LacZ-promoter fusions

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***Christopher M. Thomas*, Editor-in-chief**

LacZ–promoter fusions: the effect of growth

LacZ expression in growing cells

Downstream fusion of the reporter gene *lacZ* coding for β-galactosidase to the promoter region of a structural gene is a frequently used technique to study the expression of the gene. The rate of transcription, inferred from the β-galactosidase activity of the cells, is equated to 'intrinsic promoter strength' but, more importantly, also measures the action of *cis*- and *trans*-acting elements involved in induction and/or repression of the gene. In a recent contribution to this platform Pessi *et al.* (6) critically reviewed potential pitfalls related to the construction of transcriptional and translational fusions to make sure that the reporter β-galactosidase 'reports rather than makes the news'. In this contribution we discuss another source of misinterpretation, being that promoter fusions are evaluated in growing cells, usually in the exponential phase in batch culture. A first consequence is that the rate of transcription is not the same as the level of expression, and a second is that it takes some time for the level of expression to reach a steady-state value. Though different aspects of this have been discussed in the literature (e.g. 10), we feel that there is not a general awareness of these consequences in the literature. In the following, we present a model for the kinetics of expression of a protein in exponentially growing cells that in a simple way demonstrates the difference between transcription rate and level of expression, and accounts for the pre-steady-state period. Then we will give three examples that focus on different aspects of the effect of growth on the evaluation of *lacZ*–promoter fusions.

A model for the time dependence of expression during exponential growth

In exponentially growing cells the steady-state concentration of β-galactosidase in the cells is determined by the synthesis rate and the dilution rate over newly synthesized cells, or the growth rate of the cells. In each cell the synthesis rate is constant and, therefore, the rate of synthesis in the culture will be proportional to the number of cells, which increases exponentially, i.e.

$$\frac{d[\mathrm{LacZ}]}{dt} = p \cdot N_0 \cdot e^{\mu t} \qquad (1)$$

where p is the synthesis rate per cell, N_0 the cell density at the beginning of the exponential growth phase, t is the time and μ the growth rate constant. In this approach, we have ignored any breakdown of β-galactosidase, which is probably slow anyway. The time dependence of the β-galactosidase concentration in the culture follows from integration of equation 1. In batch culture, it is unlikely that the level of expression at the beginning of growth is the same as the steady-state level (see for example ref. 7) as the cultures are usually inoculated with cells from the stationary growth phase ('overnights') or with 'uninduced' cells. In the latter case, the β-galactosidase concentration in the inoculate is zero and integration yields

$$[\mathrm{LacZ}] = \frac{p \cdot N_0}{\mu}(e^{\mu t} - 1) \qquad (2)$$

Normalizing to the amount of cells (N) present at any time point during growth results in the expression for the cell-specific β-galactosidase concentration

$$\frac{[\mathrm{LacZ}]}{N} = \frac{p}{\mu}\left(\frac{e^{\mu(t-t_{lag})} - 1}{e^{\mu(t-t_{lag})}}\right) \qquad (3)$$

The time t_{lag} was introduced to account for the lag time that precedes exponential growth in a real experiment. The initial condition of zero β-galactosidase ignores the synthesis of any β-galactosidase during the lag time.

Equation 3 shows that the β-galactosidase concentration in the cell reaches a steady state concentration p/μ after enough time has elapsed to make $e^{\mu(t-t_{lag})} \gg 1$. Importantly, the steady state concentration depends both on the synthesis rate p and the growth rate μ. The time dependence of the process by which the synthesis rate and the dilution rate over the new cells are balanced (the pre-steady state) only depends on the growth rate.

Pre-steady-state expression of β-galactosidase

The Mg^{2+}-citrate transporter CitM is the principal citrate transporter of *Bacillus subtilis*. Expression of CitM is induced by citrate and repressed by glucose, and other sugars and non-sugars present in the medium (9). Repression is mediated by carbon catabolite

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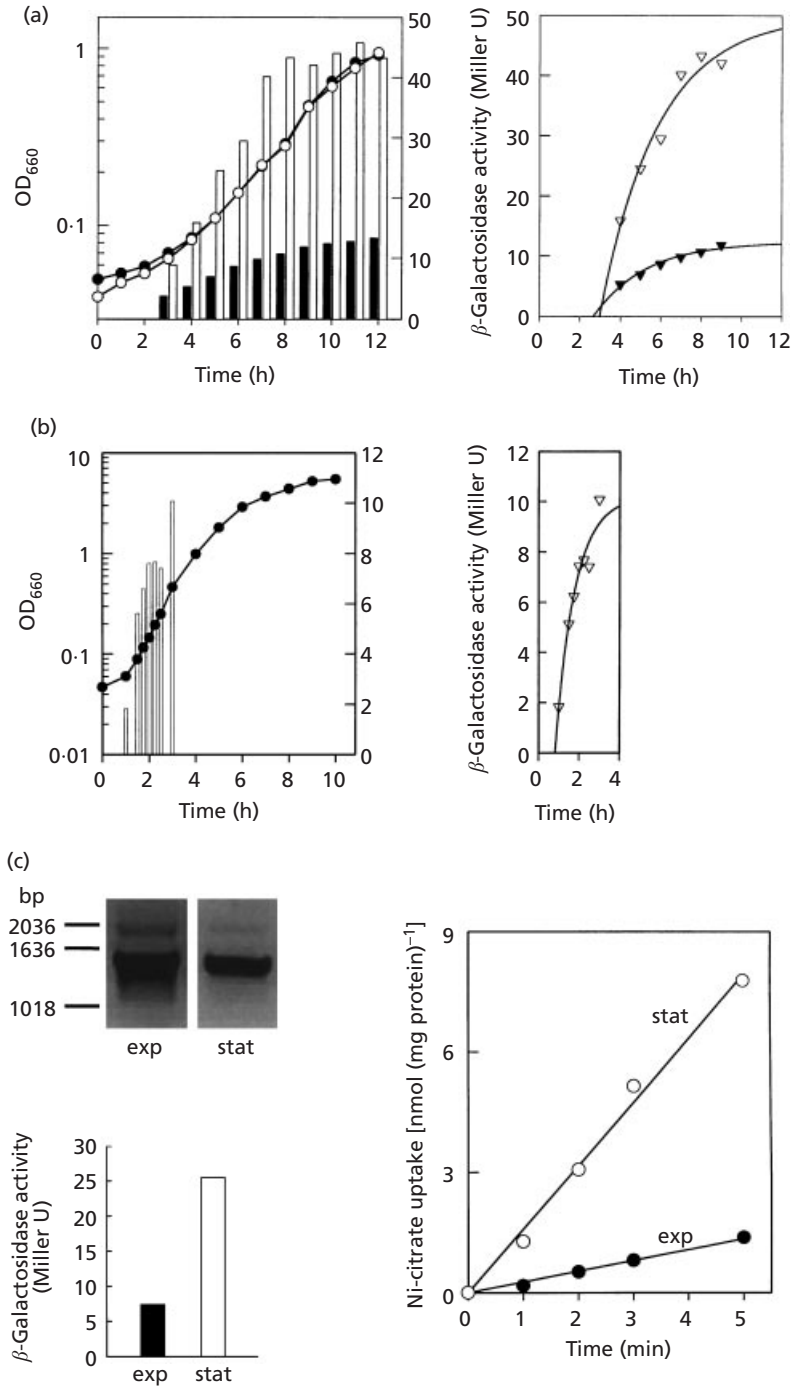

Fig. 1. (a, b). Left panels. Growth curves of *B. subtilis* strains CM002 (wild type, black circles) and CM010 (ΔCcpA, white circles) in CSE medium (a) and LB medium (b) containing 10 mM citrate. The bars indicate the β-galactosidase activity of the cells at the indicated time points (in Miller U). Right panels. Best fit of the β-galactosidase activities from the exponential part of the growth curves to equation 3. (c) RT-PCR of isolated mRNA using primers targeted at the *citM* gene (top left), β-galactosidase activity (bottom left) and Ni^{2+}-citrate uptake (right) from strain CM010 grown in LB medium supplemented with 10 mM citrate. The cells were harvested at $t = 2$ h (exp) and $t = 8$ h (stat). The position of the DNA size markers is indicated on the left of the gel picture. The expected size of the transcript is 1367 bp. For experimental details, see reference (9).

repression (ccr). The *B. subtilis* strains CM002 and CM010 contain a transcriptional fusion of the *citM* promoter region and the *lacZ* reporter gene. Strain CM002 is the wild-type background, while CM010 is deficient in CcpA, a central component of ccr in *B. subtilis* (1). The deficiency results in relief of ccr-mediated repression. CSE minimal medium containing citrate was inoculated with a preculture of the strains grown in the absence of citrate and, therefore, completely devoid of any β-galactosidase activity (Figure 1a, left panel).

In both strains, the β-galactosidase activity expressed per cell density increased gradually during the exponential growth phase and reached a maximum when entering the stationary phase. Clearly, inferring promoter activity from the level of expression would significantly depend on the time point of sampling. Fitting of the data from the exponential growth phase to equation 3 gave satisfactory results (regression coefficients of $R = 0{\cdot}99$ and $R = 0{\cdot}98$ for strains CM002 and CM010, respectively), indicating that the data is well described by the simple model presented above (Figure 1a, right panel). It follows that the β-galactosidase synthesis rates in the two strains were constant during the exponential growth phase. The increase in cell-specific β-galactosidase merely reflects the pre-steady-state period of the expression in the cells and is an intrinsic property of the system. The analysis allows for a reliable estimate of the difference in promoter activity in the two strains that is independent of the time point at which the expression was measured. The fit revealed that the β-galactosidase synthesis rate p was 3·6 times higher in strain CM010 than in strain CM002. Assuming that the difference is completely caused by the relief of ccr in the CcpA-deficient CM010 strain, this would correspond to a repression of 72 % in the wild-type strain during exponential growth on CSE medium.

β-Galactosidase expression at different growth rates

Growth of *B. subtilis* strain CM010 in LB medium is three times faster than growth in minimal CSE medium. The steady-state level of cell-specific β-galactosidase activity in the exponential growth phase (between ~ 1 and 3 h) was accordingly reached faster, in agreement with equation 3 (Figure 1b, left panel). Again, a reasonable fit of the data to equation 3 was obtained ($R = 0{\cdot}97$) indicating a constant rate of β-galactosidase synthesis during exponential growth. The steady-state level of expression of β-galactosidase, p/μ was fitted to be 10 Miller U, which was five times lower than observed during growth on minimal CSE medium. Importantly, the β-galactosidase synthesis rates, p, differed only by a factor of 1·5. Following this analysis, one might argue that a constant level of expression observed at different growth rates is the result of strict regulation of the rate of transcription by the growth rate; the level of expression is independent of the growth rate when p is

proportional to μ. In contrast, an inverse relation between the level of expression and growth rate, as observed here, would be indicative of a growth-rate-independent rate of transcription (10). Such considerations may be helpful when discussing growth-rate-dependent expression (2, 3, 4, 5, 8) and, at least, show the importance of careful use of terminology. At any time, it should be kept in mind that the rate of transcription is a complex parameter containing many different contributions.

β-Galactosidase expression and growth phase

The dominant effect of growth on the evaluation of *lacZ*-promoter fusions is nicely demonstrated when mRNA levels and protein levels are compared in CM010 cells from the mid-exponential and the early-stationary growth phase in LB medium. In the stationary growth phase, the level of mRNA in the cells as determined by RT-PCR was significantly lower than observed in the exponential growth phase (Figure 1c, top left). This situation may arise from a lower rate of transcription, increased messenger instability or a combination of these. Many effects may cause the lower rate of transcription, i.e. a change in the energetic state of the cells, inactivation of the transcription machinery, reduced induction (e.g. depletion of the inducer during growth), or downregulation of transcription. Surprisingly, the cell-specific β-galactosidase activity was a factor of 3·4 *higher* in the cells from the stationary phase than in the cells from the exponential growth phase (Figure 1c, bottom left). Measurement of the Mg^{2+}-citrate transporter uptake activity in the same cells revealed an increase of a factor of six, indicating that the increase is not an artefact of the *lacZ* reporter gene fused behind the *citM* promoter region (Fig. 1c, right panel). The paradoxical relationship between the decreased mRNA level and increased protein levels should be analysed in the context of the almost complete lack of growth of the cells in stationary phase. Then, the decreased rate of protein synthesis from the lower amount of mRNA may still exceed the very low, if any, rate of dilution caused by cell division, resulting in a net increase of protein content per cell. While the rate of transcription goes down, the level of expression goes up. Importantly, changing levels of expression when cells shift from the exponential to the stationary growth phase as observed here do not necessarily indicate (a change in) regulation of expression by *trans*-acting elements.

Conclusion

In growing cells, the rate of transcription is not the same as the level of expression. The latter is determined by both the rate of transcription and the growth rate. The model presented in equation 3 (above) gives a quantitative account of the relation between rate of expression end level of expression and indicates that the pre-steady state of expression is solely dependent on the growth rate. Two questions should be kept in mind when analysing *lacZ*–promoter fusions by measuring the specific β-galactosidase activity of the cells. One: has the expression level reached the steady-state value? Occasionally, the exponential growth phase in batch culture may be too short to reach the steady state. Then, the steady-state level follows from extrapolation of the levels of expression during the exponential growth phase using equation 3. Two: when comparing β-galactosidase activities, are the growth rates in the two situations the same? If not, expression levels should be corrected for the growth rates to make a reliable comparison between the transcription rates. In conclusion, reliable evaluation of *lacZ*–promoter fusions requires measurement of the time course of β-galactosidase activity during the exponential growth phase and the growth rate.

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The genomes of *Pseudomonas* encode a third HU protein

HU proteins are ubiquitous DNA-binding factors that, along with other so-called nucleoid-associated proteins, are involved in the structural maintenance of the bacterial chromosome and other events that require DNA bending (1). In contrast to the structurally related integration host factor (IHF) protein, HU proteins bind DNA in a sequence-independent manner, although both produce changes in DNA structure (8, 13). In *Escherichia coli*, HU is one of the most abundant of this kind of protein, with a postulated binding site in the chromosome every 200 bp. In *Pseudomonas putida*, HU has been implicated in the transcriptional activation of the *Pu* and *Ps* promoters of the toluene degradative plasmid TOL (12). Previous work in our laboratory led to the purification and characterization of two HU-like factors named HupB and HupN (2). They were first identified biochemically on the basis of sequence-independent DNA binding and DNA-bending activity. While independent knockouts of each yield no apparent phenotype *in vivo*, simultaneous mutation of the two is lethal to the cell. This observation implies a redundancy in function, as is the case with the HupA and HupB proteins of *E. coli* (9). In principle, these results ruled out the existence of any other HU-like protein, at least on the basis of functional screenings.

These assumptions were challenged, however, during the course of a genome-wide search for nucleoid-associated proteins of *P. putida* KT2440 (http://www.tigr.org) using the Genewise 2.0 program. This software can scan suboptimal DNA sequences using a Hidden Markov Model like that stored in Pfam database (3). This procedure is very sensitive, as it can properly handle small errors in DNA sequence (such as frameshifts due to artefactual insertions or deletions). In addition, the same software accurately finds weak similarities. This is because the program extracts all the information contained in an alignment of known members of the family, instead of using a single protein or a consensus for the search (7). Our choice was the use of the PF0012 model from the Pfam database (3), which was contrived on the alignment of all known IHF and HU-like proteins. By this