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Look and See if It Is Time To Induce Protein Expression in *Escherichia coli* Cultures†

K. Danielle Kelley,‡ Lorenzo Q. Olive,‡ Arina Hadziselimovic, and Charles R. Sanders*

Department of Biochemistry and Center for Structural Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-8725. ‡These authors contributed equally to this work.

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ABSTRACT: It is shown that Methyl Red can be used as an indicator dye that changes color in *Escherichia coli* culture as a result of time- and cell density-dependent bleaching by azoreductase produced by the bacteria. For cell cultures that are being used to express a recombinant protein, this phenomenon can be exploited to provide a simple visual cue that cell cultures have reached an appropriate growth phase for addition of an agent to induce protein expression, such as isopropyl thiogalactoside.

Since the advent of the modern era of preparative molecular biology in the 1980s, cultures of *Escherichia coli* have been widely used to overexpress recombinant proteins. The most commonly used methods involve the growth of liquid cultures to midlogarithmic phase followed by addition of a gene transcription-inducing agent, typically IPTG. The most commonly used method for determining when midlog phase has been reached is to monitor light scattering of cultures by determining the apparent absorption of light at 600 nm. This can be a time-consuming and onerous task, requiring repeated measurements for each culture flask until an appropriate OD_{600} is reached (usually when the OD_{600} is between 0.5 and 1.0). To see if a visual colorimetric method could be devised to determine when midlog phase is reached, *E. coli* cultures were grown in the presence of a variety of dyes and/or indicators (Table S1 of the Supporting Information) at concentrations that confer a visually obvious color to the medium. For these tests, we used BL21(DE3) *E. coli* harboring a pET21b vector encoding the 99-residue C-terminal domain of the amyloid precursor protein (C99)[1] (*1*). When pH 7.0-buffered M9 cultures were then incubated with rotary shaking at 37 °C, no change in culture color was observed for most dyes. However, in the case of 100 mL or 1 L cultures grown in the presence of 20 mg/L Methyl Red, it was observed that when the OD_{600} reached 0.67 ± 0.1 or 0.75 ± 0.1, respectively, the cultures completed a change in color from orange to a pale yellow (Figure 1 and Figure S1 of the Supporting Information). Similar results were obtained for BL21(DE3) cells harboring an empty pET21b plasmid. Tests with 20 mg/mL Methyl Red in 1 L cultures of a different strain of *E. coli* (WH1061) harboring a different recombinant plasmid [pSD0005 encoding diacylglycerol kinase (*2*)] completed the same orange-to-colorless change at an OD_{600} of 0.87 ± 0.25, although some calibration of culture conditions was required to ensure that the color change occurs when OD_{600} reaches the 0.5−1.0 range (see the Supporting Information). For each strain or vector, it is especially important to optimize the volume of the starter culture used to inoculate the fresh dye-containing M9 medium.

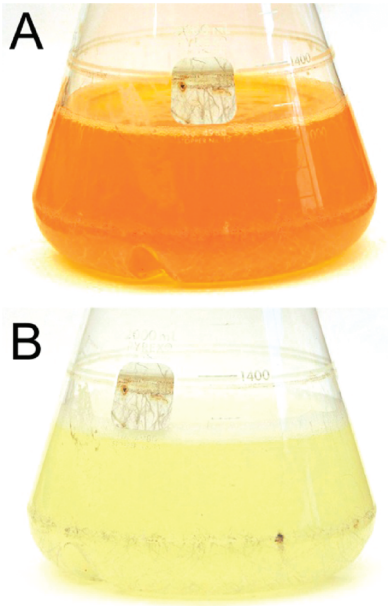

FIGURE 1: M9 minimal medium BL21(DE3) cell culture (1 L) expressing C99 in the presence of 20 mg/L Methyl Red. (A) Culture immediately after inoculation with a 25 mL overnight LB culture. (B) Culture after growth with shaking at 37 °C to an OD_{600} of 0.69.

M9 cultures (1 L) of BL21(DE3) with C100 and WH1061 with DAGK were grown in the presence or absence of Methyl Red, and protein expression was induced by IPTG when Methyl Red-containing cultures went colorless or when the OD_{600} of dye-free cultures reached 0.6−1.0. In either case, expression was allowed to proceed for several hours followed by harvesting of the cells and purification of the recombinant proteins according to published methods (*1*, *3*). For both C99 and DAGK, it was found that the expression levels were comparable to expression levels in cells grown and induced using identical methods but without Methyl Red.

Direct measurement of the kinetics of cell culture growth for BL21(DE3) with C99 grown in the presence and absence of Methyl Red revealed that cells grow a little slower and to a lower final density when cultured in the presence of Methyl Red (Figure S2 of the Supporting Information), suggesting that the presence of the dye stresses the cells. This was supported by the observation that cells would not grow in the presence of 30 mg/L dye as opposed to the usual 20 mg/L dye. We also conducted an experiment

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*To whom correspondence should be addressed. Telephone: (615) 936-3756. Fax: (615) 936-2211. E-mail: chuck.sanders@vanderbilt.edu.

[1]Abbreviations: ABA, 2-aminobenzoic acid; C99, 99-residue C-terminal domain of the human amyloid precursor protein; DAGK, *E. coli* diacylglycerol kinase; DMPD, *N*,*N*′-dimethyl-*p*-phenylenediamine; TLC, thin layer chromatography.

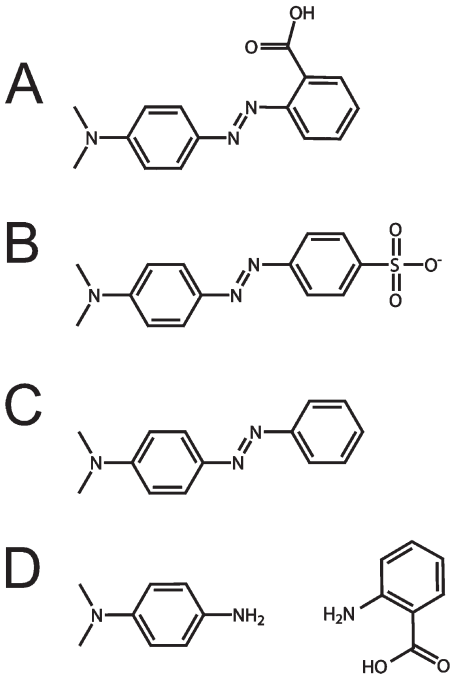

FIGURE 2: (A) Methyl Red. (B) Methyl Orange. (C) Methyl Yellow. (D) *N*,*N*′-Dimethyl-*p*-phenylenediamine (DMPD) (left) and 2-aminobenzoic acid (ABA) (right). DMPD and ABA are products of cleavage of Methyl Red by azoreductase.

in which cells were grown in the presence of 10 mg/L Methyl Red, in which case the color change was observed to occur only at a much higher OD_{600} (1.6) for WH1061 with DAGK. This suggests that the rate of dye bleaching is not directly proportional to *E. coli* biomass. Rather, it appears that the process responsible for bleaching the dye is induced in response to dye-induced stress and at levels that are proportional to the concentration of the dye.

Additional tests indicated that this method can, following reoptimization of conditions, be applied to M9 media at different pH values (e.g., pH 6.25 and 7.7), strain−protein combinations [e.g., RosettaBlue/pAH13 expressing human peripheral myelin protein 22 (*4*)], and culture temperatures (e.g., 20 °C). We found that this method can also be employed using Luria Broth medium.

It has previously been shown that Methyl Red can be cleaved and decolorized by a stress-induced azoreductase found in *E. coli* and other microorganisms (*5*−*7*). This flavoenzyme converts Methyl Red into *N*,*N*′-dimethyl-*p*-phenylenediamine (DMPD) and 2-aminobenzoic acid (ABA) (see Figure 2). Using thin layer chromatography (TLC), we confirmed that the disappearance of Methyl Red in *E. coli* cultures was accompanied by the appearance of compounds that exhibit the same R_f on two-dimensional thin layer chromatography (Figure 3) and the same response to ultraviolet light as ABA and DMPC standards (ABA fluoresces, and DMPD initially absorbs but then turns brown). We found that *E. coli* did not bleach Methyl Orange or Methyl Yellow (Figure 2), at least not at a rate sufficient to generate a color change during the time course of growing a culture to the postlog phase.

To conclude, it appears that Methyl Red can be used as a colorimetric indicator to provide a visual cue that *E. coli* cultures harboring a recombinant expression plasmid have reached an optimal phase of growth for induction of protein expression. For each strain and recombinant protein, significant calibration is required to determine exact conditions for obtaining useful and

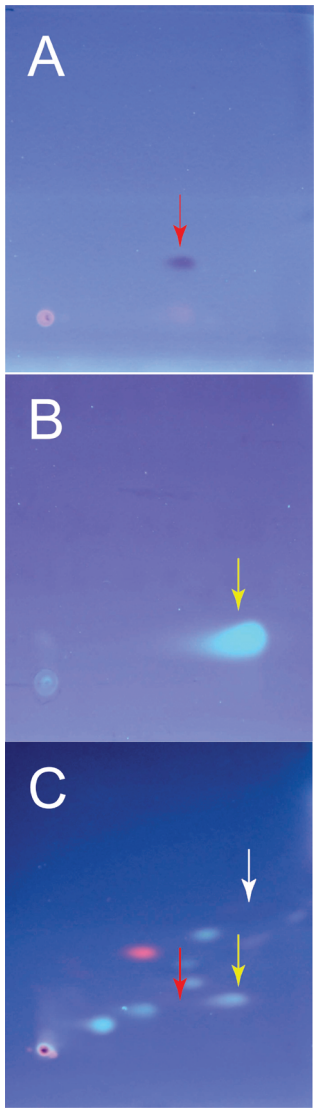

FIGURE 3: Two-dimensional thin layer chromatograms under 302 nm ultraviolet light. Glass-backed silica gel plates were eluted in two directions: first horizontally with ethyl acetate and then vertically with 96% chloroform and 4% methanol. The origin in each case is the lower left spot. (A) *N*,*N*′-Dimethyl-*p*-phenylenediamine (DMPD) standard. $R_{f,1} = 0.45$, and $R_{f,2} = 0.2$. (B) 2-Aminobenzoic acid (ABA) standard. $R_{f,1} = 0.68$, and $R_{f,2} = 0.12$. (C) Chloroform extract of the supernatant from the M9 minimal medium *E. coli* culture after color change. Red and yellow arrows indicate locations predicted for spots from DMPD and ABA, respectively. The white arrow indicates where Methyl Red would appear on the plate if it were present. A spot in C corresponding to ABA is evident. However, the DMPD spot is difficult to detect under UV light. Nonetheless, when plates A and C were allowed to sit at room temperature, it was seen (not shown) that the DMPD spot of plate A turned brown and that a brown spot also appeared on plate C at the expected location for DMPD (red arrow).

reproducible results. Given that reoptimization of the method is required for any new strain, recombinant plasmid−protein, and/or culture conditions, we suggest that this approach will be most useful for investigators who are routinely and repetitively expressing the same protein, as is often the case when a protein is being subjected to long-term biochemical or structural biological studies, or to biotechnological exploitation.

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SUPPORTING INFORMATION AVAILABLE

Full methodological details, Table S1, Figure S1, and Figure S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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