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Effects of the *Vitreoscilla* Hemoglobin Gene on the Expression of the Ferritin Gene in *Escherichia coli*

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To investigate the effects of the *Vitreoscilla* hemoglobin (*VHb*) gene on the production of a heterologous protein, a comparative expression system for VHb and ferritin was constructed. First, the VHb gene was inserted into the downstream and upstream regions of the ferritin gene to construct pHF2 and pHF3, respectively. Next, the two plasmids pACHB1 and pVUTFH10, having the *VHb* gene and the ferritin gene respectively, were constructed in order to express the two genes in different plasmids by using a coplasmid expression system. It was observed that the cell growth was improved in all strains containing the *VHb* gene. Furthermore, in our coplasmid expression system, the presence of the *VHb* gene increased production of the ferritin by 1.8 times, as much as that in a strain not having the *VHb* gene.

Keywords: Bacteria, Expression, Ferritin, Hemoglobin, *Vitreoscilla.*

Introduction

Vitreoscilla, a filamentous bacterium in the Beggiatoa family, synthesizes a soluble hemoglobin (VHb) protein consisting of two identical subunits, each with a molecular mass of ca. 16 kDa (Wakabayashi *et al.*, 1986). The functional expression of the *VHb* gene is known to improve the aerobic growth of recombinant *E. coli* in oxygen-poor conditions, but the precise molecular processes are still unclear. One possible reason is that the hemoglobin enables the organism to survive in oxygen-limited environments by functioning as an oxygen storage-trap and by facilitating oxygen diffusion (Wakabayashi *et al.*, 1986). In general, bacterial cells containing recombinant plasmids require more oxygen during their growth than the plasmid-free cells. This disadvantage could be solved by the overproduction of VHb within these cells (Khosla and Bailey, 1988; Khosravi *et al.*, 1990).

The natural promoter of the *VHb* gene was known to be highly active in *E. coli* under oxygen-limited conditions (Khosla and Bailey, 1988). This might be due to the molecular recognition of an oxygen regulated promoter element such as the global transcriptional activator of fumarate nitrate reduction (FNR) protein (Tsai *et al.*, 1995). The FNR protein is known to control the expression of various genes and to positively regulate the promoter activity of the *VHb* gene in *E. coli* (Green *et al.*, 1991; Tsai *et al.*, 1995; Ahn and Choe, 1996).

In a previous study, the *VHb* gene has been reported to increase the production of foreign recombinant proteins such as the bacterial α-amylase in *E. coli* (Khosravi *et al.*, 1990). Also, the expression of the *VHb* gene has been proven to be superior to horse myoglobin or yeast flavohemoglobin for enhancing *E. coli* growth (Kallio *et al.*, 1996). The expression of the tadpole ferritin (*TFH*) gene had been succeeded in *E. coli* (Kim and Kim, 1994; Lee *et al.*, 1996).

In this study, we constructed a comparative expression system for VHb and ferritin to further define the molecular processes of the *VHb* gene for the production of a heterologous protein. The effects of the transcriptional activator of the FNR protein on the ferritin gene expression in *E. coli* are also discussed.

Materials and Methods

Strains and plasmids *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) was used as a host strain for plasmids. Plasmids pUC8, pBluscript IIKS (−) (Short *et al.*, 1988) and pACYC184 (Rose, 1988) were used as cloning vectors. Plasmid pACYC184 has a p15A replication origin and presents a low copy number. Plasmid pVUTFH10 is an expression vector for tadpole ferritin upon

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induction with IPTG (Kim and Kim, 1994). Plasmid pUC8:15 has the *VHb* gene and is a gift from Prof. D. A. Webster (Illinois Institute of Technology, USA). The *E. coli* JM109 cells, transformed with the plasmids pVUTFH10, pHF2, pHF3, and pACHB1/pVUTFH10, are denoted as strains TFH-10, HF2, HF3, and ACHB1/TFH, respectively.

Construction of plasmids In order to define the molecular mechanisms of the *VHb* gene for the production of a heterologous protein, a comparative expression system for VHb and ferritin was constructed as shown in Fig. 1. The 2.2 kb *Hin*dIII–*Sal*I fragment of the *VHb* gene in pUC8:15 was treated with the Klenow fragment for blunt end ligation and then inserted into the Klenow fragment-treated pVUTFH10 after digestion with *Nde*I to construct the plasmid pHF2. In pHF2, the 2.2 kb fragment containing the *VHb* gene was separated by 210 bp from the ferritin gene. The construction of the plasmid pHF3 was initiated by the insertion of the above Klenow fragment-treated *VHb* gene into the Klenow fragment-treated pBluscript IIKS(−) after digestion with *Xho*I. To insert the ferritin gene into this resultant plasmid, the 0.95kb *Bam*HI fragment containing the ferritin gene in pVUTFH10 was inserted into the *Bam*HI site of pBluscript IIKS(−). In this plasmid, the 0.95 kb fragment containing the ferritin gene was separated by 7 bp from the *VHb* gene. Plasmid pACHB1 was constructed by the insertion of the 2.2 kb *Hin*dIII–*Sal*I fragment of the *VHb* gene into the 3.2 kb *Hin*dIII–*Sal*I backbone fragment of pACYC184. The construction of the plasmid pTFH-I was initiated by replacing the *Bam*HI–*Sal*I fragment of pUC19 with the corresponding *Bam*HI–*Sal*I fragment containing the *lac* I^q gene of pSS6 (Park *et al.*, 1998). The 0.95 kb *Bam*HI fragment of the ferritin gene was then inserted into the *Bam*HI site of pUC19 containing the *lac* I^q gene.

Expression of *VHb* and ferritin genes *E. coli* JM109 harboring the plasmids pVUTFH10, pHF2, pHF3, pACHB1/pVUTFH10, and pACHB1/pTFH-I were grown at 37°C in LB supplemented with ampicillin (50 μg/ml). Expression from the *tac* promoter was induced, when necessary, by the addition of IPTG at the point of A_{600} of 0.5. The strains were incubated for an additional 6 h at 37°C. The natural promoter of the *VHb* gene was known to be highly active in *E. coli* under oxygen-limited conditions, and the *VHb* gene was strongly expressed under our experimental conditions. Cellular proteins were analyzed by 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). A Northern blot analysis was performed to investigate the expression of the ferritin gene at the mRNA level, according to the method of Sambrook *et al.* (1989).

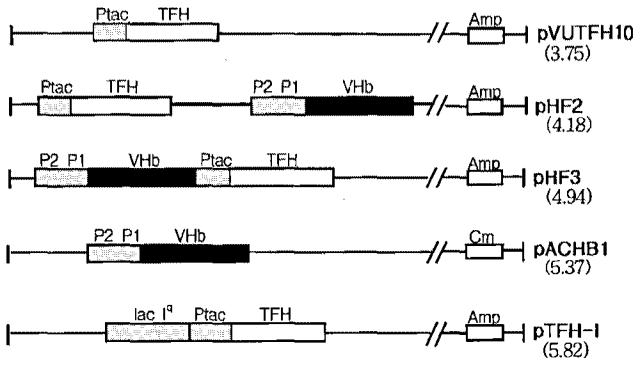

Fig. 1. Physical map of the plasmids. The plasmid pVUTFH10 is the ferritin gene expression vector. VHb, *Vitreoscilla* hemoglobin gene; TFH, Tadpole ferritin gene.

Preparation of antiserum and Western blotting Antiserum to the *Vitreoscilla* hemoglobin was raised in New Zealand white rabbits by a series of subcutaneous injections of purified *Vitreoscilla* hemoglobin (500 μg) each suspended in Freund's adjuvant (50%). The IgG fraction of the antiserum was isolated from the resultant rabbit serum by applying it to the Protein A column (Bio-Rad). The antiserum to tadpole ferritin was prepared as described previously (Chang *et al.*, 1995). The immunological detection of proteins on nitrocellulose was performed using the method described by Burnette (1981).

Measurement of VHb and ferritin production The production of VHb and ferritin was measured by using a densitometer (Molecular Dynamics PD-120, USA). The total amount of ferritin produced by each strain was indicated by the peaks, and the total amount of ferritin produced is represented by the sum of the areas under each peak, adding up to a total of 100% peak area. In addition, the VHb protein produced in *E. coli* has been previously identified by the CO-difference spectra (Dikshit and Webster, 1988).

Results and Discussion

To investigate the effects of the *VHb* gene on the production of ferritin, a comparative expression system for VHb and ferritin was constructed. Physical maps of the plasmids constructed are shown in Fig 1. The plasmid pVUTFH10 is a vector expressing tadpole ferritin upon induction with IPTG. For pHF2, the *VHb* gene was inserted into the downstream region of the ferritin gene of pVUTFH10. The plasmid pBluscript IIKS(−) was used to construct pHF3. The *VHb* gene was inserted into the upstream region of the ferritin gene. For a coplasmid system, pACHB1 was constructed by the insertion of the *VHb* gene into the plasmid pACYC184. The plasmid pTFH-I was constructed by the insertion of the *lac* I^q gene into the upstream region of the ferritin gene in pVUTFH10. Recombinant colonies were selected on LB agar plates containing ampicillin. On the other hand, the two recombinant plasmids pACHB1 and pVUTFH10 were cotransformed into the *E. coli* JM109. These transformed cells containing both recombinant plasmids were selected by two antibiotics, ampicillin and chloramphenicol.

The growth of *E. coli* JM109 containing the plasmids was examined (Fig. 2). The growth of each strain was similar until when the midlog phase was reached. At this time, the strains of HF2, HF3, and ACHB1/TFH begin to outgrow TFH-10. This shows that cell growth is improved in all strains containing the *VHb* gene. This result is also consistent with the previous proposal that the expression of the *VHb* gene in *E. coli* increases the efficiency of ATP

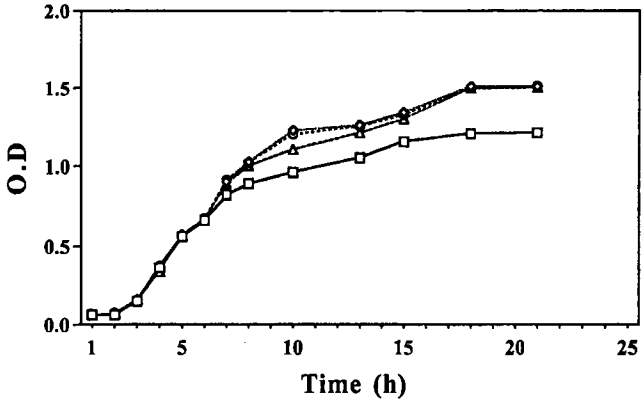

Fig. 2. The growth curves of the strains TFH-10, HF2, HF3, and ACHB1/TFH. Strains carrying each plasmids were grown in LB medium containing ampicillin (50 μg/ml), and the aliquots of each culture were taken at time intervals as indicated for the determination of the absorbance at 600 nm. □, TFH-10; ○, HF2; ◇, HF3; △, ACHB1/TFH.

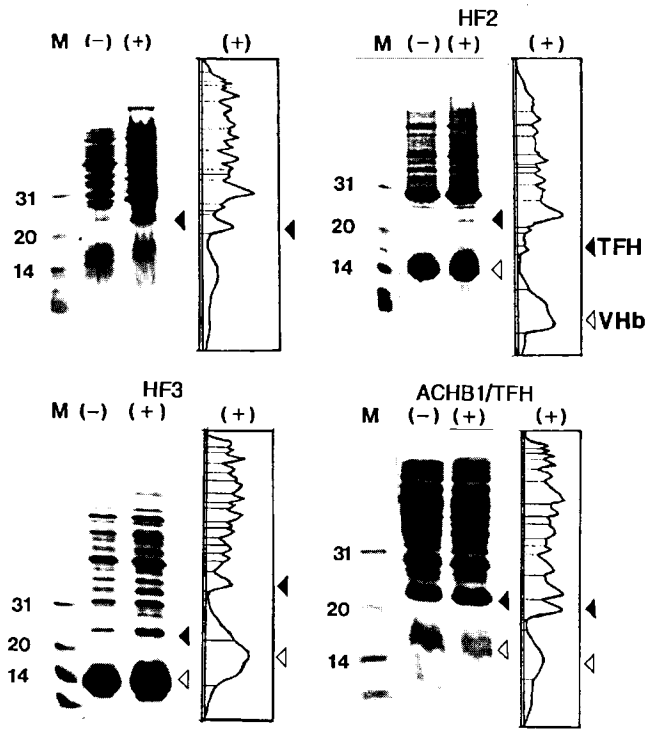

Fig. 3. SDS-polyacrylamide gel electrophoresis of VHb and ferritin (TFH) produced under different expression systems. *E. coli* JM109 carrying each of the plasmids was grown and induced with 0.1 mM IPTG. Equal amounts of the aliquots of each culture were harvested and the fractions of the lysates were applied to 12% SDS-PAGE. M, Molecular weight size marker; (−), without IPTG; (+), with IPTG. Each densitogram corresponds to the gel band shown on the left.

production in oxygen-limited growth environments (Khosla and Bailey, 1988; Khosla *et al.*, 1990; Kallio *et al.*, 1994).

The synthesis of proteins from *E. coli* JM109 cells harboring both the *VHb* and ferritin genes was examined upon induction with IPTG. Cell lysates from different expression systems were analyzed on 12% SDS-PAGE (Fig. 3). The results were also analyzed quantitatively by using a densitometer. Through these analytical processes, strains containing the *VHb* gene were shown to have an increased amount of total protein. Our result is consistent with the report by Khosla *et al.* (1990) where the cells producing VHb synthesized more proteins under oxygen-limited growth conditions.

Ferritin production was variable depending on the expression systems as shown in Fig. 3. In HF2 and HF3, VHb was overproduced as compared to ferritin. One possible explanation for the overproduction of VHb may be due to the oxygen limitations present within the cultures. In recombinant plasmids containing the *VHb* gene, the FNR binding site is located in the upstream region of the *VHb* gene. In HF3, where the *VHb* gene is located in the upstream region of the ferritin gene, VHb was produced in a similar fashion as in HF2. However, ferritin production was increased by 4 times to that of HF2. It may be caused by the high transcriptional activity of the *VHb* gene under the oxygen-limited conditions since the ferritin gene is only 4 bp away from the *VHb* gene. In ACHB1/TFH, the VHb production was decreased and ferritin production was largely improved. The expression of the two protein genes in the ACHB1/TFH was analyzed by the Northern blot analysis, revealing that the ferritin mRNA levels increased, but not the VHb mRNA levels (data not shown). Therefore, it is thought that the expression of the *VHb* gene, being harbored in a multicopy plasmid, might have been influenced at the transcriptional level. In the strain ACHB1/TFH, ferritin was overproduced even in the absence of IPTG. Also, it was shown that upon the insertion of the *lac* I^q gene into the plasmid pVUTFH10, both VHb and ferritin were markedly reduced as they are irrelevant to IPTG induction (Fig. 4). The repressor protein produced from the *lac* I^q gene affected the ferritin gene as well as the *VHb* gene.

The expression pattern of the *VHb* and ferritin genes was further analyzed in relation to cell growth. The results from the SDS-PAGE analysis (Fig. 5A) were correlated with the results from the Western blot analysis (Fig. 5B). During the experimental period, the expression pattern of the ferritin gene was similar to that of the *VHb* gene as shown in Fig. 5. This result shows that the expression of the ferritin gene without IPTG induction depends on the expression of the *VHb* gene. Both the natural *VHb* gene promoter and the *tac* promoter of ferritin gene appear to be closely related to each other. The nucleotide sequences of the promoter regions are compared in Table 1. The Pribnow box of the promoter region (TATAA) of the *VHb* gene is absolutely identical to that of the *tac* promoter (De Boet *et al.*, 1984). The −35 sequence region of the *VHb*

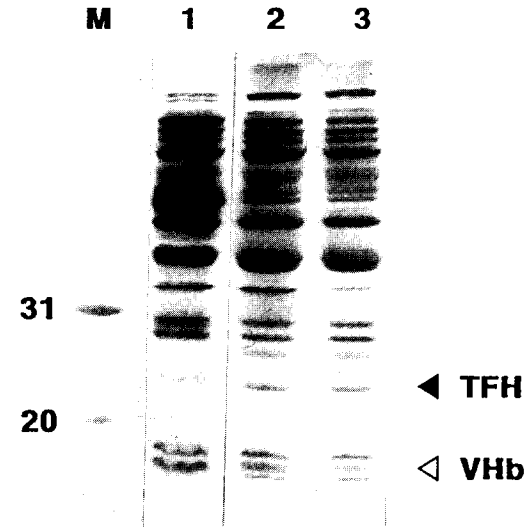

Fig. 4. Patterns of the SDS-PAGE analysis of VHb and ferritin in the strain containing the plasmid harboring the *lac* I^q gene. M, Molecular weight size marker; 1, JM109; 2, without IPTG; 3, with IPTG.

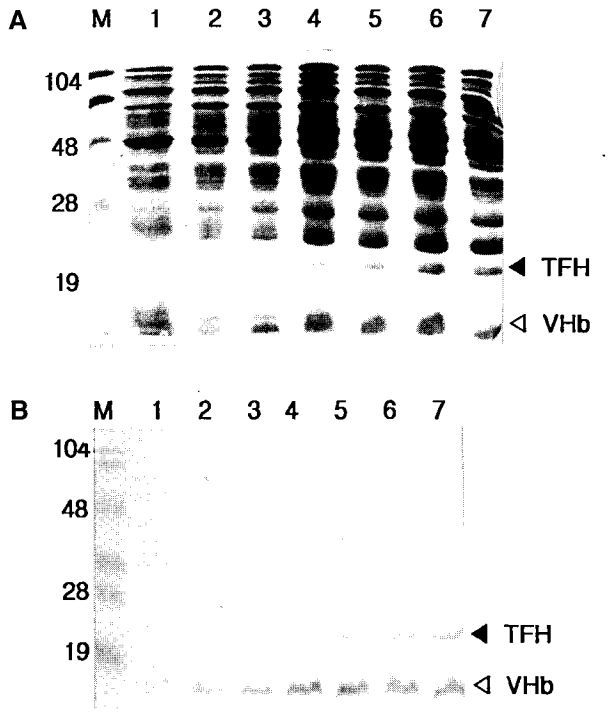

Fig. 5. Patterns of SDS-PAGE (A) and Western blot analysis (B) of the expression of the *VHb* and ferritin genes in the strain ACHB1/TFH. Lanes 1 through 7 were samples in the culture medium at OD_{600} = 0.38, 0.7, 0.93, 1.05, 1.29, 1.36, and 1.52, respectively. Strain ACHB1/TFH was grown with no induction.

gene promoter is TTTTAA, which half matches with that of the ferritin gene promoter, which is TTGACA. The *VHb* gene can be expressed to a very high level in *E. coli* despite the lack of the consensus −35 sequence region

Table 1. Sequence comparison of the promoter region among the genes of *VHb*, α-amylase, and ferritin (*TFH*).

	VHb	α-Amylase	*TFH*
SD sequence	GAAGA	AAGGGGGA	AGGA
−10 region	TATAA	TATAA	TATAA
−35 region	TTTTAA	TTGAAA	TTGACA
	→	→	
FNR binding site	TTGAT	TTGCA	Nil
	←	←	
	ATTAA	TGCAA	

(Joshi and Dikshit, 1994). In particular, the FNR binding site located 48 bp upstream of the VHb transcription initiation site is known to increase VHb expression through its promoter activity (Tsai *et al.*, 1995). The regulatory site is absent in the *tac* promoter of the ferritin gene. This may further explain the reason why VHb was produced more compared to ferritin in HF2 and HF3. For comparison, the promoter region of the α-amylase gene is discussed as the *VHb* gene has been used to improve α-amylase production in recombinant *E. coli* (Khosravi *et al.*, 1990; Liu *et al.*, 1992; Kallio and Bailey, 1996). Alpha-amylase was produced 3.3 times more by expressing the *VHb* gene. In this case, the FNR binding site is present in the 5′-noncoding region of the α-amylase gene promoter, and it is assumed that the transcriptional activity of the promoter was further influenced by the FNR protein.

The amounts of ferritin production was compared among different strains in terms of their relative ferritin value (Fig. 6). The relative ferritin value per ml of culture was estimated at a culture time of 9 h, since differences were observed in cell densities between the VHb-absent cell and the VHb-containing cells. ACHB1/TFH produced 1.8 times as much ferritin as TFH-10, whereas HF2

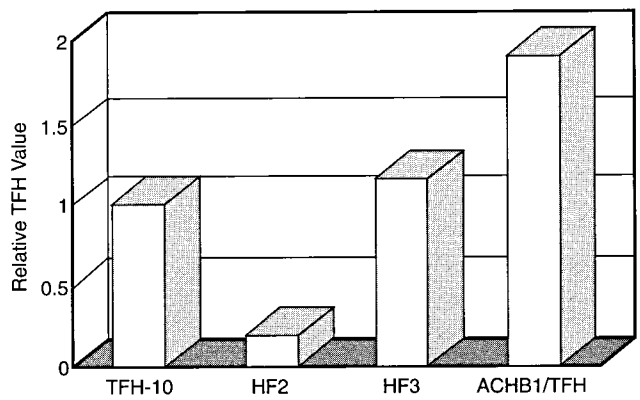

Fig. 6. Comparison of the production of the ferritin (TFH) protein by TFH-10, HF2, HF3, and ACHB1/TFH. For the relative ferritin values/ml of culture, aliquots of cell lysates were sampled 9 h after innoculation. In each case, the values are the average of five independent determinations.

produced ferritin with a very low yield. The relative ferritin values of HF3 and TFH-10 were similar.

In summary, we constructed a comparative expression system to investigate the effects of the *VHb* gene on the expression of the ferritin gene in *E. coli*. In our coplasmid expression system, the presence of the *VHb* gene increased the cellular production of ferritin 1.8 times more via improving cell growth and increasing the production of the target protein.

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