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Viewpoint

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Natural LacI from *E. coli* Yields Faster Response and Higher Level of Expression than the LVA-Tagged LacI

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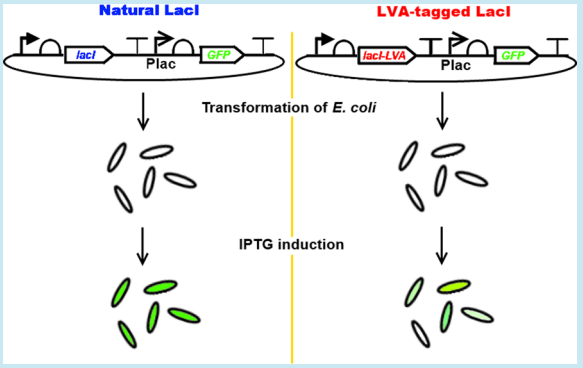
Supporting Information

ABSTRACT: The *lac* promoter is one of the most commonly used promoters for expression control of recombinant genes in *E. coli*. In the absence of galactosides, the *lac* promoter is repressed by its repressor protein LacI. Since the *lac* promoter is regulated by a repressor, overexpression of LacI is necessary for regulation when the promoter is introduced on a high-copy plasmid. For that purpose, a modified variant of LacI, a LVA-tagged LacI, was submitted to the Registry of Standard Biological Parts and has been used for more than 500 constructs since then. We have found, however, that natural LacI is superior to the LVA-tagged LacI as controller of expression.



Since its discovery the *lac* operon has provided fundamental mechanistic insight into gene regulatory circuits. More than half a century ago Francis Jacob and Jacquos Monod proposed that the *lac* promoter was repressed by an unknown protein and that the repression could be relieved by the presence of galactosides (including isopropyl *β*-D-1-thiogalactopyranoside (IPTG)).[1] Five years later in 1966, the *lac* repressor protein (LacI) was isolated confirming the mechanism suggested by Jacob and Monod.[2] Since then, the *lac* promoter has become a powerful tool—not only for controlling expression but also for overexpression of recombinant genes. For instance, in the synthetic biology competition iGEM (international Genetically Engineered Machine), the *lac* promoter has been used in more than a thousand constructs.[3]

In 2003, when the *lacI* coding sequence was added to the Registry of Standard Biological Parts, the designers added a LVA degradation tag to the C-terminus of LacI, in order to improve the switch time from high to low performance.[4] The LVA tag is a short peptide sequence, originally from *ssrA*,[5] marking the protein of interest for degradation by cellular proteases.[6] Addition of the LVA-tag to LacI *(LacI-LVA)* increases the turnover of the protein,[7] which then in turn should improve the expression response time. However, this was never tested.

Our team (as many before us) realized that natural levels of LacI in *E. coli* were not sufficient to repress the *lac* promoter when it was introduced on a high-copy plasmid (pSB1C3). Consequently, we decided to express the available LacI-LVA from the plasmid as well. We found it to be functional as a repressor, although a poor response was observed after IPTG induction. An important aspect of the iGEM competition is to improve genetic tools already available. Therefore, we decided to test the LVA-tagged lacI against the natural LacI. In this study, we show that the natural LacI (a BioBrick we built and submitted to the Registry of Standard Biological Parts) is superior to LacI-LVA as a controller of gene expression.

RESULTS AND DISCUSSION

To investigate the expression from the *lac* promoter, a *dxs-GFP* fusion with a flexible linker was used. *dxs* is the coding sequence for a protein in the methylerythritol phosphate pathway, which was used in our setup. Flow cytometry was used to measure the GFP signal as a reporter of the *lac* promoter activity above a given threshold in addition to the mean fluorescence of the population. Figure 1 is a schematic overview of plasmids pSDU1 *(without LacI)*, pSDU2 *(LacI-LVA)*, and pSDU3 *(LacI)*, which were used to assay the activity of the *lac* promoter.

Efficiency of LacI-LVA Functionality. In order to test if the LacI-LVA construct was functional, cells of *E. coli* MG1655 carrying either pSDU1 *(without lacI)*, pSDU2 *(LacI-LVA)*, or no plasmid (WT, to set background reference) were assayed by flow cytometry. Cultures were grown to exponential phase ($OD_{600} \approx 0.2$). At this point of growth, IPTG was added to induce *dxs-GFP* transcription. MG1655/pSDU1 *(without lacI)*

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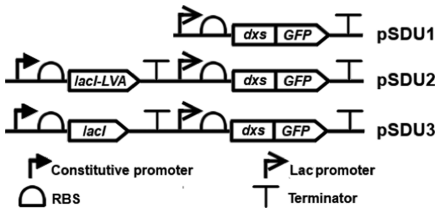

Figure 1. Three different plasmids were used to assay the function of the LacI variants. All three backbones are pSB1C3. pSDU1 (*without LacI*) contains a dxs-GFP fusion with a flexible linker under the control of the *lac* promoter. In addition, pSDU2 (*LacI-LVA*) and pSDU3 (*LacI*) contain *lac*I-LVA and wild-type *lacI*, respectively, under the control of a constitutive promoter. *dxs* is the coding sequence for a protein in the methylerythritol phosphate pathway, which was used in our setup.

showed constitutive GFP expression, from the *lac* promoter, both with and without IPTG. Thus, this construct did not show any significant response to IPTG. This suggests that the natural levels of LacI are not sufficient to fully repress the *lac* promoter. MG1655/pSDU2 (*LacI-LVA*) showed repression of the *lac* promoter, which was relieved upon addition of 1 mM IPTG (Figure 2). This suggests that LacI-LVA was functional; however, fluorescence did not reach the same level as the unrepressed strain, MG1655/pSDU1 (*without lacI*).

Natural LacI Is Superior to LacI-LVA. In order to compare the functionality of natural wild-type LacI with LacI-LVA, an experiment similar to the experiment described in Figure 2 was conducted with MG1655 carrying pSDU2 (*LacI-LVA*) or pSDU3 (*LacI*). Measurements for MG1655/pSDU2 (*LacI-LVA*) replicated previous findings. MG1655/pSDU3 (*LacI*) showed repression of the *lac* promoter, which was relieved upon addition of 1 mM IPTG. The response was much faster and maximal activity was higher after induction of pSDU3 (*LacI*) compared to pSDU2 (*LacI-LVA*). Not only was a larger fraction of the MG1655/pSDU3 (*LacI*) population induced, the induced MG1655/pSDU3 (*LacI*) cells were also more fluorescent than the induced MG1655/pSDU2 (*LacI-LVA*) cells (Figure 3).

LacI Response to Different IPTG Concentrations. Instant maximum expression of a gene is not always desirable; therefore, we wished to examine whether a differentiated response could be obtained by using different concentrations of IPTG. MG1655/pSDU2 (*LacI-LVA*) and MG1655/pSDU3 (*LacI*) were induced with concentrations of IPTG ranging from 0.05 to 0.5 mM. MG1655/pSDU2 (*LacI-LVA*) did not respond to a 10-fold concentration difference in IPTG (Figure 4B). In contrast, MG1655/pSDU3 (*LacI*) showed a proportional dose−response relationship with increasing concentrations of IPTG (Figure 4A), enabling control of the timing of expression levels dependent on IPTG concentrations.

Conclusion. From the above results, we conclude that the natural LacI is superior to the LVA-tagged LacI. This conclusion is based on four aspects: (1) Using natural LacI, a more homogeneous expression in the population was observed (i.e., all cells became fluorescent). (2) The level of expression in individual cells was 3−4 fold higher using the natural LacI compared to LVA-tagged LacI. (3) Using natural LacI, a significantly faster response was observed upon addition of the inducer IPTG. (4) A dose−response relationship was observed for the natural LacI with different concentrations of IPTG, which was not observed for the LVA-tagged LacI.

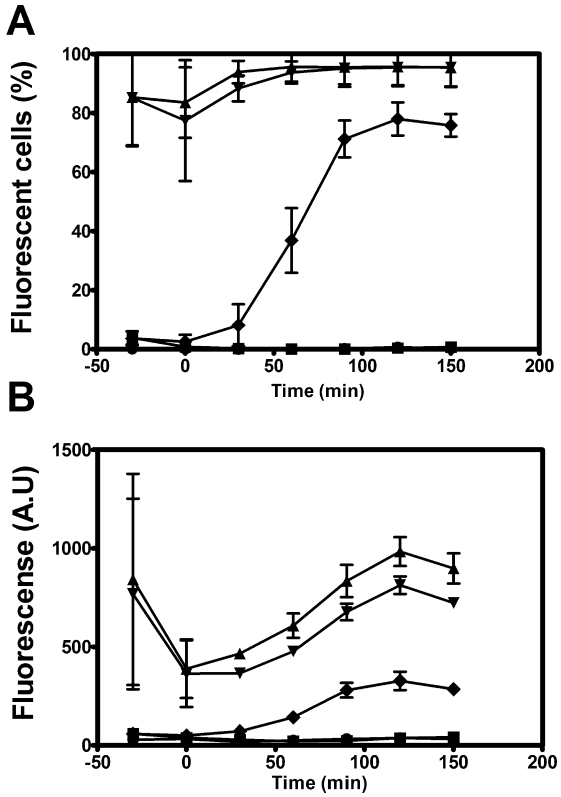

Figure 2. Fluorescent measurements for 0 mM IPTG MG1655 (●), 0 mM IPTG MG1655/pSDU1 (*without LacI*) (▼), 1 mM IPTG MG1655/pSDU1 (*without LacI*) (▲), 0 mM IPTG MG1655/pSDU2 (*LacI-LVA*) (■), and 1 mM IPTG MG1655/pSDU2 (*LacI-LVA*) (♦). All strains were grown to exponential phase and induced with 1 mM IPTG (if indicated) at time 0 min. (A) Percentages of the population that fluoresced. Almost the entire population (80−95%) of MG1655/pSDU1 (*without LacI*) cultures, both uninduced and induced, were fluoresced at all times of measurement. The entire population of uninduced MG1655/pSDU2 (*LacI-LVA*) cultures were unfluorescent at all times of measurement. The induced pSDU2 (*LacI-LVA*) strain had an increasing population that became fluorescent and reached a maximum of approximately 70−75% of the population fluorescing. (B) Mean fluorescence of fluorescent cells. Uninduced and induced pSDU1 (*without LacI*) strain show similar tendencies. The induced pSDU2 (*LacI-LVA*) strain shows increasing fluorescence after induction, but it did not reach as high mean fluorescence as the MG1655/pSDU1 (*without LacI*) cultures. All experiments were conducted in triplicate.

METHODS

Plasmid Construction. The expression assay plasmids were constructed using the USER cloning technique[8] to build the GFP fusion under the control of the *lac* promoter. Subsequent addition of the natural and LVA-tagged LacI with constitutive promoter was performed using the BioBrick assembly standard (RFC[10]). USER enzyme, Phusion High Fidelity Polymerase and restriction enzymes (New England Biolabs) and PFu Turbo Cx Hotstart Polymerase (amplification of DNA for USER cloning) (Agilent Technologies) were used. pSDU1 (BBa_K1088008) is composed of BBa_R0010 (Lac promoter), BBa_J15001 (RBS), BBa_K1088000 (*dxs*), BBa_K105012 (linker), and BBa_E0040 (GFP). pSDU2 (BBa_K1088009) is composed of BBa_J23106 (constitutive promoter), BBa_B0030 (RBS), BBa_K1088023 (*lacI-LVA*), BBa_B1002 (terminator), and BBa_K1088008 (pSDU1).

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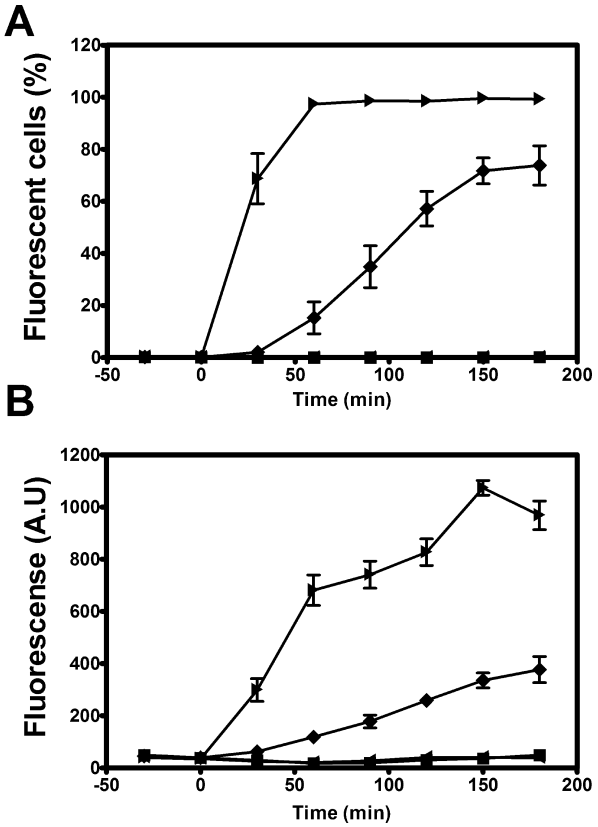

Figure 3. Fluorescent measurements for 0 mM IPTG MG1655/pSDU2 (*LacI-LVA*) (■), 1 mM IPTG MG1655/pSDU2 (*LacI-LVA*) (♦), 0 mM IPTG MG1655/pSDU3 (*LacI*) (◀), and 1 mM IPTG MG655/pSDU3 (*LacI*) (▶). All strains were grown to exponential phase and induced with 1 mM IPTG (if indicated) at time 0 min. Figure 3 A) Percentage of population that fluoresced. MG1655/pSDU2 (*LacI-LVA*) showed same tendency as in Figure 2. Uninduced MG1655/pSDU3 (*LacI*) shows same tendency as uninduced MG1655/pSDU2 (*LacI-LVA*). The entire MG1655/pSDU3 (*LacI*) population became fluorescent between 30 and 60 min after induction, and reaches its maximum much faster than the induced MG1655/pSDU2 (*LacI-LVA*). (B) Mean fluorescence of fluorescent cells. MG1655/pSDU2 (*LacI-LVA*) showed same tendency as in Figure 2. Induced Mg1655/pSDU3 (*LacI*) had approximately 3-fold higher maximum fluorescence compared to the induced MG1655/pSDU2 (*LacI-LVA*).

pSDU3 (BBa_K1088026) is composed of BBa_J23106 (constitutive promoter), BBa_B0030 (RBS), BBa_K1088018 (lacI), BBa_B1002 (terminator), and BBa_K1088008 (pSDU1). Sequences can be seen in Supporting Information S1, S2, and S3 (pSDU1, pSDU2, and pSDU3, respectively). Primers (Sigma-Aldrich) can be seen in Supporting Information Table S1.

Flow Cytometry. MG1655 wild-type strain or plasmid carrying strains were grown from OD_{600} = 0.005 at 37 °C in heating baths with shaking to approximately OD_{600} = 0.2 in 50 mL LB media (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) in 250 mL Erlenmeyer flasks. At this point (time = 0 min), the cultures were induced with IPTG (Sigma-Aldrich). Flow cytometry was performed on a BD FACSAria II flow cytometer (Becton, Dickinson, and company). Data from 10^5 events per sample were collected and analyzed using BD FACSDiva software (Becton, Dickinson and company). Strains were distinguished first on the basis of forward scatter area (FSC-A) and side scatter area (SSC-A) and second on the basis of forward scatter area (FSC-A) and GFP fluorescence area (GFP-A).

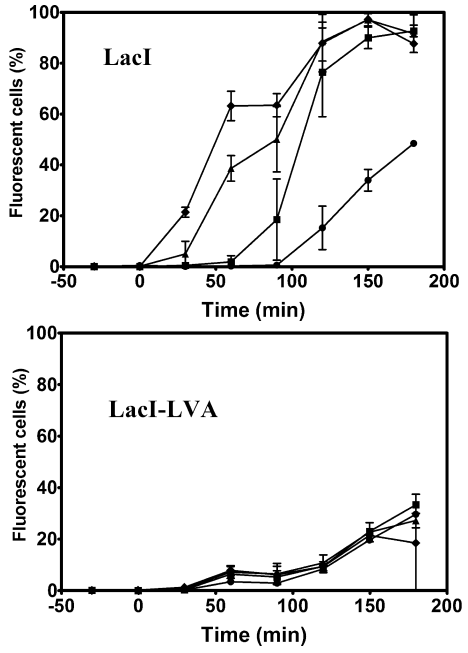

Figure 4. Percentage of population that fluoresced for MG1655/pSDU2 (*LacI-LVA*) and MG1655/pSDU3 (*LacI*). All strains were grown to exponential phase and induced with 0.05 (●), 0.125 (■), 0.25 (▲), or 0.5 (♦) mM IPTG at time 0 min. Figure 4 LacI) MG1655/pSDU3 (*LacI*) shows disguisable responses to the different concentrations of IPTG. LacI-LVA) MG1655/pSDU2 (*LacI-LVA*) shows similar responses to the different concentrations of IPTG.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Author Contributions

P.R.A., S.F., M.H., M.H.K., K.J., A.K., T.J.K., T.K., N.C.M., S.I.S., H.W., and A.A. contributed to data acquisition and data analysis. P.R.A., K.J., and A.A. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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