Johnson PE: **Assembly PCR oligo maker: a tool for designing oligodeoxynucleotides for constructing long DNA molecules for RNA production.** *Nucleic Acids Research* 2005, **33**:W521-W525.
50. DeLisa MP, Li JC, Rao G, Weigand WA, Bentley WE: **Monitoring GFP-operon fusion protein expression during high cell density cultivation of *Escherichia coli* using an on-line optical sensor.** *Biotechnology and Bioengineering* 1999, **65**:54-64.
51. Zhang QY, Tiersch TR, Cooper RK: **Inducible expression of green fluorescent protein within channel catfish cells by a cecropin gene promoter.** *Gene* 1998, **216**:207-213.

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