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OXYGEN DEPENDENT REGULATION OF VITREOSCILLA GLOBIN GENE: EVIDENCE FOR POSITIVE REGULATION BY FNR

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SUMMARY: *Vitreoscilla* globin (*vgb*) gene, encoding for *Vitreoscilla* haemoglobin (VtHb) has been cloned and functionally expressed in heterologous bacterial hosts. Analysis of *vgb* gene expression and the study on *vgb-xylE* transcriptional fusion revealed that *vgb* promoter is preferentially activated in response to oxygen limitation in *Vitreoscilla* and other heterologous bacterial hosts. Microaerobic mode of induction in various hosts, provided evidence for a common regulatory factor involved in activation of *vgb* promoter under hypoxic condition. Primary structure analysis of *vgb* upstream regulatory region indicated the presence of a possible binding site for the transcriptional activator, FNR. Further, the *E.coli* mutant lacking *fnr* gene product was not able to activate *vgb* promoter under microaerobic condition, suggesting the involvement of FNR or FNR-like proteins in modulating its activity. The possibility of a second level of control by CRP is also indicated. Oxygen responsive nature and regulatory characteristics of *vgb* promoter offers a novel system for the expression of gene in heterologous bacterial hosts in an oxygen dependent manner.

Vitreoscilla haemoglobin (VtHb), previously known as soluble cytochrome o, was positively identified as the first known bacterial haemoglobin on the basis of its oxygen binding properties and sequence similarity with leghaemoglobins (1). The haemoglobin content of *Vitreoscilla* increases almost 50-fold when the oxygen concentration of the growth medium falls below 10% of atmosphere (2). During the course of molecular cloning of *Vitreoscilla* globin (*vgb*) gene, the natural *vgb* promoter was identified as a strong promoter in *E.coli* (3,4). Expression of *vgb* gene as well as transcriptional fusion of *vgb* promoter with reporter genes like CAT and *xylE* (5,6,7) indicated that *vgb* promoter is maximally activated under hypoxic condition through a molecular mechanism involving recognition of an upstream oxygen

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regulated promoter element, by as yet unknown transacting factor(s). Oxygen responsive regulatory element(s) that control expression of *vgb* functions similarly, in *Vitreoscilla* as well as *E.coli* (5). In addition to its strength and regulatory characteristics, the specific and advantageous feature of *vgb* promoter is its high level induction in *E.coli* at the onset of hypoxic condition. Our initial study utilizing broad host range plasmid vector carrying *vgb-xylE* transcriptional fusion indicated that *vgb* promoter is also active in several other gram-negative hosts (7). We further extended our study to determine the strength of *vgb* promoter and its regulatory characteristics with respect to oxygen availability in various heterologous bacterial hosts. Although, the mechanism which fine tunes *vgb* gene expression under microaerobic condition is unknown at present, analysis of *vgb* promoter and its flanking region displayed possible binding site for the transcriptional activator, FNR which prompted us to explore the possibility of FNR involvement in the regulation of *vgb* promoter.

Members of the FNR family can be identified as multi-redox center proteins which control the master switch in regulation of several genes in response to oxygen availability, and are structurally related to cAMP receptor protein, CRP (8). Molecular mechanism underlying oxygen and/or redox sensing and transcriptional control by FNR is not fully understood but regulatory proteins related to FNR have been identified from diverse bacterial species (8,9). Recent report on HMP (haemoglobin-like protein) in *E.coli* (10) having combination of distinct haemoglobin-like and FNR-like domains within the protein structure, sets an interesting example of novel partnership between two functionally distinct proteins to generate proteins with unique structural and functional properties.

The present study was conducted to study the regulatory characteristics of *vgb* promoter in various heterologous hosts in response to oxygen availability. This paper reports the novel characteristics of *vgb* promoter and demonstrates that it can be regulated by oxygen, in a broad spectrum of bacterial hosts, with varying strength. Primary structure analysis of *vgb* promoter and utilization of *fnr* mutants provided positive evidence for the involvement of FNR or FNR-related proteins in transcriptional activation of *vgb* gene promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions: The bacterial strains and plasmids utilized in this work are listed in Table 1. All *E.coli* strains were grown in

Table 1. Bacterial Strains and Plasmids

Strains and Plasmids	Relevant Characterstics	Source or ref.
E.coli		
XL1-Blue	*recA*, *fnr*$^+$, *crp*$^+$	Stratagene
HB101	*recA*, *fnr*$^+$, *crp*$^+$	
JM105	*fnr*$^+$, *crp*$^+$	Promega
JRG1728	*lac*$^-$, *fnr*$^-$	18
MC1000	*lac*$^-$, *crp*$^-$	19
P. aeruginosa		11
PAO2003		
P. putida		ATCC
ATCC 12633		
A.vinelandii		ATCC
ATCC 475		
Plasmids		
pUC8:15	Ap^r, pUC8 carrying *vgb* gene	3
pVDX18	Ap^r, *xyl E* (promoterless)	11
pKD49	Ap^r, pVDX18 carrying *vgb-xylE fusion*	5
pKD50	Ap^r, pVDX18 carrying *vgb* gene	This study

Ap^r: ampicillin resistant.

LB medium. Growth conditions for *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Azotobacter vinelandii* were according to ATCC recommendations. Supplements were added when required at the following concentrations: Ampicillin (Ap) 50μg/ml and tetracycline (Tc) 12.5μg/ml. Adjustment of oxygen level in shake flask was done according to the method adopted by Narro et al (11) or procedure described previously (5). All restriction and DNA modifying enzymes were obtained from Promega (WI, USA) or New England Biolabs (Beverly, USA).

DNA manipulations: Standard recombinant techniques were applied for genetic manipulations (12). Detailed procedure for RNA isolation and *vgb*-specific transcript analysis have been described previously (5,7). Plasmid pKD 49 has been created (7) for *vgb-xylE* transcriptional fusion on broad host plasmid vector pVDX18 (13). Plasmid pKD 50 was constructed after cloning HindIII fragment of plasmid pUC8:15 (3), carrying entire *vgb* gene on pVDX18. pKD 49 and pKD 50 were introduced into various bacterial hosts by triparental filter mating using *E.coli* HB101 harboring pRK 2013 as helper.

Enzyme and protein assay: Haemoglobin content of cells was determined by taking carbon monoxide difference spectra of whole cells as described previously (3). Promoter activity in the cells carrying the reporter plasmids was determined by assaying the catechol-2,3-dioxygenase (CDO) activity as recommended by Konyecsni and Deretic (13). One unit of catechol-2,3-dioxygenase is defined as the amount of enzyme which converts 1μmole catechol to 2-hydroxymuconic semialdehyde (a yellow product with molar extinction coefficient of 4.4×10^4 at 375 nm) per min. The protein concentration of cell extracts was determined by the method of Bradford (14).

RESULTS AND DISCUSSION

Microaerobic induction of *vgb* and *vgb-xylE* fusion in heterologous hosts: In our earlier attempt to study the mode of *vgb* expression (6), we observed that *vgb* promoter was able to express in several gram-negative bacterial hosts. To analyze the regulatory characteristics of oxygen sensitive promoter further in various heterologous hosts, we introduced the broad host range plasmids pKD 49 and pKD 50 (fig.1), into *E.coli*, *P.aeruginosa*, *P. putida* and *A.vinelandii*. *vgb* promoter activity was monitored by the level of haemoglobin (VtHb) and fusion product formation under different oxygen levels (Table 2), which indicated that *vgb* promoter gets activated under oxygen limitation, albeit with varying strength, in different heterologous hosts. 10–50 fold increase in the production level of VtHb and reporter gene product was recorded when DO level decreased to 5% or less, compared with the same strain growing under high aeration. However, magnitude of *vgb* and *xyl*E activation varied in different hosts as compared to *E.coli*, possibly reflecting the differences in transcription and translational signals among different gram-negative bacteria. Overall data indicates that the microaerobic induction of *vgb* promoter was possible in heterologous system and probably a common oxygen dependent activation mechanism is operating in controlling the *vgb* promoter activity.

Primary structure of *vgb* promoter–Presence of putative FNR binding site: To further understand the regulatory properties of *vgb* promoter, we analyzed its primary structure. The nucleotide sequence of *Vitreoscilla* globin gene and its

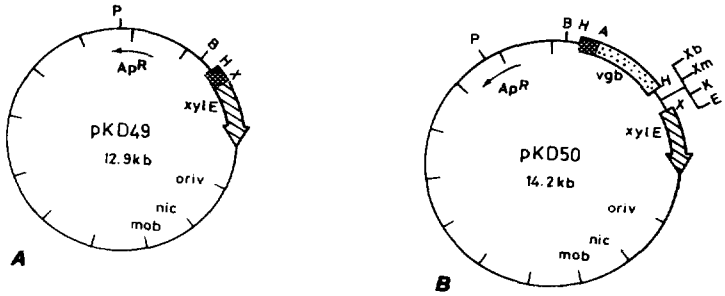

Fig. 1. Map of broad host range plasmids carrying *vgb-xylE* transciptional fusion (A) and entire *vgb* gene (B). Details in Materials and Methods. Dotted rectangle: *vgb* gene, Filled rectangle: *vgb* promoter, Shaded arrow: *xylE* gene. Abbreviations used: Ap^r,ampicillin resistance; *xylE*, gene encoding Catechol-2,3-dioxygenase; *vgb*, gene encoding *Vitreoscilla globin*; *ori v*, origin of replication; *nic*, relaxation nic site; *mob*, determinant for plasmid mobilisation. Sites for restriction enzymes are denoted by A–AflII; B–BamHI; E–EcoRI; H–HindIII; K–KpnI; P–PstI; Xb–XbaI and Xm–XmnI.

Table 2. Expression of *vgb* and *vgb-xylE* transcriptional fusion in various heterologous hosts

Strain	Total VtHb content* nmole/ g wet wt		CDO activity** U/mg protein	
	aerobic	microaerobic	aerobic	microaerobic
E.coli HB101	16	158	15	614
P.aeruginosa	24	286	54	1015
P.putida	26	245	38	816
A.vinelandii	44	114	91	315

* for estimation of VtHb content pKD50 containing *vgb* has been introduced and Hb content was determined by CO-difference spectra.
** *vgb-xylE* transcriptional fusion product was determined in terms of CDO activity.

regulatory region has been reported (15) and transcriptional start site was determined using S1 protection analysis (5). Nucleotide sequence of entire promoter region is shown in Fig.2. It contains a perfect Pribnow box but lacks a consensus -35 sequence inspite of being expressed to a very high level in *E.coli*. Scanning of *vgb* promoter region revealed possible FNR and CRP recognition sites. Presumptive FNR binding site overlaps -35 polymerase binding site and resides around 25-30 bp upstream with respect to transcription start site. For activated genes, the interval between FNR and transcriptional start site is 40-50 bp upstream which probably dictates the pattern of gene expression (8). Presence of FNR consensus upstream of *vgb* promoter indicates the possibility of FNR involvement in controlling its oxygen

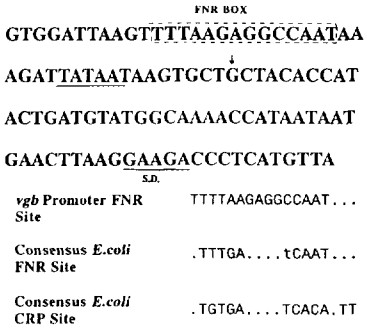

Fig. 2. Primary structure of *vgb* gene promoter: Pribnow box and ribosome binding site (S.D.) is underlined. Putative FNR binding site is boxed and the transcriptional start site is designated by an arrow. *vgb* FNR box is compared with *E.coli* FNR and CRP recognition sites.

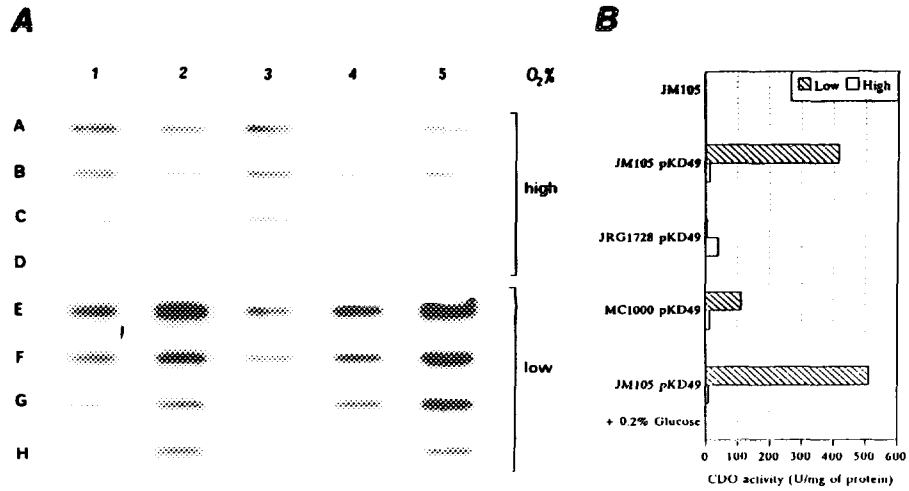

Fig. 3. *vgb* promoter activation in response to microaerobiosis: (A) Autoradiogram of slot blot of total RNA (A and E - 4μg, B and F - 2μg, C and G - 1μg, D and H - 0.5μg) isolated from strains grown under high- or low-oxygen conditions. Nick translated *vgb* gene from pUC 8:15 was used as a probe. Strains used: 1. XL1-Blue 2. XL1-Blue (pUC 8:15) 3. JRG 1728 (pUC 8:15) 4. MC1000 (pUC 8:15) 5. XL1-Blue (pUC 8:15) grown in presence of 0.2% Glucose. (B) Results of *xylE* assay: Cells were grown under varying oxygen conditions and assayed for catechol-2,3-dioxygenase. Activity expressed in U/mg of protein.

dependent activity. Besides that, a sequence resembling GTGAGTTA motif recognised by CRP on *lac* promoter (16) is also present upstream of FNR box and may have possible relevance in promoter regulation as predicted earlier (6).

FNR dependent microaerobic induction of *vgb* and *vgb-xylE* fusion: In order to resolve the role of FNR in controlling the *vgb* promoter activity, we studied the expression of *vgb* gene and *vgb-xylE* transcriptional fusion in strains carrying deletion in *fnr* and *crp* genes. Promoter activity was monitored by measuring the relative level of *vgb* specific transcript in various strains under aerobic and microaerobic conditions (fig. 3). Slot blot analysis and densitometric determination of *vgb*-specific mRNA indicated 30 to 50 fold increase in transcript level in *vgb* containing wild type E.coli, XL-Blue (fnr^+, crp^+) and MC 1000 (crp^-) when grown under oxygen limiting condition. However, level of transcript activation was slightly lower in crp^- strain. In contrast, JRG 1728 lacking *fnr* gene did not show any stimulation in *vgb* specific transcript level under identical condition. Weak *vgb* specific hybridization signal in *E.coli* (Fig. 3, lane 1) lacking *vgb* may be due to the presence of its native oxygen binding haemoglobin like protein (HMP), recently identified in *E.coli* (17). Extensive sequence homology has been found between N-terminal segment of HMP

and *Vitreoscilla* haemoglobin. Lack of promoter activation under oxygen limitation in *fnr* deleted strain demonstrated the requirement of *fnr* gene product for oxygen dependent regulation of *vgb* promoter.

We further confirmed this observation by analyzing the expression of reporter gene, *xylE*, in various strains with respect to oxygen availability. Promoter activity was monitored by the level of CDO activity, the product of *xylE*. pKD 49 when introduced into JRG 1728, did not show any improvement over basal level of CDO activity after exposure to oxygen limiting condition (fig. 3). CDO activity increased 30 to 50 fold higher in case of wild type *E.coli* and MC 1000, activation being lower in case of *crp* mutant. This indicates that CRP may be required to activate the *vgb* promoter to its full potential but may not be an absolute requirement for oxygen dependent regulation. It is possible that CRP may be participating indirectly in controlling *vgb* promoter as predicted earlier (7). Microaerobic mode of activation was retained when *E.coli* was grown in presence of glucose. This may be because cell growth is enhanced in presence of glucose which may result in the depletion of intracellular glucose and thus enables cells to synthesise enough CRP to mediate *vgb* promoter activation. Above results demonstrated the obligate requirement of *fnr* gene product for oxygen sensitive activation of *Vitreoscilla* globin gene promoter.

FNR-like proteins have been identified in several bacterial species: *HlyX* from *Actinobacillus pleuropneumoniae* (18) , *Anr* from *Pseudomonas aeruginosa* (19) and ORF 240 from *Rhizobium leguminosarum* (20). *FnrN* of *R. leguminosarum* can complement the FNR function in *E.coli* (20). Therefore, activation of *vgb* promoter in various bacterial hosts in an oxygen dependent manner may be mediated by their respective FNR-like proteins, the binding sites of heterologous FNR proteins being more or less similar. In *E.coli*, *fnr* gene is expressed under aerobic and anaerobic condition but the protein is active when oxygen is limiting (22). Our experimental evidences clearly indicate involvement of FNR or FNR-related protein(s) in oxygen responsive control of *Vitreoscilla* globin gene promoter. Furthermore, optimal activation of *vgb* promoter may also require CRP. Co-ordinate regulation of *ansB* promoter by FNR and CRP has been reported recently (23). Experiments are underway to explore this system by gel retardation and DNA foot printing analysis. Besides providing an excellent system for studying DNA-protein interaction, it also provides an unique and useful expression system which can be efficiently exploited for oxygen dependent expression of genes in heterologous systems of commercial implications.

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REFERENCES

[1] Wakabayashi, S., Matsubara H. and Webster, D.A. (1986) Nature 322, 481-483.

[2] Boerman, S and Webster, D.A. (1982) J. Gen. Appl. Microbiol. 28, 35-43.

[3] Dikshit, K.L. and Webster, D.A. (1988) Gene 70, 377- 386.

[4] Khosla, C, and Bailey, J.E. (1988) Mol. Gen. Genet. 214, 158-161.

[5] Dikshit, K.L., Spaulding, D., Braun, A and Webster D.A. (1989) J. Gen. Microbiol. 135, 2601-2610.

[6] Khosla, C. and Bailey, J.E. (1989) J. Bacteriol. 171, 5995-6004.

[7] Dikshit, K.L., Dikshit, R.P. and Webster, D.A. (1990) Nucl. Acids Res. 18, 4149-4155.

[8] Guest, J.R. (1992) J. Gen. Microbiol. 138, 2253- 2263.

[9] Bannan, J.D., Moran, M.J., MacInnes, J.I., Soltes, G.A. and Friedman, R.A. (1993) J. Bacteriol. 175, 7228-7235.

[10] Andrews, S.C., Shipley, D., Keen, J.H., Findlay, J.B.C., Harrison, P.M. and Guest, J.R. (1992) FEBS Letters, 302, 247-252.

[11] Narro, M.L., Adams, C.W. and Cohen, S.N. (1990) J. Bacteriol. 172, 4549-4554.

[12] Sambrook, J., Fritsch, E.F. and Maniatis, J. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, NY.

[13] Konyecsni, W.M. and Deretic, V. (1988) Gene, 74, 375-386.

[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

[15] Dikshit, K.L., Dikshit, R.P. and Webster, D.A. (1991) In Structure and function of Invertebrate oxygen carriers. (Eds. S.N. Vinogrador and Oscar H. Kapp.) pp 313-321. Springer Verlag, New York.

[16] Crombugghed, B., Busby, S. and Henri, B. (1984) Science 224, 831-838.

[17] Vasudevan, S.G., Armarego, W.L.F., Shaw, D.S., Lilley, P.E., Dixon, N.E. and Poole, R.K. (1991) Mol. Gen. Genet. 226, 49-58.

[18] Green,J.D., Sharrocks, A.D., MacInnes, J.I. and Guest, J.R. (1992) Proc. R. Soc. B. 248, 79-84.

[19] Sawers, G. (1991) Mol. Microbiol. 5, 1469-1481.

[20] Schluter, A., Palschkowski, T. Unden, G. and Priefer, U. B. (1992) Mol. Microbiol. 6, 3395-3404.

[21] Spiro, S. and Guest, J.R. (1987) J. Gen. Microbiol. 133, 3279-3288.

[22] Spiro, S., Roberts, R.E. and Guest, J.R. (1988) Mol. Microbiol. 3, 601-608.

[23] Jennings, M.P. and Beacham, I.R. (1993) Mol. Microbiol. 9, 155-164.