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Inclusion of an upstream transcriptional terminator in phage display vectors abolishes background expression of toxic fusions with coat protein g3p

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Abstract

Expression of toxic gene products affects bacterial cell growth and phage display, causing a strong selection against plasmid maintenance and integrity. During phage propagation steps, in particular, phagemid instability can dramatically affect diversity of antibody libraries or even lead to the deletion of antibody genes. We constructed a modified phage display vector by introducing a strong transcriptional terminator upstream of the *lac* promoter, which, together with glucose suppression of its CAP-dependent activation, very efficiently represses product formation before induction.

Keywords: Recombinant antibodies; *E. coli*; Filamentous phage; Antibody libraries

1. Introduction

It has often been observed that the strong selection pressure generated by toxic gene products leads to high frequency of recombination events, resulting in genetic instability of the vector system used (Brown and Campbell, 1993; Wülfing and Plückthun, 1993). Once the gene encoding the toxic product is deleted, bacteria harboring plasmids free of this gene gain a growth advantage over bacteria still expressing the product, and quickly overgrow the culture.

Phage display vectors which go through many generations and pass through single stranded forms during their life cycle suffer most acutely from the occurrence of recombination events (Smiley and Benkovic, 1994; Kingsbury and Junghans, 1995). Due to the growth advantage of deletion mutants, these can become over-represented very easily, thereby reducing or completely eliminating the possibility of successfully enriching the displayed molecules by panning procedures. Since antibodies differ in their toxicity for the producing bacterial cell (Knappik and Plückthun, 1995), a successful maintenance of library diversity depends on minimizing the growth disadvantage of certain clones by not producing the toxic antibody fragment or fusion protein until required.

The problem can be overcome by stringent repression of g3 fusion protein expression during all propagation steps, combined with low level expression during phage production steps. Therefore, a precisely controlled expression system is a prerequisite if this goal is to be achieved.

2. Experimental and discussion

2.1. *Control of* lac-*based promoter systems by repression*

The most common expression systems used for phage display are based on the *lac* promoter (Barbas et al., 1991; Hoogenboom et al., 1991). In the past, tighter control of this promoter was achieved in different ways: (a) coexpression of a plasmid-encoded *lac*-repressor (Ørum et al., 1993; Ge et al., 1995; Hayashi et al., 1995), (b) use of *lacI*^{Q} strains (Ørum et al., 1993), (c) addition of glucose to prevent CAP-cAMP dependent stimulation

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Abbreviations: CAP, catabolite activator protein; cAMP, cyclic adenosine 3′,5′-monophosphate; g3p, M13 coat protein 3; IPTG, isopropyl-β-D-thiogalactopyranoside; OD, optical density; PAGE, polyacrylamide-gel electrophoresis; POD, peroxidase; scFv, single-chain Fv fragment; SDS, sodium dodecyl sulfate; t, terminator.

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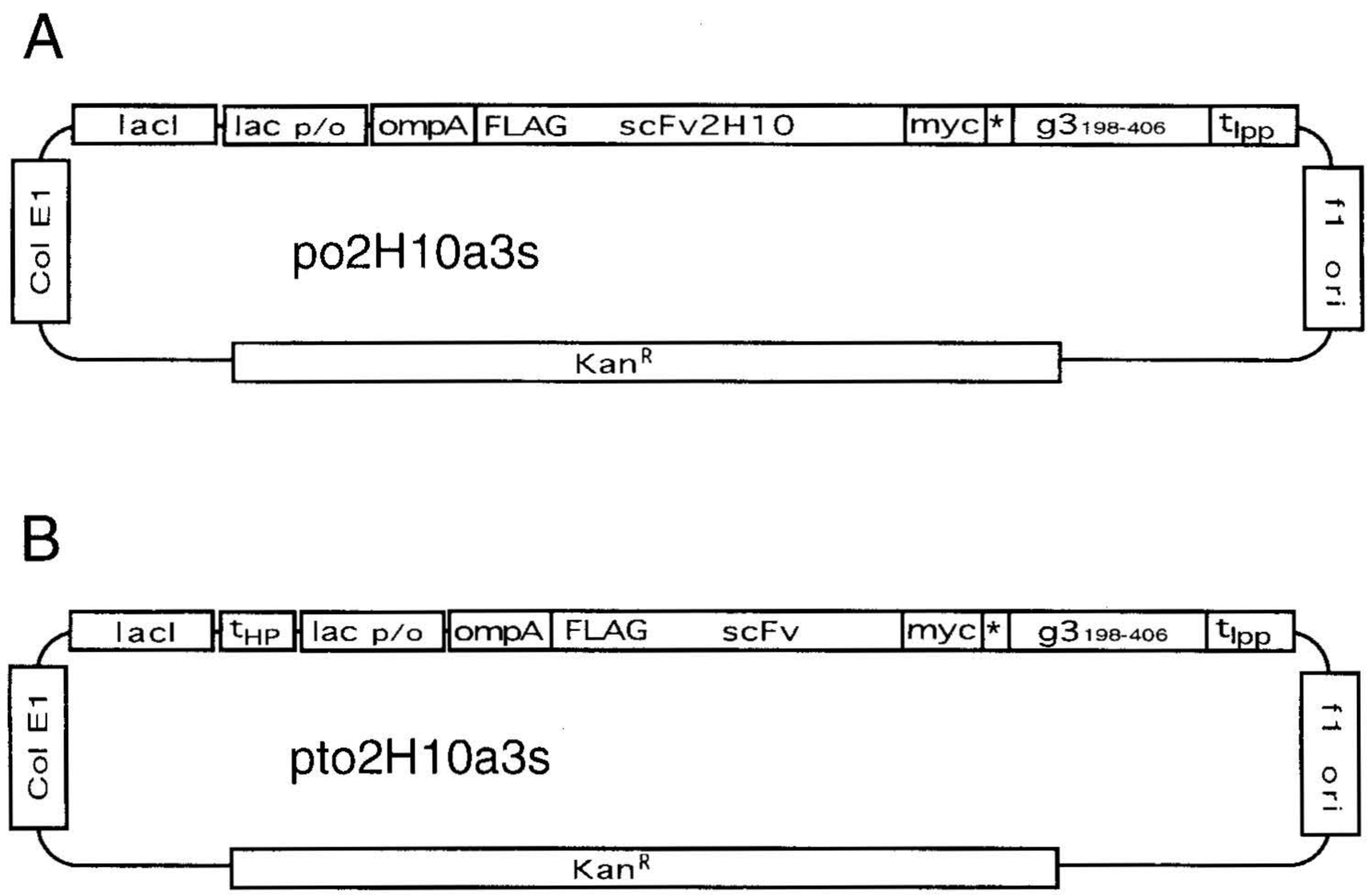

Fig. 1. (A) phagemid po2H10a3s and (B) phagemid pto2H10a3s. *lacI*, gene encoding the *lac* repressor; t_{HP}, t_{HP} terminator; *lac p/o*, *lac* promoter/operator region; *ompA*, signal sequence of bacterial outer membrane protein A; *FLAG*, four amino acid (DYKD) detection tag (Knappik and Plückthun, 1995); *scFv*, gene encoding the scFv fragment of the benzoyl-ampicillin binding antibody 2H10 (Nieba et al., 1996) in the orientation V_L-$(G_4S)_3$-V_H; *myc*, peptide tag (EQKLISEEDL) recognized by the mouse monoclonal antibody 9E10 (Oncogene Science, Uniondale, NY, USA); *, amber codon; $g3_{198-406}$, gene encoding the C-terminal portion of M13 coat protein 3 starting at amino acid 198; Kan^R, kanamycin resistance (transcription level down-regulated).

of RNA polymerase binding to the *lac* promoter (Peterkofsky and Gazdar, 1971; De Bellis and Schwartz, 1990; Hoogenboom et al., 1991; Ge et al., 1995; Tagami and Aiba, 1995). While each of the three strategies has been found to have a beneficial effect, they are apparently not always sufficient for tight repression and reliable handling of large phage display libraries.

To make the negative effect of background expression clearly visible, we used the scFv fragment of the antibody 2H10 (Nieba et al., 1996) fused to the C-terminal part of g3p as a model system. This single-chain antibody and its gene3 fusion belongs to the group of proteins causing more than average lysis in *E. coli*. We refer to this phenomenon as 'toxicity'. Fig. 1A shows the phagemid po2H10a3s, which expresses the scFv2H10-gene3 fusion protein under *lac* promoter control and encodes a *lacI* gene as well as the wt CAP-binding site, which provides glucose regulation. In Fig. 3 it can be clearly seen that the phagemid po2H10a3s still gives rise to detectable product formation in the non-induced state, even when methods (a)–(c) from above ($lacI^Q$ strain XL1-Blue, phagemid encoded *lac* repressor, glucose repression) were combined (Fig. 3A, culture 3). Furthermore XL1-Blue cells, harboring the repressed phagemid po2H10a3s start to lyse after growth for several hours at 37°C (Fig. 3B, culture 3).

The most likely explanation for the observed background expression is the occurrence of independent transcriptional start points upstream of the *lac* promoter region. Read-through transcription from cryptic promoter sequences has previously been observed in T7 RNA polymerase expression systems and was eliminated

```
5'
CTGGAAAGCGGGCAGTGAGCGGTACCCGATAAAAGCGGCTTCCTGACAGGAGGCCGTT
Leu Glu Ser Gly Gln *                          tHP  terminator
      lacI

TTGTTTTGCAGCCCACCTCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAG
                                CAP binding site

                                          ------------> mRNA      3'
GCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACA
   -35                        -10            lac-operator
```

Fig. 2. *Lac* promoter region with inserted t_{HP} terminator sequence (bold). The terminator sequence, taken from Nohno et al. (1986), was introduced into the phagemid po2H10a3s by site directed mutagenesis according to Kunkel et al. (1987). The resulting vector was called pto2H10a3s (Fig. 1B).

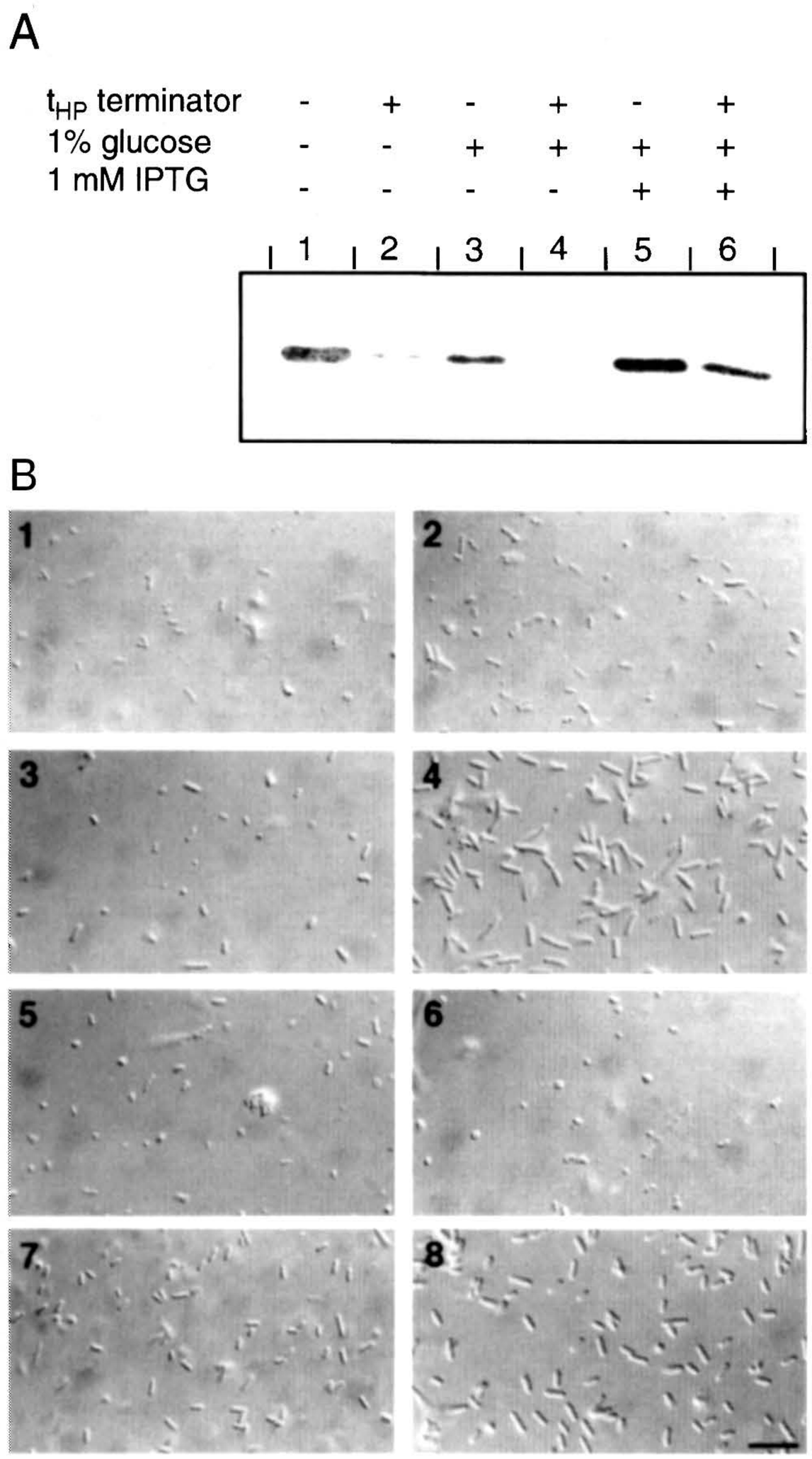

Fig. 3. Product formation due to unsuppressed background expression clearly affects growth behavior of *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA). This negative effect can be overcome by the insertion of an upstream terminator in combination with glucose repression. XL1-Blue cells harboring po2H10a3s (no t_{HP} terminator) or pto2H10a3s (with t_{HP} terminator) were used to inoculate 2 × YT (1 l: 16 g bacto tryptone, 10 g yeast extract, 5 g NaCl), Kan 25 μg/ml or 2 × YT, 1% glucose, Kan 25 μg/ml, respectively (culture 1, no terminator, no glucose; culture 2, with terminator, no glucose; culture 3, no terminator, 1% glucose; culture 4, with terminator, 1% glucose). All cultures were grown in shake flasks at 37°C. At $OD_{600} = 0.5$, culture 3 and 4 were divided into two aliquots. One aliquot was induced with 1 mM IPTG (culture 5, no terminator, 1% glucose, 1 mM IPTG; culture 6, terminator, 1% glucose, 1 mM IPTG), whereas the other one was kept in the non-induced state. As a control plasmid-free bacteria were grown in 2 × YT (culture 7) and 2 × YT, 1% glucose (culture 8) under the same conditions. The experimental data shown in Fig. 3A and B have been repeated three times leading to the same results. (**A**) To perform a Western Blot analysis 100 μl of culture 1–6 were harvested by centrifugation 3 h after reaching $OD_{600} = 0.5$. The bacterial cell pellets were resuspended in reducing SDS sample buffer and subjected to 0.1% SDS-12% PAGE. Since a FLAG sequence is present at the N-terminus of scFv2H10, detection was carried out essentially as described in Knappik and Plückthun (1994) by a monoclonal anti FLAG antibody, but using precipitating BM Blue POD Substrate (Boehringer-Mannheim) for development. Since po2H10a3s and pto2H10a3s contain an amber codon in front of gene3 (indicated by an asterisk in Fig. 2), which is only partially suppressed in XL1-Blue, the main expression product is the scFv fragment of 2H10. This rather than the gene3 fusion protein was chosen to quantify background expression. (**B**) To visualize cell lysis caused by background expression differential interference contrast microscopy was used. Eighteen hours after inoculation 20 μl of each culture was spread on an object slide. In order to avoid cell disruption plasticine was used as spacer between object and cover slide (bar = 10 μm).

by the introduction of a transcriptional terminator upstream of the promoter of interest (Nishihara et al., 1994; Brown and Campbell, 1993).

2.2. Prevention of background expression

In order to prevent expression initiated at sites other than the *lac* promoter we inserted a strong terminator (t_{HP}) (Nohno et al., 1986) between the *lacI* gene and the CAP binding site of the *lac* promoter (Fig. 2). Under the same experimental conditions as described above, this modification clearly reduces product formation to below the level of detection (Fig. 3A, culture 4). Even in the absence of glucose (Fig. 3A, culture 2), the terminator suppresses expression more efficiently than glucose alone does in its absence (Fig. 3A, culture 3). Moreover, this effect proved to be general for the expression and phage display of various other proteins tested (data not shown).

In order to document negative effects on cell physiology, caused by expression of toxic gene products, micrographs of bacterial cultures were taken 18 h after inoculation. Differential interference contrast microscopy data clearly visualized that tight suppression of toxic gene products has a very beneficial effect on bacterial cell growth and prevents cell lysis in uninduced shake flask cultures (Fig. 3B, culture 4 versus 3). Only bacteria harboring the phagemid pto2H10a3s (Fig. 1B), where product formation is repressed by the t_{HP} terminator sequence and addition of 1% glucose, show cell growth indistinguishable from plasmid-free bacteria (Fig. 3B, culture 4 versus 8). In an aliquot of the same culture, product formation is readily induced upon IPTG addition (Fig. 3A and B, culture 6), indicating that pto2H10a3s can be used to express scFv-gene3 fusion protein under non-repressing conditions for phage display.

It can be seen that as soon as scFv-gene3 fusion protein is produced, either due to background expression or to induction by IPTG, bacterial cell physiology is negatively influenced resulting in reduced cell density and increasing amounts of cell debris in cultures 1, 2, 3, 5, 6 in comparison to cultures 4, 7, 8 (Fig. 3B). The occurrence of this effect depends on the simultaneous presence or absence of the t_{HP} terminator and 1%

glucose, and the microscoopy data can serve as an independent corroboration for the data presented in Fig. 3A.

If cultures 5 and 6 are compared, it can be seen that the total yield of recombinant protein 3 h after induction is slightly decreased due to the incorporation of the t_{HP} terminator sequence. This can be partly explained by the exclusion of any contribution to expression caused by additional upstream promoter sequences. For phage display, however, tight suppression during propagation steps and low expression of scFv gene3 fusions during display steps are most crucial for the preservation of library diversity. In our hands the production level of the modified *lac* promoter was very satisfactory for phage display and can be further enhanced, if necessary, either by eliminating the amber codon between scFv and gene3, or by removing 1% glucose directly before induction or, more generally, by replacing parts of the natural *lac* promoter region with stronger promoters and Shine-Dalgarno sequences (e.g., *lacUV5*, SD*T7g10*) to be used for expression plasmids (data not shown).

3. Conclusions

The general success of this method suggests that the observed background transcription of the *lac* promoter, in the context of a natural *lacI-lac p/o* arrangement with CAP site present, does indeed come from transcriptional starts further upstream, for example from the *lacI* promoter region or cryptic promoter sequences somewhere else on the phagemid. Furthermore, micrographs of uninduced *E. coli* cultures harboring a typical phage display vector indicated that tight regulation of *lac* promoter controlled expression is very important for securing of comparable growth conditions for less toxic and more toxic coat protein fusions during library propagation steps. In our hands, the cloning, phage panning and mutagenesis of antibody fusion proteins, which are known to affect cell growth and cause plasmid instability to different extents, have been greatly facilitated by modifying the expression system. Therefore, we propose that the incorporation of a strong terminator sequence upstream of the *lac* promoter will in general simplify the handling of various phage display vector systems.

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