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Most of us feel, from time to time, that other authors have not acknowledged the work of our own or other groups or have omitted to interpret important aspects of their own data. Perhaps we have observations that, although not sufficient to merit a full paper, add a further dimension to one published by others. In other instances we may have a useful piece of methodology that we would like to share.

The Editors hope that readers will take full advantage of this section and use it to raise matters that hitherto have been confined to a limited audience.  

Christopher M. Thomas, Editor-in-chief

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**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.

\[
\frac{d[\text{LacZ}]}{dt} = p \cdot N_o \cdot e^{\mu t}
\]  

where \( p \) is the synthesis rate per cell, \( N_o \) the cell density at the beginning of the exponential growth phase, \( t \) is the time and \( \mu \) 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

\[
[\text{LacZ}] = \frac{p \cdot N_o}{\mu} (e^{\mu t} - 1)
\]  

Normalizing to the amount of cells (N)

\[
\frac{[\text{LacZ}]}{N} = \frac{p}{\mu} (e^{\mu t} - 1)
\]  

The time \( t_{\text{lag}} \) 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 \( p/\mu \) after enough time has elapsed to make \( e^{\mu t_{\text{lag}}} \geq 1.0 \). Importantly, the steady state concentration depends both on the synthesis rate \( p \) and the growth rate \( \mu \). 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\(^{++}\)-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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**GUIDELINES**

Communications should be in the form of letters and should be brief and to the point. A single small Table or Figure may be included, as may a limited number of references (cited in the text by numbers, and listed in alphabetical order at the end of the letter). A short title (fewer than 50 characters) should be provided.

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and CM010 contain a transcriptional fusion repressor (ccr). The
β-galactosidase activity (bottom left) and Ni-citrate uptake (right) from strain CM010 grown in LB medium is three times faster than growth in minimal CSE medium. Importantly, 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 release 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* = 0.97) 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

![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 (Figure 1b, left panel). (c) RT-PCR of isolated mRNA using primers targeted at the citM gene (top left), β-galactosidase activity (bottom left) and Ni²⁺-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].](image-url)
In growing cells, the rate of transcription is proportional to $\mu$. 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, 6) 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.

**$\beta$-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 $\beta$-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 relationship 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 $\beta$-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 $\beta$-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 two situations. In conclusion, reliable evaluation of lacZ-promoter fusions requires measurement of the time course of $\beta$-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 vitro, 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 detects 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
Fig. 1. (a) Alignment of the HupP proteins of *Pseudomonas.* CLUSTAL W (16) was employed to align the sequences found in the genome-wide searches described in the text. Note the nearly complete conservation of the sequence. (b) Phylogenetic tree of HU-like proteins of the Proteobacteria, the chromosomal sequences of which are known. The HU-like proteins were also aligned with CLUSTAL W. The evolutionary distances were calculated with the Protdist program. The tree shown was generated with the neighbour-joining algorithm of the PHYLIP 3.5 package. Note that HupN and HupP clearly cluster away from the archetypal HU-α and HU-β subfamilies.

procedure, five potential HU/IHF proteins were found. Four had already been described and characterized by experimental methods. Two of these ORFs encode the two subunits of the IHF protein of *P. putida* (5). Two others match the HupB and HupN protein sequences (2). But, in addition, a fifth gene was found that clearly encoded an extra HU-like protein. This so-far unknown protein was used as a query with the HU-like protein. This so-far unknown was found that clearly encoded an extra sequences (2). But, in addition, a fifth gene others match the HupB and HupN protein of the novel HU-like gene in the four are still incomplete, the degree of conservation blast procedure, five potential HU/IHF proteins were scanned for the presence of HU-like proteins using similar procedures, most had were found. Four had already been described (16 species) or two (6 species) HU similarities among correspondents in these species, and the presence of an orthologue in the four are not trivial to screenings to detect either the function or the *P. aeruginosa* chromosomes (an entirely encoded genome sequence; 15), indicated that such a sequence determines a true protein. The corresponding gene was thereby named *hupP*, since it appeared to be habitually present in several *Pseudomonas* species. Further, we argue that *hupP* is an authentic gene encoding a genuine and thus-far unidentified HU-like protein.

The capacities of *hupP* are not trivial to predict. The failure of *in vivo* and *in vitro* screenings to detect either the function or the sequence of the *hupP* gene of *P. putida* (2) and *P. aeruginosa* (6) is intriguing. In the case of *P. putida*, such a screening was based on the enrichment of DNA-binding proteins from a cell lysate that stimulated a β-recombination reaction dependent on non-specific DNA binding. This procedure exclusively yielded the HupN and HupB products. Moreover, the lack of viability of double *hupB/hupN* mutants of *P. putida* suggests that their redundant functions in multiple cellular events cannot be replaced by *hupP*. The possibility that *hupP* is a pseudogene, devoid of coding capabilities, is unlikely. Unlike typical pseudogenes, both the *hupP* sequence and its surrounding chromosomal context are conserved in the *Pseudomonas* species examined. In addition, each of the four *hupP* genes are flanked by a sigma-70 dependent promoter and a strong Rho-independent terminator. This is predicted by the NNPP (http://www.fruitfly.org/seq_tools/promoter.html) and Terminator (4) programs. Such a semblance is a feature rarely present in pseudogenes and argues that the corresponding sequence can be perfectly expressed. It thus appears that the *hupB*, *hupN* and *hupP* genes of *Pseudomonas* encode unequivocally distinct HU variants. One possibility is that HupB provides housekeeping HU activity (i.e., facilitating non-specific DNA bending) while HupN and/or HupP have more specialized functions as the result of a recent horizontal gene-transfer event.

Such an uneven number of HU types in the same cells is somewhat surprising. When 31 completed and published eubacterial genomes were scanned for the presence of HU-like proteins using similar procedures, most had only one (16 species) or two (6 species) HU proteins. When all the HU-like proteins from the group Proteobacteria that were found in the analysis of completed genomes were compared (Fig. 1b), the HupN and HupP proteins from the genus *Pseudomonas* were present in independent clusters. The *hupP* gene was previously annotated in the *P. aeruginosa* genome as *hupA*, a name that could be misleading, since the *hupP* genes of *Pseudomonas* clearly branch out from the HU-α group (Fig. 1b). In contrast, the HupB protein is included in a more populated group that comprises, for instance, the reference HU-α specimen of *E. coli*. This classification is compatible with those described previously (10, 11) and can shed some light on the possible origin of the three HU-like proteins present in *Pseudomonas*. The tree of Fig. 1(b) hints at HU-like proteins originating in a common ancestor (10, 11) which may have been maintained as such in some organisms of the same subfamilies. The ancestor of that kind could have then diverged in the γ group to give rise to the HU-α (i.e. *Enterobacteriaceae*, *Vibrionaceae*) and HU-β (i.e. *Pseudomonadaceae*, *Xanthomonas*) branches. An enticing possibility is that, later in evolution, the enterobacteria could have horizontally acquired an extra copy of the HU-β type, thus giving rise to the heterodimeric HU that is emblematic of this family (10). In this context, it would be reasonable that HupP appeared in *Pseudomonas* through the duplication of HupB (after
the proposed gene-transfer event to the enterobacteria), and rapidly evolved for attaining a specialized function. The origin of HupN is more ambiguous, since it pertains to a wider class of less delineated HU species (Fig. 1b). HupN could have been procured from other soil microorganisms (i.e. Rhizobiaceae) and maintained as a backup for the equivalent functions of HupB (2).

In conclusion, three HU-like proteins were found to be encoded in the genomes of four Pseudomonas species. Two, HupN and HupP, clearly diverge from the other known members of the HU protein family, and are so far restricted to Pseudomonas. The appearance of three different HU-like proteins in a bacterial genome is not yet reported for other genera and could reflect a degree of regulatory complexity related to the adaptability of this genus to a wide range of environmental conditions (14). Although related to the other HU factors in its sequence and predicted structure, HupP may form a class of its own within the larger protein family.

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