Available online at www.sciencedirect.com
SCIENCE DIRECT
Protein Expression and Purification 48 (2006) 28–31
Protein Expression & Purification
www.elsevier.com/locate/yprep

Protein expression enhancement in efflux-deleted mutant bacteria

Thuc T. Le, Călin C. Guet, Philippe Cluzel *

The Institute for Biophysical Dynamics, James Franck Institute, Center of Integrative Sciences Building, The University of Chicago, 929 E. 57th St, Chicago, IL 60637, USA

Received 14 November 2005, and in revised form 17 November 2005
Available online 19 December 2005

Abstract

We applied a single-cell assay to characterize how transcription dynamics affects protein expression levels of a tetracycline-inducible gene expression system. Transcriptional activity of the tetracycline promoter in response to a steady level of inducer is steady in ΔacrAB efflux mutant but pulsating in wildtype *Escherichia coli* cells. We found that the expression level of the green fluorescent protein is several folds higher in ΔacrAB efflux mutant than in wildtype cells.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Efflux pump; Single cell; GFP; Inducible vector

Fluorescence correlation spectroscopy (FCS)[1] is a non-invasive biophysical technique used to measure in real-time protein concentration, protein–protein, and protein–RNA interactions within a single living cell [1–4]. This technique is sensitive to the fluctuation of the fluorescence intensity of molecules diffusing in and out of the volume of detection. The variance about the mean of the fluorescence intensity signal is equal to the inverse number of molecules per unit time present in the volume of detection. Moreover, this technique is also sensitive to the size of the fluorescent molecules. Large molecules diffuse slower than small molecules through the volume of detection, and typical diffusion times are measured by performing the autocorrelation function of the fluorescence intensity. We have previously showed that the combination of FCS with a simple genetic system can be used to profile the activity of any promoter in a prokaryotic cell in real time at the RNA level. We reported that real-time RNA profiling allowed us to characterize two distinct transcriptional dynamics associated with the activity of the *tet* promoter in two bacterial strains [4]. Here, we show that transcription dynamics can be a key parameter to control protein expression level. Our hypothesis is that distinct transcriptional dynamics will lead to distinct protein expression levels [5–7].

In our previous study, we used a dual-plasmid system to monitor in real time the transcription activity of the *tet* promoter in a single *Escherichia coli* cell. We constructed a synthetic gene coding for one ribosomal binding sequence and two ms2-RNA binding sequences under the control of a *tet* promoter on one plasmid (pZE31ms2). We used another plasmid to pre-express the MS2 coat protein fused to GFP (pZS12MS2-GFP). We monitored with FCS the expression of ms2-RNA transcripts as a function of time after aTc induction. When a ms2-RNA transcript binds to a ribosome and two MS2-GFP proteins, the resulting ms2-RNA/ribosome/$(MS2\text{-}GFP)_2$ complex diffuses 30-fold slower than the free MS2-GFP fusion protein. The FCS technique allows us to distinguish RNA-bound from free diffusing MS2-GFP molecules, and consequently to infer the concentration of RNA transcripts.

First, we grew cells in M9 liquid media (M9 minimal salt supplemented with 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.4% glycerol, and 0.5% Casamino acids) at 30 °C. We pre-expressed MS2-GFP to allow sufficient time for GFP to mature by inducing cells with IPTG overnight. We diluted the overnight culture and allowed cells to grow back to an

* Corresponding author.
E-mail address: cluzel@uchicago.edu (P. Cluzel).

[1] *Abbreviations used:* FCS, fluorescence correlation spectroscopy; aTc, anhydrotetracycline; GFP, green fluorescent protein; λP_R, lambda promoter.

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.11.018

optical density of ∼0.2 (OD 600 nm). We mixed cells in M9 medium with low-melting point agarose and immobilized them on a glass coverslip coated with antibodies against bacterial flagellin. Anti-flagellin antibodies bind to bacterial flagella and bring cells to the surface of the coverslip. Low-melting point agarose helps to firmly immobilize cells at the glass surface. This attachment is critical because it prevents undesirable jittering of cells, which reduces the signal-to-noise ratio in FCS measurements. To coat the coverslip with anti-flagellin antibodies, we treated the coverslip with silane (2% 3-aminopropyltriethoxysilane, 8% water, and 90% ethanol), then with the linker glutaraldehyde, and finally with anti-flagellin antibodies. We also added casein to the surface to block unreacted glutaraldehyde functional groups on the coverslip. We allowed cells to undergo one cell division on the glass surface before measuring *tet* promoter activities. Immediately after cells completed their first division (completion of septum formation) on the glass surface, we induced cells with 400 ng/ml anhydrotetracycline (aTc).

In wildtype *E. coli* cells (Frag1B), we previously reported that inducible transcription by a steady level of anhydrotetracycline (aTc) exhibits a pulsating dynamics that coincides with the cell cycle. In contrast, in the Frag1A mutant cells, where the AcrAB multi-drug efflux system controlling the intracellular concentration of inducer was defective, we observed a steady transcription activity across generations (Fig. 1) [4].

Because aTc is a known substrate for the AcrAB efflux system [8], it is conceivable that AcrAB modulates the effective aTc concentration in *E. coli* wildtype cells. These observations suggest that AcrAB multi-drug efflux system plays a role in controlling the dynamics of transcription activity of a *tet*-inducible promoter. Since the deletion of the efflux pump system suppressed the transcription pulses and caused over-expression of induced RNA in mutant cells, we hypothesize that the associated protein concentration expressed from a *tet* controlled promoter is also higher in mutant cells than in wildtype cells. Using the RNA concentration profiles measured from single bacteria with FCS, we estimated the ratio of the associated protein expression between mutant and wildtype cells. In a crude estimate, we assumed that the protein concentration was proportional to the time integral of the induced RNA concentration (Fig. 2A) defined as, $k_P \int_0^t \mathrm{RNA}\,dt$, where k_P is a proportionality factor associated with the rate of translation. We estimated the ratio of induced protein concentration between mutant and wildtype cells by computing the ratio of the area under the RNA concentration curves from 0 to 140 min between mutant and wildtype cells in Fig. 1. We found this ratio to be approximately 2.5.

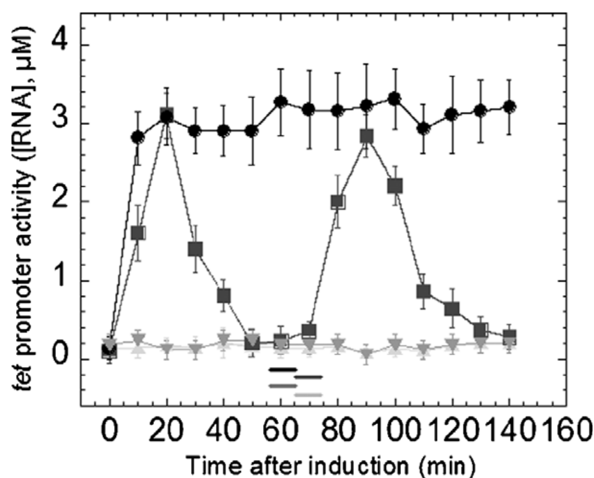

Fig. 1. Real-time concentration profiles of RNA transcripts under the control of a *tet*-inducible promoter in a single *E. coli* cell. A wildtype Frag1B cell (dark gray, solid squares) and an efflux-deleted mutant Frag1A cell (black, solid circles) induced with 400 ng/ml aTc immediately after cell division ($t = 0$ min). Non-induced control, Frag1B (gray, inverted solid triangles), and Frag1A (light gray, solid triangles). Error bars represent uncertainties in the fit parameters extracted from the autocorrelation function. Horizontal bars indicate cell division. RNA transcripts were expressed from pZE31ms2 plasmids. Frag1A: F-, rha-, thi, gal, lacZam, ΔacrAB::kan^R, $P_{N25/tetR}$, $P_{lacIq/lacI}$, Sp^R. Frag1B: F-, rha-, thi, gal, lacZam, $P_{N25/tetR}$, $P_{laciq/lacI}$, Sp^R.

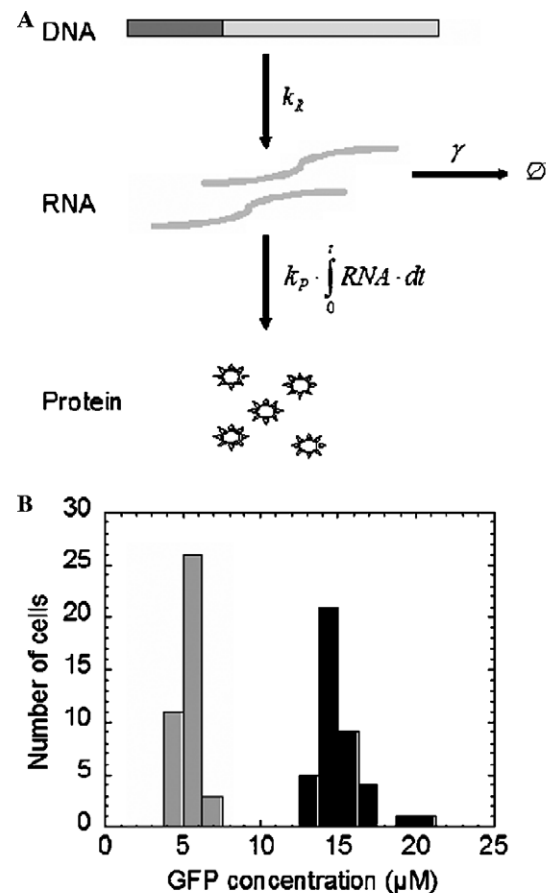

Fig. 2. Predicting protein expression levels in wildtype and ΔacrAB efflux mutant *E. coli* cells. (A) Schematic representation of RNA and protein expression. k_R represents the effective transcription rate, which varies in wildtype cells but is constant in efflux mutant cells. γ is the RNA decay rate and k_P is the translation rate. We assume that the protein degradation rate is negligible over the timescale of our experiments. (B) Distribution of GFP concentration from the *tet* promoter in single cells. GFP expression under the control of a *tet* promoter induced with 400 ng/ml aTc. Wildtype (gray) and ΔacrAB efflux mutant cells (black).

Next, we experimentally investigated how different transcription dynamics from a *tet*-inducible promoter affect the level of protein expressed in wildtype and mutant cells. We used the green fluorescent protein (GFP) as a fluorescent reporter to study the output of the *tet*-inducible gene expression system. The *gfp* gene was under the control of a *tet*-inducible promoter and carried by a pZ-based high copy number plasmid [9], the pZE31-GFP plasmid. We transformed the efflux mutant Frag1A and wildtype Frag1B cells with this plasmid. Because efflux mutant Frag1A cells grew slowly in the presence of antibiotics [10,11], we compared the growth and GFP expression levels in efflux mutant Frag1A and wildtype Frag1B cells in the absence of any antibiotic. We grew the cells (without antibiotic) at 30 °C in M9 minimal medium to an optical density (600 nm) of 0.3. We then induced the cells with 400 ng/ml of aTc and harvested them after 4 h. We mixed cells in M9 medium with low-melting point agarose and immobilized them on a glass coverslip coated with antibodies against bacterial flagellin. We measured the level of induced GFP with FCS in 40 single cells as previously described [2]. FCS measurements consist of measuring the fluorescence intensity from a volume of detection smaller than the size of the cell. The fluctuations of the emitted fluorescence are directly associated with the GFP molecules diffusing in and out of this volume. In this technique, the variance of the fluorescent signal is inversely equal to the number of GFP molecules present in the volume of detection per unit time. This approach yields direct measurements of GFP concentration. We found that the induced concentration of GFP was on average three times larger in efflux mutant Frag1A than in wildtype Frag1B cells (Fig. 2B). This experimental observation is consistent with our estimation based on the RNA expression profiles in Fig. 1.

To check if the deletion of the AcrAB efflux pump affects cell growth and protein expression level, we also characterized the expression of GFP from a constitutive lambda promoter (λP_R). We transformed efflux mutant Frag1A and wildtype Frag1B cells with a plasmid expressing GFP from a lambda promoter, pZE1RGFP [12]. Frag1A and Frag1B cells lack lambda repressors (C_I); therefore, GFP was constitutively expressed from this lambda promoter. We used a multi-well fluorimeter Wallac Victor^2 to monitor cell growth and GFP expression level in the absence of any antibiotic and inducer [12]. We found that the growth rate of Frag1A and Frag1B cells carrying these plasmids were identical (data not shown). We also observed that the levels of expressed GFP were indistinguishable between wildtype Frag1B and ΔacrAB mutant Frag1A cells (Fig. 3). These observations suggest that the deletion of the AcrAB efflux pump has no effect on cell growth or activity of a non-inducible, constitutive promoter.

The use of a non-invasive FCS-based technique combined with genetic manipulation allows us to identify the Frag1A mutant strain that enhances protein expression of simple inducible systems. It is conceivable that deletions of other efflux pump systems should also contribute to protein expression enhancement. The use of such mutant strains should be significant for improving the production yield of a large range of therapeutic proteins.

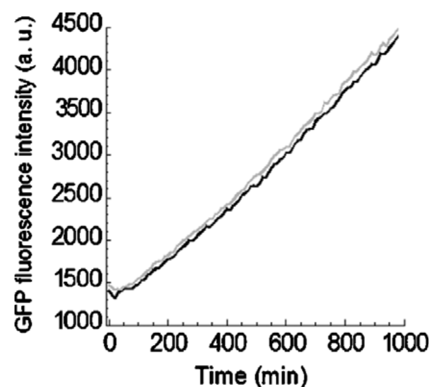

Fig. 3. GFP expression from a constitutive λP_R promoter. GFP expression levels in arbitrary fluorescence intensity units as a function of time. Wildtype Frag1B cells (dark gray). ΔacrAB efflux mutant Frag1A cells (black). We added diluted cultures of cells transformed with pZE31GFP plasmid into a 96-well plate at 200 μl per well and covered each well with 100 μl of hydrocarbon oil to prevent evaporation. We grew cells with constant shaking (1 mm, orbital). We monitored cell growth with absorbance measurements at 600 nm and GFP expression levels by measuring the emission fluorescence at 510 nm (excitation light at 485 nm). Fluorescence intensity was normalized with cell density (OD 600 nm). The doubling time was about ~80 min.

Acknowledgments

We thank T. Pan, T. Emonet, and W. Epstein for discussion and critical reading of the manuscript. This work was partially supported by NIH and MRSEC grants. C.C. Guet was supported by the Yen fellowship.

References

[1] M. Auer, K.J. Moore, F.J. Meyer-Almes, R. Guenther, A.J. Pope, K.A. Stoeckli, Fluorescence correlation spectroscopy: lead discovery by miniaturized HTS, Drug Discov. Today 3 (1998) 457–465.
[2] P. Cluzel, M. Surette, S. Leibler, An ultrasensitive bacterial motor revealed by monitoring signaling proteins in single cells, Science 287 (2000) 1652–1655.
[3] D. Magde, W.W. Webb, E. Elson, Thermodynamic fluctuations in a reacting system—measurement by fluorescence correlation spectroscopy, Phys. Rev. Lett. 29 (1972) 705.
[4] T.T. Le, S. Harlepp, C.C. Guet, K. Dittmar, T. Emonet, T. Pan, P. Cluzel, Real-time RNA profiling within a single bacterium, Proc. Natl. Acad. Sci. USA 102 (2005) 9160–9164.
[5] M.B. Elowitz, S. Leibler, A synthetic oscillatory network of transcriptional regulators, Nature 403 (2000) 335–338.
[6] N. Friedman, S. Vardi, M. Ronen, U. Alon, J. Stavans, Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria, PLoS Biol. 3 (2005) e238.
[7] K. Hakkila, M. Maksimow, A. Rosengren, M. Karp, M. Virta, Monitoring promoter activity in a single bacterial cell by using green and red fluorescent proteins, J. Microbiol. Methods 54 (2003) 75–79.
[8] X.Z. Li, H. Nikaido, Efflux-mediated drug resistance in bacteria, Drugs 64 (2004) 159–204.

[9] R. Lutz, H. Bujard, Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I-1-I-2 regulatory elements, Nucleic Acids Res. 25 (1997) 1203–1210.

[10] L.M. McMurry, M. Oethinger, S.B. Levy, Overexpression of marA, soxS, or acrAB produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*, FEMS Microbiol. Lett. 166 (1998) 305–309.

[11] H. Nikaido, Multidrug efflux pumps of gram-negative bacteria, J. Bacteriol. 178 (1996) 5853–5859.

[12] C.C. Guet, M.B. Elowitz, W.H. Hsing, S. Leibler, Combinatorial synthesis of genetic networks, Science 296 (2002) 1466–1470.