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ORIGINAL PAPER

D. G. Kang · Y. K. Kim · H. J. Cha

Comparison of green fluorescent protein expression in two industrial *Escherichia coli* strains, BL21 and W3110, under co-expression of bacterial hemoglobin

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Abstract *Vitreoscilla* hemoglobin (VHb) has been successfully used to enhance production of foreign proteins in several microorganisms including *Escherichia coli*. We compared the expression of an oxygen-dependent foreign protein, green fluorescent protein (GFP) under co-expression of VHb in two typical industrial *E. coli* strains, BL21 (a B derivative) and W3110 (a K12 derivative), which have different metabolic properties. We employed the *nar* oxygen-dependent promoter for self-tuning regulation of VHb expression due to the natural transition of dissolved oxygen (DO) level during culture. We observed several interesting and differing behaviors in cultures of the two strains. VHb co-expression showed a positive influence on expression, and even on solubility, of GFP in both strains; while strain BL21 had the higher GFP expression level, W3110 showed higher solubility of expressed GFP. GFP expression in strain BL21 was very largely affected by variation of aeration environments, but W3110 was not significantly impacted. We surmised that this arose from different oxygen utilization abilities and indeed the two strains showed different patterns of oxygen uptake rate. Interestingly, the VHb co-expressing W3110 strain exhibited a peculiar increasing pattern of GFP expression during the late culture period even under low aeration conditions and this enhancement was more obvious in large-scale cultures. Therefore, this strain could be successfully employed in practical large-scale production cultures where DO levels tend to be limited.

D.G. Kang · Y.K. Kim · H.J. Cha (✉)
Department of Chemical Engineering and Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790–784, Korea
e-mail: hjcha@postech.ac.kr
Tel.: +82-54-2792280, Fax: +82-54-2795528

Introduction

Many metabolic engineering approaches have attempted to enhance growth and recombinant protein production in *Escherichia coli*. Such approaches should consider the special growth conditions present at high cell density. It can be expected that oxygen will probably be limited under these conditions. Co-expression of the gene encoding bacterial hemoglobin VHb from *Vitreoscilla* sp. has been used to enhance foreign protein formation under microaerobic conditions (Dikshit and Webster 1988; Khosla and Bailey 1988; Dikshit et al. 1990; Khosravi et al. 1990; Chen et al. 1994; Pendse and Bailey 1994; Kallio and Bailey 1996; Tari et al. 1998; Enayati et al. 1999).

In the present work, we compared the expression patterns of green fluorescent protein (GFP), which is dependent on oxygen molecules for active formation of its chromophore structure (Heim et al. 1994), in two *E. coli* strains, BL21 (a derivative of *E. coli* B) and W3110 (a derivative of *E. coli* K12), that are widely used as industrial hosts for production of foreign protein, in VHb co-expressing environments. We mainly performed cultures in shake flasks to be able to easily vary many environmental conditions for comparison of the two strains. *E. coli* strains derived from K12 are known to have different metabolic properties from those derived from *E. coli* B (Shiloach et al. 1996; van de Walle and Shiloach 1998). Unique attributes of GFP as a reporter protein are: (1) it requires no co-factors or staining for fluorescence (Chalfie et al. 1994), (2) the fluorescence is readily visible from outside the cells, (3) it does not represent a large metabolic burden to the host, and (4) in vivo quantification is possible by simply measuring fluorescence intensity (Cha et al. 1999, 2000).

Unique to our system was an alternative promoter for *vhb* transcription, namely another oxygen-dependent inducible promoter, *nar*, which is maximally induced under microaerobic conditions in the presence of nitrate ion. This has many advantages because of its high expression level and the lack of a need for chemical induction (Li and DeMoss 1988; Lee et al. 1996; Han et al.

1998). Also, we expected self-tuning regulation of VHb expression under the *nar* promoter due to the natural transition of dissolved oxygen (DO) levels throughout the duration of the culture. It was reported previously that the *vhb* promoter is initially induced as DO levels drop to 40% of air saturation (Hughes et al. 1989; Khosla and Bailey 1989) and therefore its regulation might not be sufficiently tight to enable self-tuning at low DO levels. On the other hand, introducing the *nar* promoter enables more tight regulation (Lee et al. 1996) as it was still sufficiently repressed at DO levels below 20% (Han et al. 1998), and maximally induced when DO was 1–2% of air saturation (Lee et al. 1996).

Materials and methods

Strains and media

E. coli TOP10 (*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*) (Invitrogen, USA) was used for constructing recombinant plasmids. *E. coli* BL21 (*F′ ompT hsdSB* (r_B^- mB^-) *gal dcm*) (Novagen, USA) and W3110 (*F⁻ mcrA mcrB IN(rrnD-rrnE)1 λ⁻*) (ATCC 27325) were used for expressing the proteins. Luria-Bertani (LB) rich medium [5 g/l yeast extract (Sigma, USA), 10 g/l, Tryptone (Sigma), and 10 g/l NaCl] and M9 minimal medium (12.8 g/l $Na_2HPO_4{\cdot}7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 3 mg/l $CaCl_2$, 1 mM $MgSO_4$) were used for plasmid construction and cell culture, respectively. $NaNO_3$ (10 g/l) was added to the medium for induction of the *nar* promoter and 0.1 mM $FeSO_4$ was added as a metal cofactor for VHb protein. Plasmid-containing cells were grown in medium supplemented with 50 μg/ml ampicillin (Sigma).

Construction of expression plasmids

Plasmid pTG was constructed as a GFP control vector by inserting the polymerase chain reaction (PCR)-amplified (DNA Thermal Cycler; Eppendorf, USA) gfp_{uv} fragment from the pGFPuv plasmid (Clontech, USA) into the *Nhe*I-*Hin*dIII sites of pTrcHis A (Invitrogen), which contains a histidine affinity ligand in the appropriate orientation (Fig. 1). The *Bgl*II-*Acc*651-digested PCR-amplified *nar* promoter from *E. coli* K-12 (ATCC 29425) genomic DNA was subcloned in the pTG plasmid. This plasmid was denoted pTGN. The *Acc*651-*Eco*RI-digested PCR-amplified *vhb* gene from genomic DNA of *Vitreoscilla stercoraria* (ATCC 15218), was subcloned in the plasmid pTGN. The *vhb* gene sequence was obtained from GenBank (M27061 or M30794; http://www3.ncbi.nlm.nih.gov/Entrez). This vector was named pTGNV (Fig. 1). All PCR primers used in this study are listed in Table 1.

Cell culture and analytical methods

The plasmids pTG and pTGNV were introduced into *E. coli* BL21 and W3110. All seed cultures were performed in M9 medium containing 0.5% glucose and 50 μg/ml ampicillin at 37°C and 250 rpm. Culture experiments for GFP expression were performed in 250 ml or 1 l flasks. Samples were taken at intervals from each culture and the optical density at 600 nm (OD_{600}) was measured on a UV-vis spectrophotometer (Shimadzu, Japan). At the mid-growth phase, the cultures were induced by the addition of 1 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) to express GFP. From this induction point, fluorescence intensity of GFPuv was also measured using a fluorescence spectrophotometer (Shimadzu).

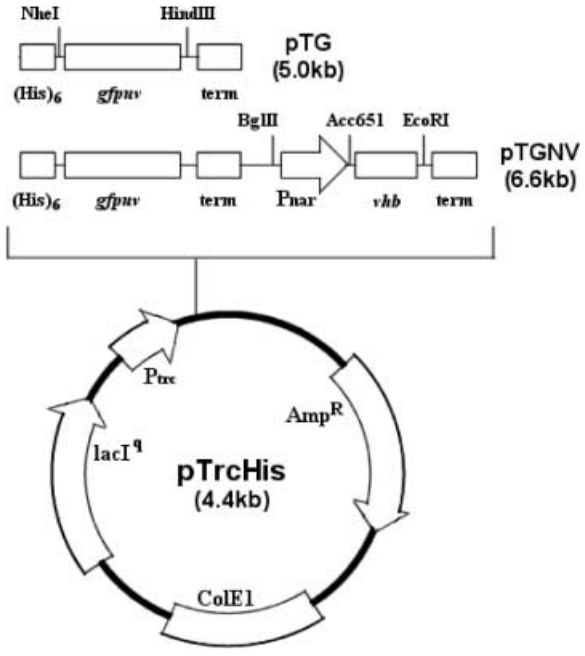

Fig. 1 Gene maps of recombinant plasmids pTG and pTGNV. P_{trc} *trc* Promoter, Amp^R ampicillin resistance gene, $lacI^q$ overexpressed *lac* repressor, *ColE1* replication origin, $(His)_6$ hexahistidine affinity ligand, P_{nar} *nar* promoter, *term* transcriptional termination sequence

Table 1 Primers used in this work for the construction of the plasmid pTG and pTGNV

Primer	Sequence (5′→3′)
gfp_{uv}-1	cggctagcatgagtaaaggagaagaac
gfp_{uv}-2	cgaagctttcattatttgtagagctcatc
nar-1	ccgccgagatctttgattttctatatcgcc
nar-2	gcgcggtaccctcctgtgggagcctgtcgg
vhb-1	gattggtaccgatgttagaccagcaaacc
vhb-2	cggaattcttcacccgcttgagc

Measurement of oxygen uptake rate

The cultures for measurement of oxygen uptake rate (OUR) were performed in a 5 l cylindrical glass bioreactor (BioTron, Korea) with a working volume of 2 l. Growth medium was M9 minimal medium with 0.5% glucose. Fermentations were run at 37°C, an agitation rate of 250 rpm, and an aeration rate of 2 vvm. During fermentations, we calculated values of OUR at intervals until cell density reached mid-exponential phase (OD_{600} = ~1.5) by measuring the slope (%DO/min) of the linear decrease of DO shortly after aeration was stopped by closing the air-valve (Blanch and Clark 1997). DO was measured using an O_2 sensor (Mettler-Toledo Process Analytical, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect GFP and VHb hemoglobin polypeptides. Samples were mixed with sample buffer [0.06 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol (Sigma, USA), and 0.01% bromophenol blue (Sigma)], incubated at 100°C for 5 min, centrifuged briefly, and loaded onto a 12%

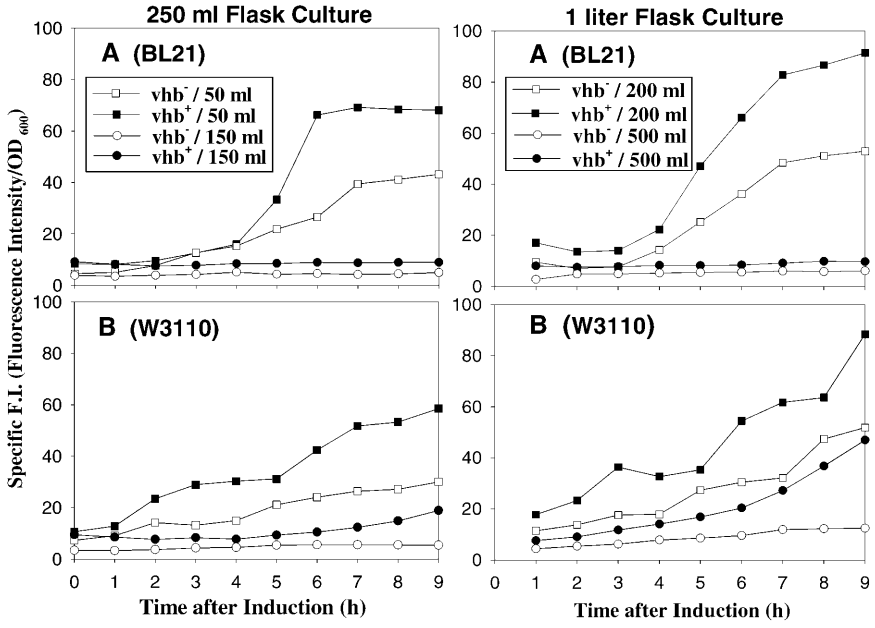

Fig. 2 Time course of specific fluorescence intensity in 250 ml flask cultures (**A**, **B**) with 50 ml (*squares*) or 150 ml (*circles*) working volume and 1,000 ml flask cultures (**C**, **D**) with 200 ml (*squares*) or 500 ml (*circles*) working volume for BL21 (**A**, **C**) and W3110 (**B**, **D**) under VHb non-expressing (*open symbols*) or VHb-expressing (*closed symbols*) conditions

slab gel. After electrophoresis, the gel was stained with Coomassie blue (Sigma). The stained gel was scanned, and the digitized image was stored and analyzed using Gel-Pro Analyzer software (Media Cybernetics, USA).

Results

Effect of surface aeration under VHb co-expression

To evaluate the effect of surface aeration on GFP expression under co-expression of VHb, we performed 250 ml flask cultures with 50 ml and 150 ml working volumes using M9 minimal medium with 0.5% (w/v) glucose as shown in Fig. 2A, B. The presence of VHb had a significant impact on GFP fluorescence in both strains. When we compared the specific fluorescence intensities [GFP fluorescence intensity divided by cell density (OD_{600})], we found both VHb co-expressing (*vhb*$^+$) *E. coli* strains had about twice the fluorescence of the VHb non-expressing (*vhb*$^-$) strains in all cultures. Interestingly, the 50 ml cultures with high surface aeration had much higher fluorescence intensities than the 150 ml cultures with low surface aeration where GFP expression was very low during the whole culture period regardless of VHb co-expression.

In the case of BL21 in 50 ml culture (Fig. 2A), the specific fluorescence intensity sharply increased when approaching the stationary growth phase (from about 4 h after induction). However, in the case of W3110 in 50 ml culture (Fig. 2B), the specific fluorescence intensity gradually increased from initial induction time. We performed large-scale 1,000 ml flask cultures with 200 ml working volumes to confirm these different patterns (Fig. 2C, D). When compared to the small-scale cultures using 250 ml flasks, we obtained almost the same profiles except that GFP yield was enhanced in both strains. From the large-scale cultures using 1,000 ml flasks, it was clear that each strain exhibits its own particular pattern of foreign protein expression regardless of VHb co-expression (Fig. 2C, D); BL21 showed a sudden increasing pattern from early stationary growth phase, but W3110 exhibited a gradual increasing pattern from the induction point. We also confirmed that BL21 cultures with low surface aeration had minimal expression of foreign GFP and that this was independent of VHb expression. However, the *vhb*$^+$ W3110 showed somewhat different patterns under the low surface aeration environment; increase of GFP expression was shown from the late culture period (Fig. 2B) and this peculiar pattern was clear in large-scale culture with 500 ml working volume (Fig. 2D).

To elucidate the possible reasons for these obviously different patterns of foreign GFP expression in the two *E. coli* strains, we measured the OUR for each *vhb*$^+$ strain in bioreactor experiments and investigated oxygen utilization patterns. As shown in Fig. 3, the specific OUR of *vhb*$^+$ BL21 strain was higher by about 16–38% than that of *vhb*$^+$ W3110. Due to lower oxygen uptake during the cell growth, the W3110 culture had a higher DO level. Therefore, BL21 and W3110 strains have different oxygen utilization patterns.

In vivo visualization of GFP and VHb

As shown in Fig. 4A, green fluorescence was easily detected from the cell culture without cell disruption under illumination by UV due to special features of GFP. From in vivo visualization of GFP fluorescence, both *vhb*$^+$ strains had brighter intensities than *vhb*$^-$ strains. Also, BL21 had a brighter intensity than W3110. These observations agreed with the culture profiles shown in Fig. 2. Because there is a linear correlation between fluorescence and amount (Cha et al. 2000), we could perform a

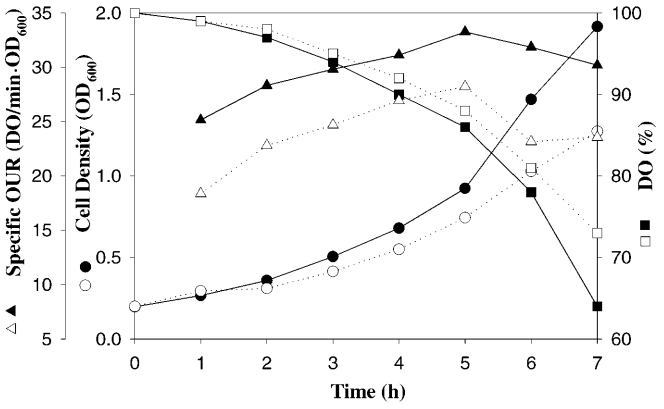

Fig. 3 Oxygen uptake rate (OUR) (*triangles*), dissolved oxygen (DO) (*squares*), and cell density (*circles*) in *vhb*+ BL21 (*closed symbols*) and *vhb*+ W3110 (*open symbols*) cultures using a bioreactor (2 l working volume)

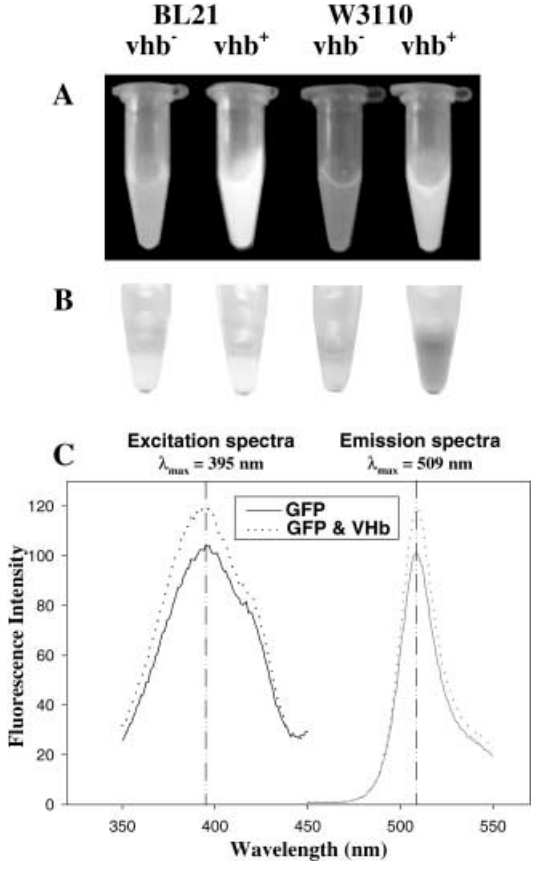

Fig. 4 **A** Green fluorescence-expressing soluble supernatant samples under UV light and **B** the same samples under normal light. **C** Comparison of excitation and emission spectra between GFP alone and GFP with VHb. The analyses were performed using culture samples at 9 h after induction in 250 ml flask cultures with a 50 ml working volume

facile and non-invasive comparison of VHb effect according to strain and culture conditions by simple in vivo visualization of green fluorescence.

The *vhb*+ W3110 strains exhibited a red color under illumination by normal light in all culture conditions

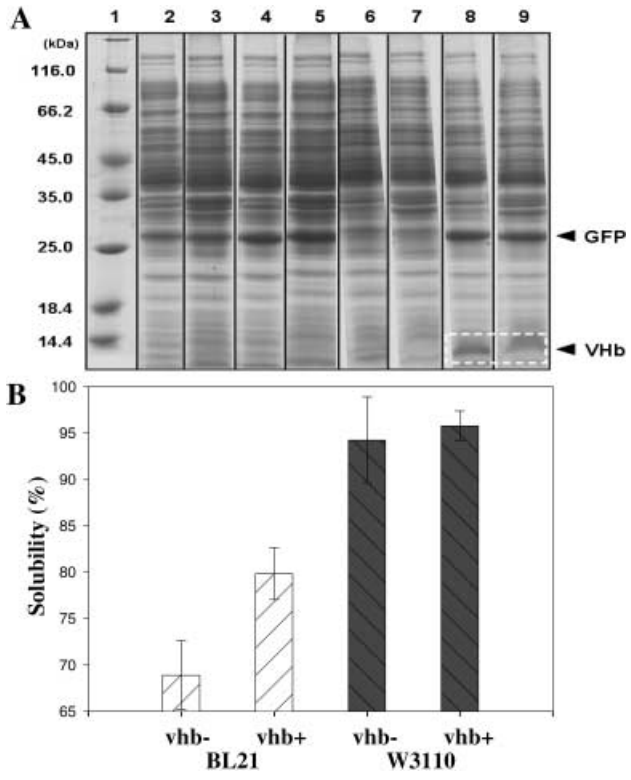

Fig. 5 **A** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of cell lysis supernatants and whole cell samples. Lanes: *1* Protein molecular weight marker, *2* soluble *vhb*– BL21, *3* total *vhb*– BL21, *4* soluble *vhb*+ BL21, *5* total *vhb*+ BL21, *6* soluble *vhb*– W3110, *7* total *vhb*– W3110, *8* soluble *vhb*+ W3110, *9* total *vhb*+ W3110. **B** Solubility of GFP in BL21 and W3110 cultures under VHb non-expressing and co-expressing conditions. Solubility was defined as the fraction of soluble protein per total protein. The analyses were performed using culture samples 9 h after induction in 250 ml flask cultures with a 50 ml working volume. Duplicate analyses were performed for each sample; average values of the duplicates were reported

(Fig. 4B); the red color is known to come from the 'heme' structure of VHb. This red color did not interfere with the fluorescence excitation and emission spectra of GFP (Fig. 4C). However, in the case of *vhb*+ BL21 strain, the red color was barely detectable. Actually, SDS-PAGE analysis revealed that W3110 expressed much more VHb than BL21 as shown in Fig. 5A (see dotted box in lanes 8 and 9).

Effect of VHb on GFP solubility

We performed SDS-PAGE analysis of cell lysis supernatants and whole cell samples to investigate solubility of expressed GFP in the two strains under VHb co-expression (Fig. 5A). Solubility was calculated as the quantity of soluble GFP in cell lysis supernatant divided by the total quantity of GFP in the whole cell sample. Fig. 5B showed that strain *vhb*– W3110 had much higher (about 50%) solubility than the *vhb*– BL21. Therefore, even though the *vhb*– BL21 had a higher expression level, re-

covery yield of GFP from the *vhb*– W3110 strain could be better due to the much higher solubility. When VHb was co-expressed, the solubility in BL21 increased about 20%. This was a positive effect of VHb, but was somewhat different than that previously reported about inclusion body formation of a foreign protein by VHb overexpression (Rinas and Bailey 1993). Note that we employed the *nar* promoter, which is not able to direct overexpression of a foreign protein. In the case of W3110 strain, VHb co-expression did not lead to a large difference (within error range) in the GFP solubility.

Discussion

VHb co-expression had a significant impact on foreign GFP expression in both BL21 and W3110 strains. We suspect that this resulted from an increase of oxygen uptake due to active VHb. This might have been coupled with a more efficient transfer of oxygen to GFP resulting in increased specific fluorescence. However, these enhancements of GFP expression by VHb co-expression could be clearly observed only in high surface aeration cultures even though there was also some increase in minimal expression in low surface aeration cultures. High aeration is generally regarded as very important for foreign protein expression. Therefore, from these results, we surmise that the initial aeration environment is critical for foreign protein expression, especially for GFP, in both strains; *E. coli* cells seem to use mainly cellular oxygen just for growth and maintenance under low aeration conditions, and this results in minimal GFP expression during the entire culture regardless of VHb co-expression. Interestingly, we found that *vhb*+ W3110 had a peculiar increasing pattern of GFP expression during the late culture period even under low surface aeration conditions and this enhancement was more obvious in large-scale cultures. Therefore, the *vhb*+ W3110 strain could be successfully employed in practical large-scale production cultures such as high cell density fed-batch where DO levels are easily limited because this strain can express foreign proteins even in low aeration environments.

In flask cultures with low and high surface aerations, strain BL21 showed very large differences in absolute levels as well as in transient changes of GFP expression. However, W3110 strains were generally more consistent with and without *vhb*, and at various culture volumes. These particular expression patterns were strain-dependent but not related to VHb co-expression, as clearly revealed in large-scale flask cultures; BL21 showed a sudden increasing pattern from early stationary growth phase, but W3110 exhibited a gradual increasing pattern from the induction point. These results demonstrated that BL21 was significantly affected by changes in aeration of the culture medium. From measuring oxygen uptake rate of two *vhb*+ strains, we found that BL21 strain needs more oxygen than W3110 for its cellular metabolism. We suspected that these different oxygen utilization patterns caused some notably different behavior in the cultures of the two *E. coli* strains. Also, from the results of VHb visualization and SDS-PAGE analysis, we surmised that W3110 has lower cellular oxygen levels than BL21 even under the same culture conditions, because we have introduced the *nar* promoter that can be expressed only in a low cellular oxygen environment. This observation agreed with the oxygen utilization pattern of W3110 compared to that of BL21. From these results, we could conclude that W3110 needs a lower level of oxygen for its growth and metabolism and thus it is not greatly affected by variation of aeration environments and the foreign protein could be gradually expressed from the induction point even in a low aeration environment. Because we performed the preliminary comparison cultures in shake flasks to easily change cultural environments, it was not possible to measure DO levels for a detailed investigation on the action of the oxygen-dependent *nar* promoter to express VHb in the two strains. These investigations are underway by performing cultures in bioreactors with DO monitoring.

Even though W3110 and BL21 had very different expression levels of VHb, from observation of the red color and SDS-PAGE analysis, both *vhb*+ strains showed a 2-fold enhancement of specific GFP fluorescence intensity compared to *vhb*– strains. The cellular mechanisms of VHb are not yet known exactly; however, from these results, we surmised that the effect of VHb on cellular metabolism was not proportional to the amount of VHb.

To summarize, we compared foreign GFP expression in two typical *E. coli* production strains, BL21 and W3110, under co-expression of VHb using the oxygen-dependent *nar* promoter. We observed some significantly different features in the two strain cultures with VHb co-expression. From the point of view of foreign protein expression levels, BL21 could be a better strain in which to employ the VHb co-expression technique. However, regarding the protein production process, W3110 might be a better strain for the VHb strategy because it requires lower levels of oxygen molecules for its cellular metabolism, including foreign protein expression; this is a significant advantage for practical large-scale production of foreign proteins.

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