Relationship Between Opacity of Transformed E. coli Colonies and Over-Expression of the Recombinant Transcript


ABSTRACT

Following transformation with recombinant plasmid clones, E. coli BL21(DE3) often produced extremely opaque colonies on a standard semisolid agar plate, as compared with the translucent colonies produced by normal, untransformed bacteria. A standard BL21(DE3) culture consisted of two kinds of cells: one kind produced translucent colonies and the other produced opaque colonies upon transformation by recombinant plasmids. The translucent-generating phenotype often switched to the opaque-generating phenotype, which was irreversible. Opacity in the BL21(DE3) background was correlated to a higher preinduced level of T7 RNA polymerase, presumably through a stable and inheritable genetic change. In all E. coli strains tested, a robust transcription of the recombinant gene from the plasmid clone was found to be an essential prerequisite for very high opacity; translation of the RNA was not required. The degree of opacity was also determined by the nature of the insert in a given strain background. Colony opacity generally, but not invariably, correlated with a smaller colony size on semisolid agar and a reduced growth rate in liquid culture.

INTRODUCTION

In the last few years, we have cloned and expressed a number of prokaryotic and eukaryotic proteins in various strains of Escherichia coli, including the popular BL21(DE3). During these studies, we have often come across two kinds of transformant colonies on LB-ampicillin plates: opaque and translucent. The translucent colonies were virtually indistinguishable from standard untransformed E. coli cells. However, the opaque colonies were only encountered in transformed cultures. E. coli transformed with pFLAG-1 and pMal-c2-based recombinant clones have, on the other hand, been reported to be predominantly translucent (1). Although the exact mechanism of such variations in colony morphology remains unknown, I will summarize our observations, which extend earlier findings and lead to a number of generalized rules. These findings should assist in the selection of the right transformants and future directions of research in this novel area.

MATERIALS AND METHODS

Preparation of Cell Extracts

Growth and selection of bacteria in LB broth or LB-agar plates were carried out by standard procedures. E. coli BL21(DE3) and pET vectors were obtained from F.W. Studier, Brookhaven National Laboratory (8), and the pFLAG vector was obtained from Scientific Imaging Systems, Eastman Kodak (New Haven, CT, USA). Opacity or translucency of bacterial colonies on LB plates (containing 100 µg ampicillin [Amp] per mL, where indicated) was observed by holding plates against fluorescent ceiling lights and comparing against non-transformed translucent E. coli colonies.

To induce protein expression, cultures were grown in LB-Amp to an A590 of approximately 0.3. Isopropyl-β-d-thiogalactopyranoside (IPTG) was then added to half of the culture, and growth continued for another 3 h. Cells were then collected by centrifugation and lysed by lysozyme-EDTA as described earlier (7). This lysate is referred to as “total extract”; where mentioned, 10 µL of this total extract were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (6,7).

Quantititation of intracellular T7 RNA polymerase activity was carried out as follows. S100 extracts were prepared by centrifugation of the total extracts at 120 000×g for 1 h at 4°C. Different amounts (2–4 µL) of the S100 extracts were then used as the source of T7 RNA polymerase in a 20-µL transcription assay containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol (DTT), 100 ng of supercoiled pET3a-RSV-P plasmid DNA, 20 µg/mL rifampicin, 0.25 mM each of ATP, CTP and GTP, 20 µM of [α-32P]-UTP (10 µCi), incubated at 37°C for 30 min. 32P-labeled RNA was quantitated by a DE81 paper-binding assay as described (3).

RESULTS AND DISCUSSION

High Opacity is a Result of Over-Expression of the Cloned Gene in the Bacterial Cell

When the cloned gene is not expressed or under-expressed, the bacterial transformant colonies remained translucent. This is based on the following evidence: (i) We have cloned a number of protein genes (PPα, PP5, respiratory syncytial virus [RSV] P protein and Piry virus P protein) in the expression vector pET3a (2,4,7) between the NdeI and BamHI sites such that transcription of these genes originates from the phage T7 promoter (8). When these clones were introduced into DH5α, HB101 or C600 (i.e., E. coli strains that do not make T7 RNA polymerase and therefore do not transcribe genes cloned in pET3a), only translucent colonies resulted. However, when introduced into BL21(DE3) (a...
specially constructed strain in which T7 RNA polymerase is expressed under the control of the lac operator/promoter from a chromosomally integrated λDE3 prophage), these same plasmid clones produced a mixture of translucent and opaque colonies (in a roughly 1:6 ratio), depending on the nature of the recombinant gene [see section entitled Individual BL21(DE3) Cells May Be Predisposed to Form Opaque or Translucent Colonies Upon Transformation].

When the same gene, e.g., PPλ, was subcloned into pUC19 and introduced into common E. coli strains, e.g., JM101, DH5α, HB101 or even BL21 (DE3) such that the expression of PPλ was directly under the control of lac operator/promoter present in the plasmid (and did not require T7 RNA polymerase), nearly all the colonies were opaque (Table 1). Upon analysis by SDS-PAGE, the extracts of uninduced cells were found to contain high amounts of PPλ protein, at essentially the same level as found in IPTG-induced cells (Figure 1). This was true with both wild-type PPλ and a phosphatase-defective PPλ mutant. We presume that the higher basal level of PPλ in these transformants is due to titration of the lac repressor by the multicopy pUC19 plasmid (5). To test this hypothesis, we have cloned the same PPλ genes in pFLAG. In this vector, as in pUC19, the cloned genes are under the control of an inducible promoter (ptac); however, pFLAG also contains the lac repressor gene (lacI), which is constitutively expressed. The multicopy lacI gene builds up a higher concentration of the repressor, resulting in a tighter repression and hence a very low basal transcription of the cloned gene. As anticipated, transformation of JM101 with the pFLAG clones resulted in translucent colonies, which is in agreement with the previous report (1). Spotting of IPTG on the plates, however, did produce highly opaque colonies in and around the spot. Interestingly, transformation with pFLAG vector alone (without insert) produced colonies that were slightly opaque at the center, as reported earlier (1); although, they were not nearly as opaque as the IPTG-induced colonies containing cloned genes. These findings are summarized in Table 1, which shows that a strong opacity is generally correlated with a robust expression of the recombinant gene regardless of the host genetic background (e.g., note the +IPTG column of vectors with insert). It appears that the pFLAG vector (without insert) has a general tendency to produce some background opacity, which can be observed even in BL21(DE3) (Table 1). We have no explanation for the somewhat lesser background opacity of pFLAG in BL21(DE3) compared to other strains; however, it may be relevant to note here that BL21 is an E. coli B strain, while all others are K.

(ii) Six independent opaque and two translucent colonies of BL21(DE3) containing the pET3a clone of the PPλ (4) were grown in LB-Amp and induced with IPTG. The total cell ex-

![Figure 2. Expression of recombinant proteins in opaque and translucent BL21(DE3) cells. Total extracts of IPTG-induced (+) and uninduced (-) BL21(DE3) cells containing pET3a-PPλ clone were analyzed by SDS-PAGE, followed by staining of gel with Coomassie blue. The reason behind doublet in some lysates is unknown but could result from proteolytic processing of PPλ. Lane M shows purified PPλ.](image-url)
tracts of the induced and the uninduced controls were then analyzed by SDS-PAGE. Results presented in Figure 2 show that all opaque colonies (1–6) had a significantly higher basal expression of the recombinant PPλ protein (-IPTG lanes) compared with the translucent ones (7 and 8). The phosphatase activity of PPλ in these extracts, as measured by pNPP hydrolysis, was proportional to the amount of the PPλ protein (data not shown). In spite of this difference in the preinduced level of PPλ protein, maximal synthesis, induced by IPTG, was comparable in both kinds of cultures, suggesting that the lower basal level in translucent cells was most likely due to a tighter repression of plac and not due to an inherent defect in the transcriptional activity of this promoter or the T7 promoter. This is further supported by the fact that plasmids isolated from the opaque colonies again produced a mixture of opaque and translucent colonies upon transformation of BL21(DE3).

(iii) To investigate whether there is a direct relationship between the extent of gene induction and opacity, translucent colonies of BL21(DE3)-pET3a-PPλ were dilution-streaked for single colony on an LB-Amp plate, and then a

Table 1. Relationship Between Colony Opacity and Expression of Recombinant Genes

<table>
<thead>
<tr>
<th>E. coli Strain</th>
<th>Vector</th>
<th>Relative Opacity of Colonies&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Without Insert</td>
<td>With Insert (PPλ)</td>
</tr>
<tr>
<td>BL21(DE3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pET3a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pUC19</td>
<td>-</td>
</tr>
<tr>
<td>BL21(DE3)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>pET3a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pUC19</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pFLAG</td>
<td>+</td>
</tr>
<tr>
<td>JM101, DH5α, HB101</td>
<td>pUC19</td>
<td>-</td>
</tr>
<tr>
<td>JM101, DH5α, HB101</td>
<td>pET3a</td>
<td>-</td>
</tr>
<tr>
<td>JM101, DH5α, HB101</td>
<td>pFLAG</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>The relative opacity is only approximate, based on visual comparison of colonies on LB-Amp plates. The two kinds of BL21(DE3) cells, with predispositions to form translucent or opaque colonies, have been described in the text. <sup>d,e</sup>These results confirm previous studies by Austin et al. (1).
A drop of 100-mM IPTG solution was spotted at the center of the plate. After an overnight incubation, all colonies within and in the vicinity of the IPTG spot were white, whereas those that were farther from the spot remained translucent. Thus, higher expression of the recombinant gene is associated with opacity.

**The Opaque Phenotype of BL21(DE3) is Generally More Stable Than the Translucent Phenotype**

In the BL21(DE3) background, the opaque colonies always “bred true”; thus, when we attempted to purify the opaque and the translucent colonies by streaking them out on LB-Amp plates, the opaque colonies produced opaque colonies only. However, about 10%–30% cells of a translucent colony will produce opaque colonies and sometimes produce sectored colonies that are half-opaque and half-translucent. The “second-generation” opaque and translucent colonies will again behave similarly; i.e., opaque will produce only opaque colonies, while translucent will produce a mixture of two kinds.

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**Figure 3. Second-generation opacity in BL21 (DE3) correlates with higher expression of recombinant protein.** Total cell extracts of the following kinds of BL21(DE3) colonies containing pET3a-PPα were analyzed by SDS-PAGE and staining: an original opaque colony (O₁) and an opaque colony (O₂) purified from it; an original translucent colony (T₁) and a translucent colony purified from it (T₂); and an opaque colony (TW) obtained from a translucent colony (T₁). (+) denotes IPTG-induced; (-), uninduced; and M, purified PPα.

**Figure 4. Predisposition of BL21(DE3) cell types to elevated expression of recombinant genes.** Specific colonies of BL21(DE3) were transformed (see text) and analyzed for PPα expression following (+) or without induction with IPTG, essentially as described for Figure 2.
Moreover, as shown in Figure 3, the opaque and translucent phenotype of the second-generation cells also correlated with high and low basal level of PPAλ, respectively.

**Individual BL21(DE3) Cells May Be Predisposed to Form Opaque or Translucent Colonies Upon Transformation**

Six independent BL21(DE3) colonies that looked indistinguishable in morphology and transparency were grown in LB, and competent cells were made thereof by standard CaCl₂ treatment. The cells were transformed with pET3a-PPA plasmid and plated on selective LB-Amp plates. All cultures except No. 4 produced essentially 100% opaque colonies, whereas No. 4 produced mostly translucent colonies with very few opaque ones. Upon subsequent purification, the translucent colonies again produced a mixture of opaque and translucent colonies. As before, translucent transformants arising from No. 4 culture contained a lower basal (preinduced) level of recombinant PPAλ than the opaque transformants obtained from all other cultures (Figure 4). Thus, we conclude that BL21(DE3) cell No. 4 is predisposed to translucency, while cells Nos. 1, 2, 3, 5 and 6 are predisposed to opacity following transformation.

At this point, we do not know the mechanism behind this apparent predisposition, although the persistence of the phenotype points to a relatively stable genetic switch. In a preliminary study, we have estimated the levels of T7 RNA polymerase in cells with opaque and translucent predispositions. Cell extracts made in identical manner from the two kinds of cultures were assayed for T7 RNA polymerase in an in vitro transcription assay using pET3a-RSV-P plasmid DNA as template in the presence of [α-³²P]UTP. Various dilutions of the extracts were used to ensure that the assay responds linearly to the amount of extract added. Results presented in Table 2 show that the cells with opaque predisposition (Nos. 1, 2 and 3) contain an average of 5–10 times higher specific activity of T7 RNA polymerase as compared to the cell with translucent predisposition (No. 4), which provides a possible explanation of the higher basal expression of pET3a-cloned genes in opaque cells. Studies are under way to determine the exact mechanism of higher expression of T7 RNA polymerase.

To exclude the unlikely possibility that our plasmid preparation contained two kinds of clone and that cells of No. 4 and No. 1 colonies were specifically accepting one kind of clone or another, we carried out a plasmid swapping experiment, in which plasmids isolated from transformed No. 4 culture (translucent) was used to transform No. 1 cells and vice versa. It was found that the phenotype of the donor colony had no effect on the predisposition of the recipient.

A further proof of the T7 polymerase effect was provided by the finding that transformation of the opaque BL21 (DE3)-pET3a-PPA cells with pLysE (8) resulted in nearly 100% translucent colonies. pLysE is a derivative of pACYC (conferring resistance to chloramphenicol) and codes for T7 lysozyme, which has been shown to specifically inhibit T7 polymerase and thus reduce basal expression of recombinant genes in the BL21(DE3)-pET3a system (8).

**For a Given BL21(DE3) Cell Type, the Opacity is Determined by the Sequence of the Over-Expressed Gene**

Expression of vesicular stomatitis virus (VSV) or RSV phosphoproteins (P) in BL21(DE3) using the same vector (pET3a) did not produce opaque colonies even in BL21(DE3) cultures Nos. 1, 3 or 5 (data not shown); although, these clones produced at least as much recombinant protein as the PPAλ clones (2, 7). The solubility of the expressed protein was probably not a factor since RSV P protein and PPAλ were synthesized as soluble proteins, whereas the VSV P protein was synthesized as insoluble inclusion bodies. Since PPAλ is a protein Ser/Thr phosphatase (4) that dephosphorylates a number of E. coli phosphoproteins (L.C. Dupuy and S. Barik, unpublished data), we considered the possibility that its expression may be toxic for E. coli, and the opacity may be somehow related to this toxicity. However, expression of site-directed mutants of PPAλ, completely defective in phosphatase activity, produced similar opaque colonies in BL21(DE3) and JM101 (Figure 1) backgrounds. This suggests that the opacity is perhaps due to some other feature(s) of the protein or its mRNA.

This last result pointed to the possibility that opacity is due to the expression of the recombinant RNA rather than the protein. To ascertain it, we have altered, by site-directed mutagenesis, the fourth (Leu/CCA) or the sixth codon of PPAλ (Leu/CCA) to a translation termination codon (UGA). As expected, these mutant clones produced the full-length PPAλ transcript when transcribed by T7 polymerase in vitro but did not produce any PPAλ protein when introduced into BL21(DE3), as tested by SDS-PAGE analysis of the total cell extract followed by Coomassie blue staining or Western blot analysis using an anti-PPAλ antibody (data not shown). However, they did produce opaque colonies when introduced into BL21

**Table 2. T7 RNA Polymerase Activity in Opaque and Translucent BL21(DE3) Cells**

<table>
<thead>
<tr>
<th>Colony No.</th>
<th>Predisposition</th>
<th>Relative T7 RNA Polymerase Activityb</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Opaque</td>
<td>-IPTG 320</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+IPTG 345</td>
</tr>
<tr>
<td>2</td>
<td>Opaque</td>
<td>-IPTG 350</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+IPTG 372</td>
</tr>
<tr>
<td>3</td>
<td>Opaque</td>
<td>-IPTG 335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+IPTG 348</td>
</tr>
<tr>
<td>4</td>
<td>Translucent</td>
<td>-IPTG 22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+IPTG 322</td>
</tr>
<tr>
<td>8</td>
<td>Translucent</td>
<td>-IPTG 34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+IPTG 367</td>
</tr>
</tbody>
</table>

BL21(DE3) colonies with predispositions to turning opaque or translucent upon transformation (numbers as in Figure 4) were grown and induced as described in Materials and Methods; note that colony No. 8 is not represented in Figure 4. T7 RNA polymerase activity was expressed as micromoles of UMP incorporated per 50 μg of S100 protein per hour using the linear range of the assay. Each number is an average of three assays differing within ±10% of one another.
Thus, the information that induces opacity is likely encoded in the mRNA sequence and not in its protein per se; although, we cannot exclude the possibility that for some other genes, proteins might contribute to opacity as well. Transformation with pET3a vector alone did not elicit opacity in any BL21 (DE3) clone. Since pET3a vector can produce a short (211-nt-long) transcript, it is possible that induction of opacity requires a minimum length (as suggested earlier; Reference 1) or a specific secondary structure in the transcript.

CONCLUSION

In conclusion, the opacity of transformed bacterial colonies depends on the quantity as well as the sequence of the recombinant transcript. It is possible that a specific secondary structure of the expressed RNA or its intracellular location results in scattering of the ambient light. Alternately, specific sequences in the RNA may interact with a cellular machinery to produce opacity. It appears that in the BL21(DE3) background, a metastable genetic switch determines the level of repression of the lac promoter, which in turn controls the level of T7 RNA polymerase in the cell. The elucidation of the switch was beyond the scope of this communication; however, it can conceivably operate through a variety of mechanisms, such as loss-of-function mutations in the lac operator or repressor, or promoter-up mutations in plac, etc. Clearly, quantitation of intracellular lac repressor concentration and analysis of the nucleotide sequence of the cognate cis-acting elements will help to distinguish these possibilities. It would be interesting to see whether the differential regulation of T7 polymerase resulted from a rearrangement of or within the λDE3 prophage. On the other hand, site-directed mutagenesis of various cloned genes will pinpoint the exact role of the recombinant RNA or its specific domains in this process. These studies are in progress.

It has been suggested that opacity is due to a slower growth rate of the colonies (1). While we have also found this to be generally true, it has been difficult to find a quantitative relationship between the degree of opacity and sluggishness of growth. At this point, any causal relationship between the two certainly remains unwarranted. Regardless of the exact mechanism, we suggest that whenever one encounters a mixture of opaque and translucent transformants, the choice of colony will be as follows: (i) In the BL21(DE3) background, screen both kinds of colonies for the expression of recombinant protein, etc., and then save the translucent colonies only, since these colonies accomplish better repression of the cloned gene and hence are less likely to exhibit problems resulting from potential toxicity of the over-expressed protein, e.g., plasmid loss, selection of under-producer mutants, etc. This is particularly important in large-scale growth where many generations...
of cell division must be achieved starting with a small inoculum. An alternative is to use a BL21(DE3) strain containing pLysE plasmid to reduce basal expression (8). (ii) In non-T7 strains commonly used for transformation, such as C600, HB101, DH5α, XL-1 Blue, Novablue, HMS174, JM101, JM109, etc., pick the more transparent colonies for vectors that encode repressors (e.g., pFLAG, pMAL-c2), as suggested earlier (1), since these colonies are more likely to contain the recombinant clone. For other vectors, pick the opaque colonies; transparent colonies, if any, may arise due to non-expressing clones or vectors without insert and can be discarded. It is imperative that this method of selection, when applicable, should only be used as a starting point and that confirmatory test of clones must come from more traditional methods of screening, such as restriction analysis, polymerase chain reaction and DNA sequencing.

REFERENCES


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