ELSEVIER

FEMS Microbiology Letters 224 (2003) 127–132

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Vitreoscilla hemoglobin promoter is not responsive to nitrosative and oxidative stress in *Escherichia coli*

Alexander D. Frey *,[1], Taija Koskenkorva, Pauli T. Kallio

Institute of Biotechnology, ETH Zürich, CH-8093 Zürich, Switzerland

Received 19 March 2003; accepted 19 May 2003

First published online 19 June 2003

Abstract

The *Vitreoscilla* hemoglobin gene (*vhb*) is expressed under oxygen-limited conditions via an FNR-dependent mechanism. Furthermore, cAMP-CRP has been implicated in its regulation. Recently, VHb protein has been reported to protect a heterologous host from nitrosative stress. In this study we analyzed the regulation of the *Vitreoscilla* hemoglobin promoter (P_{vhb}) in *Escherichia coli* under nitrosative and oxidative stress conditions. Our results show unambiguously that expression of neither VHb nor chloramphenicol acetyltransferase under the control of P_{vhb} is induced under the experimental conditions used. Thus, a clear discrepancy between in vivo function, i.e. protection against nitrosative stress, and regulation of gene expression is obvious. The regulation of P_{vhb} reported here is in clear contrast to the expression pattern of flavohemoglobins from various microorganisms, which are generally induced by nitrosative stress. However, the length of P_{vhb} is only 146 bp and therefore, we cannot rule out that additional regulatory sequences may be located in the upstream region of P_{vhb}.

Keywords: Nitrosative stress; Oxidative stress; Gene regulation; *Vitreoscilla* hemoglobin

1. Introduction

Expression of the *Vitreoscilla* hemoglobin gene (*vhb*) is under the control of the oxygen-dependent *Vitreoscilla* hemoglobin promoter (P_{vhb}) and is induced by a diminishing dissolved oxygen level [1]. Mechanistic aspects of P_{vhb} regulation have been largely characterized in *Escherichia coli* [2,3], but it is also functional in other heterologous microorganisms, such as *Pseudomonas*, *Azotobacter*, *Rhizobium etli*, *Streptomyces* sp., *Serratia marcescens*, *Burkholderia* sp. [4–7]. P_{vhb} is positively controlled by cAMP-CRP and FNR as revealed by reduced expression levels in *cya* and *fnr* mutant *E. coli* strains, respectively. A CRP binding site is positioned at −97 and an FNR binding site is centered around −41.5 within the promoter sequence [2,8]. Supplementation of cAMP to a *cya* mutant reversed the effect and increased the expression level from P_{vhb} 10-fold [2]. Despite the influence of catabolite repression, expression of reporter enzymes from P_{vhb} did not differ significantly in glucose- and glycerol-containing media under carbon-limited conditions [9]. In *fnr* mutant background expression of chloramphenicol acetyltransferase (CAT) from P_{vhb} was reduced two-fold under microaerobic conditions [8]. Expression from P_{vhb} is maximally activated both in *Vitreoscilla* and *E. coli* under microaerobic conditions, when dissolved oxygen level drops below 2% of air saturation [2].

Furthermore, repression of P_{vhb} is achieved by the addition of a complex nitrogen source such as yeast extract to the medium, resulting in a third level of regulation of P_{vhb} [9]. However, this regulation mechanism has not been studied in detail.

In bioreactor cultivations, under conditions of full P_{vhb} induction, the level of expressed β-galactosidase reporter protein accounted for up to 10% of total cellular protein [9]. The high expression level as well as the many regulatory mechanisms have rendered P_{vhb} extremely interesting for applications in biotechnological production processes

* Corresponding author. Tel.: +41 (1) 633 34 46; Fax: +41 (1) 633 10 51.
E-mail address: afrey@biotech.biol.ethz.ch (A.D. Frey).

[1] Present address: Institute of Medical Virology, University of Zürich, CH-8028 Zürich, Switzerland.

0378-1097/03/$22.00
doi:10.1016/S0378-1097(03)00434-8

since the induction of promoter activity is readily achieved by lowering dissolved oxygen concentrations in bioreactors. Thus, expensive chemical inducers, such as isopropyl-β-D-thiogalactose, can be omitted.

VHb has been expressed in several heterologous organisms and the presence of VHb could foster growth and productivity of these engineered strains under oxygen-limited conditions [10]. VHb has been suggested to deliver oxygen, thus shifting the cellular physiology to the energetically more efficient aerobic state. Recently, it has been reported that VHb and flavohemoglobins in general protect cells from nitrosative stress [11,12]. However, the NO-degrading activity of soluble protein fractions from VHb-overproducing cells was not different from that of VHb-free cell preparations in vitro, suggesting that a membrane-linked reductase could also be involved in NO detoxification in vivo [11].

The expression of flavohemoglobin genes from various bacteria is induced by several stimuli such as oxygen deprivation or oxidative and nitrosative stress. Several regulatory proteins have been implicated in the control of gene expression, such as FNR, RedD/E, Fur, NAR, MetR or stationary phase-responsive σ^S [13–15]. Activation of flavohemoglobin gene expression by reactive oxygen (ROS) and nitrogen species (RNS) is independent of the oxidative stress-responsive SoxRS and OxyR transcription factors [13,14].

In this study our goal was to clarify if expression of P_{vhb} is modulated by the presence of ROS and RNS in *E. coli*. Therefore, expression of VHb and CAT under the control of P_{vhb} was analyzed under conditions of nitrosative and oxidative stress.

2. Materials and methods

2.1. Bacterial strains and plasmids

To study the regulation of P_{vhb} by oxidative and nitrosative stress, *E. coli* K-12 MG1655 ($^{\lambda-, F-}$; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) was used, which was transformed either with pRED2, carrying the *vhb* gene under control of its native promoter (P_{vhb}), or with pOX2, harboring the *cat* gene under control of P_{vhb} (Φ(*vhb-cat*)). Both plasmids have been described elsewhere [16,17].

2.2. Growth and induction experiments

Study of P_{vhb} regulation was performed either in a modified M9 minimal medium for shake flask cultivations or in Luria–Bertani (LB) broth for deep well plate cultivations at 37°C and 300 rpm. The cultivations were essentially performed as described previously [11]. Overnight cultures were used to inoculate precultures to a starting OD_{600} of 0.05 in shake flasks, which were allowed to grow to an OD_{600} of 1. Precultures were used to inoculate cultures for the induction experiments. Shake flasks were inoculated to an OD_{600} of 0.1 and grown for 3 h to follow induction of expression from P_{vhb}. Deep well plates containing 1 ml LB were inoculated by addition of 25 μl of preculture and grown for maximally 4 h in the presence of stressors. Deep well plates were used to screen for the effects of various stressors and stressor concentrations on the induction level of P_{vhb}. The stressors used were sodium nitroprusside (SNP), paraquat (PQ) and hydrogen peroxide (H_2O_2); they were used at the concentrations indicated in the figure legends. Samples for the analysis of VHb and CAT production and to measure growth were withdrawn at the indicated time points.

2.3. Analytical procedures

Cells for analytical procedures were quickly chilled on ice and harvested by brief centrifugation at 15 000 rpm, 4°C. Cell pellets were stored at −20°C until analysis.

Samples for Western blotting were resuspended to a cell density corresponding to OD_{600} of 10 in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis loading buffer [18] and boiled for 5 min. Soluble protein fractions were separated on 15% SDS–polyacrylamide gels at 40 mA and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) overnight at 100 mA. Detection of VHb was performed as described previously using rabbit anti-VHb serum and a goat anti-rabbit IgG coupled to horseradish peroxidase (Amersham Pharmacia), both of which were used at a 1:3000 dilution [19]. Detection was performed using ECL chemiluminescence kit (Amersham Pharmacia).

Samples for CAT measurements were resuspended in a lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 7.5) and sonicated twice for 10 s. Soluble protein fractions were obtained after centrifugation at 15 000 rpm, 4°C for 10 min. Protein concentration was assayed using the Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. The CAT content of the samples was determined by an enzyme-linked immunosorbent assay (ELISA) protocol (Roche Molecular Biochemicals). CAT content is expressed as μg CAT per mg of total soluble protein.

3. Results

3.1. Screening of P_{vhb} response to stressors

Growth of various microorganisms in deep well plates has been shown not to differ from experiments performed using shake flasks, regarding mixing and oxygen transfer [20,21]. Therefore, we chose the deep well plate system to readily analyze the response of P_{vhb} to several compounds

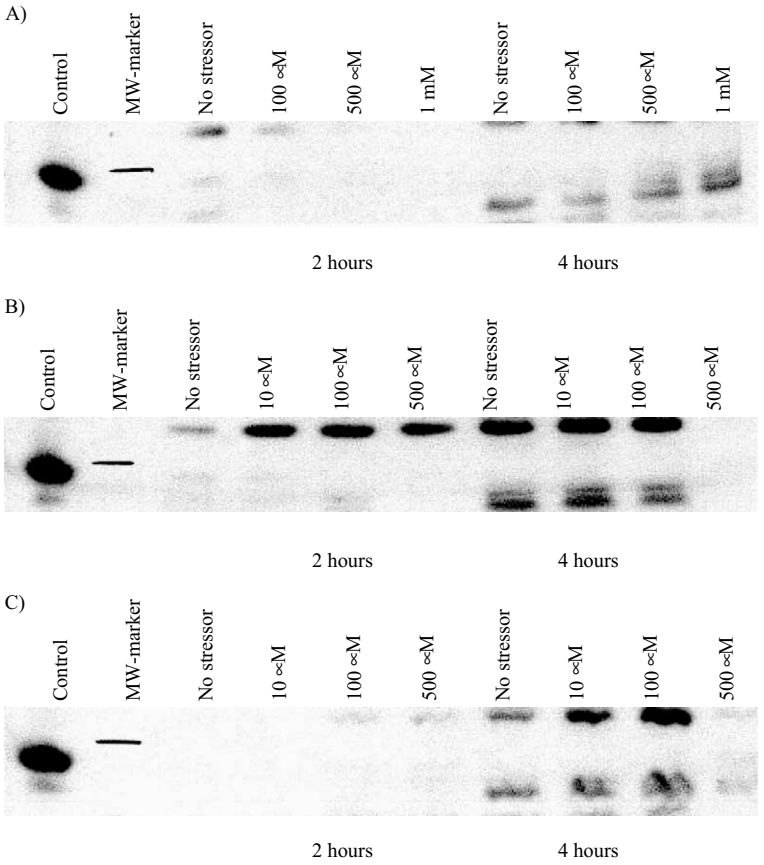

Fig. 1. Western blot analysis of MG1655:pRED2 carrying the full-length *vhb* gene under control of its native regulatory sequence (P_{vhb}). Cells were grown in deep well plates for 2 or 4 h, in the presence of SNP (A), PQ (B) and H_2O_2 (C) at different concentrations. Cell extracts for analysis were obtained by resuspending cell pellets to $OD_{600} = 10$ in SDS-loading buffer. The positive control represents a VHb-containing cell extract of *E. coli* carrying pRED2 induced by oxygen limitation. The molecular mass marker (lysozyme) has a size of 20.6 kDa.

producing either nitrosative or oxidative stress. Precultures of *E. coli* MG1655:pRED2 were used to inoculate deep well plates to a starting OD_{600} of 0.1. Stressors were added to various concentrations as specified in Fig. 1. Deep well plates were incubated for either 2 or 4 h, after which final OD_{600} was determined and samples for analysis of gene expression were withdrawn. Final OD_{600} readings after 4 h of growth are given in Table 1.

Western blot analysis to detect VHb expression 2 and 4 h post inoculation revealed that surprisingly no induction of P_{vhb} occurred in the presence of SNP (0–1 mM), PQ (0–500 μM) and H_2O_2 (0–500 μM) over the whole concentration range analyzed (Fig. 1).

3.2. Induction experiment to assess expression of VHb and CAT

Based on the above experiments conducted in deep well plates, stressor concentrations for shake flask cultivations were selected, which have been shown not to affect cell growth. Thus, cellular metabolism can be assumed not to be generally affected or stressed. Precultures were used to inoculate shake flasks, which were incubated for 3 h, during which samples were withdrawn after 0, 0.5, 1, 2, and 3 h for the analysis of the reporter gene expression. Growth was not affected by the presence of stressors with the exception of SNP, which reduced growth rate signifi-

Table 1
Final OD_{600} of *E. coli* MG1655:pRED2 grown for 4 h in deep well plates in the presence of various stressors

SNP		PQ		H_2O_2	
conc. (μM)	OD_{600}	conc. (μM)	OD_{600}	conc. (μM)	OD_{600}
0	1.49 ± 0.08	0	1.49 ± 0.08	0	1.49 ± 0.08
100	0.91 ± 0.03	10	1.49 ± 0.01	10	1.51 ± 0.04
500	0.70 ± 0.02	100	1.22 ± 0.05	100	1.38 ± 0.08
1000	0.63 ± 0.08	500	0.18 ± 0.02	500	0.75 ± 0.05

Means ± S.D. from at least four cultures are given.

Table 2
CAT activity (ng CAT μg^{-1} soluble protein) of MG166:pOX2 (Φ(*vhb-cat*)) challenged with SNP (100 μM), PQ (10 μM) and H_2O_2 (10 μM) grown in shake flasks

Time (h)	Control	SNP	PQ	H_2O_2
0	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001
0.5	0.080 ± 0.03	0.060 ± 0.016	0.095 ± 0.014	0.053 ± 0.027
1	0.009 ± 0.003	0.006 ± 0.001	0.007 ± 0.002	0.006 ± 0.001
2	0.004 ± 0.000	0.007 ± 0.002	0.004 ± 0.001	0.005 ± 0.002
3	0.004 ± 0.000	0.006 ± 0.002	0.004 ± 0.001	0.004 ± 0.000

Means ± S.D. from two independent cultures are given.

cantly compared to stressor-free control cultures (data not shown).

Shake flask experiments were conducted with MG1655:pRED2 and MG1655:pOX2, carrying Φ(*vhb-cat*). Previously, we have shown that VHb protects the host cells from nitrosative stress and mediates the detoxification of NO [11], Therefore, the use of a CAT expression strain should exclude any falsification of the expression pattern by the presence of VHb. Using this strategy, any effects on expression of a single reporter protein at the translational or posttranslational level can be circumvented. Analysis of CAT activity using an ELISA assay and determination of VHb expression by Western blotting revealed that neither of these two reporter enzymes was expressed at significant levels in *E. coli* under our experimental conditions (Table 2, Fig. 2). The highest expression levels of either CAT or VHb were observed at time point zero and no further induction was obtained in the presence of any of the stressors during the 3-h incubation

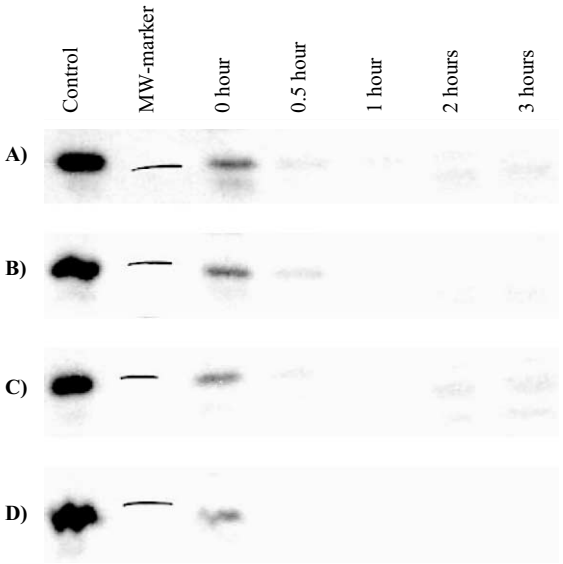

Fig. 2. Western blot analysis of MG1655:pRED2 carrying the full-length *vhb* gene under control of its native regulatory sequence grown in shake flasks for 3 h. The stressors were added to the following concentrations: SNP 100 μM (A), PQ 10 μM (B) and H_2O_2 10 μM (C). Samples for analysis were harvested after 0, 0.5, 1, 2 and 3 h. The positive control represents a VHb-containing cell extract of *E. coli* carrying pRED2 induced by oxygen limitation. D: Stressor-free culture. The molecular mass marker (lysozyme) has a size of 20.6 kDa.

period, thus suggesting that the expression of reporter proteins at time point zero is due to the background induction of the precultures. The expression patterns of VHb from pRED2 and of CAT from pOX2 correlate nicely, indicating that no further effects influence expression of these two reporter proteins at the translational or posttranslational level under the experimental conditions used.

4. Discussion

The regulation of flavohemoglobin expression in various organisms has been carefully studied, and several of these promoters have been shown to be responsive to RNS and ROS [14,22–24]. In this study we analyzed the effect of RNS and ROS on the induction of P_{vhb}. P_{vhb} has previously been shown to be induced by low oxygen concentrations via an FNR-dependent mechanism. Furthermore, CRP-cAMP has a positive effect on promoter activity [2].

Recently VHb has been shown to protect *E. coli* cells from nitrosative stress [11,12]. The biological significance of the observed protection is only given if VHb expression is upregulated in response to such compounds. However, it is not known if VHb is expressed in response to nitrosative stress in vivo. Our results unambiguously show that neither RNS nor ROS lead to enhanced expression of VHb or CAT, both of which are under the control of the native *vhb* regulatory sequence. One may conclude that VHb's protective capacity does not support the cellular stress response under the investigated conditions, since VHb is not expressed at observable levels. Thus, VHb is not able to relieve nitrosative or oxidative stress, unless it is expressed from an RNS- or ROS-responsive promoter. The P_{vhb} fragment present in both plasmids is 146 bp long. Thus, we cannot exclude the possibility that further regulatory elements are located upstream of the *vhb* promoter sequence used in this study.

In contrast to the regulation demonstrated in this study for P_{vhb}, *E. coli hmp* expression is directly stimulated by NO and PQ. Aerobic induction by NO is mediated via MetR, which has been shown to be necessary for induction of Φ(*hmp-lacZ*) by SNP and GSNO. Induction occurs upon depletion of homocysteine, which allows MetR to bind to the promoter region [25]. Under anaerobic condi-

tions, induction of *hmp* expression by NO is mediated via an FNR-dependent mechanism, an environment that in the absence of NO represses *hmp* expression [26]. Based on the observation that NO is able to reversibly inactivate FNR by nitrosylation, exposure of cells to NO derepressed a Φ(*hmp-lacZ*) fusion due to inactivation of FNR. In the *fnr* mutant no effect on the derepressed level of Φ(*hmp-lacZ*) activity was observed [26]. However, the NO-detoxifying activity of HMP is only minor under anoxic conditions and more potent NO detoxification mechanisms exist in *E. coli* [27–29]. Since FNR acts as an activator on P_{vhb}, inactivation of FNR by NO would not lead to expression of the *vhb* gene. Thus, no activation of P_{vhb} would occur under anaerobic conditions in the presence of NO. Furthermore, we could not discern a potential MetR binding site in the promoter sequence of P_{vhb}. Thus, regulation of P_{vhb} by nitrosative stress in a similar fashion as observed for *hmp* regulation in *E. coli* is unlikely.

Membrillo-Hernández et al. [23] also reported the induction of an *hmp-lacZ* fusion in the presence of PQ. Expression of Φ(*hmp-lacZ*) was induced only at PQ concentrations above 200 μM and was shown to be independent of SoxRS. Anjum et al. [30] reported a 28-fold induction of Φ(*hmp-lacZ*) after a prolonged incubation in the presence of PQ, a treatment that induces *hmp* expression to spectrally detectable levels. In our experiments we could not detect any induction of P_{vhb} when using PQ concentrations up to 500 μM, a concentration that reduced the final OD_{600} by almost 90%.

Both regulation pattern and biochemical activity of the *Ralstonia eutropha* flavohemoglobin gene (*fhp*) and protein (FHP) resemble the situation encountered with VHb. Expression of *fhp* has been shown to respond to oxygen limitation and FHP level is 20-fold upregulated [31]. The promoter region contains two potential binding motifs for NarL (centered around −108) and FNR (centered around −86) upstream of the *fhp* gene [32]. NOD activity of FHP has been reported to be 5–10-fold lower than the NO turnover mediated by flavohemoglobin from pathogenic bacteria, such as *Klebsiella pneumoniae* and *Salmonella enterica* serovar Typhi [11,33]. However, expression of FHP in *E. coli* enhanced the resistance of cells against nitrosative stress [11]. Altogether, these results suggest that FHP may protect *R. eutropha* from NO and related molecules during the denitrification process. However, steady-state NO concentrations during denitrification have been shown to be tightly controlled by NO reductases [34]. Unfortunately, no information on the expression of *fhp* in response to RNS or ROS is available.

Acknowledgements

This research was supported by ETH Zürich and Helsinki University of Technology.

References

[1] Webster, D.A. and Hackett, D.P. (1966) The purification and properties of cytochrome *o* from *Vitreoscilla*. J. Biol. Chem. 241, 3308–3315 .

[2] Khosla, C. and Bailey, J.E. (1989) Characterization of the oxygen-dependent promoter of the *Vitreoscilla* hemoglobin gene in *Escherichia coli*. J. Bacteriol. 171, 5990–6004.

[3] Dikshit, K.L., Dikshit, R.P. and Webster, D.A. (1990) Study of *Vitreoscilla* globin (*vgb*) gene expression and promoter activity in *E. coli* through transcriptional fusion. Nucleic Acids Res. 18, 4149–4155.

[4] Magnolo, S.K., Leenutaphong, D.L., Demodena, J.A., Curtis, J.E., Bailey, J.E., Galazzo, J.L. and Hughes, D.E. (1991) Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of a bacterial hemoglobin. Bio/Technology 9, 473–476.

[5] Wei, M.L., Webster, D.A. and Stark, B.C. (1998) Metabolic engineering of *Serratia marcescens* with the bacterial hemoglobin gene: Alterations in fermentation pathways. Biotechnol. Bioeng. 59, 640–646.

[6] Ramírez, M., Valderrama, B., Arrendondo-Peter, R., Sobéron, M., Mora, J. and Hernández, G. (1999) *Rhizobium etli* genetically engineered for the heterologous expression of *Vitreoscilla* sp. hemoglobin: Effect on free-living and symbiosis. Mol. Plant-Microbe Interact. 12, 1008–1015.

[7] Patel, S.M., Stark, B.C., Hwang, K.W., Dikshit, K.L. and Webster, D.A. (2000) Cloning and expression of *Vitreoscilla* hemoglobin gene in *Burkholderia* sp. strain DNT for enhancement of 2,4-dinitrotoluene degradation. Biotechnol. Prog. 16, 26–30.

[8] Tsai, P.S., Kallio, P.T. and Bailey, J.E. (1995) Fnr, a global transcriptional regulator of *Escherichia coli*, activates the *Vitreoscilla* hemoglobin (*vhb*) promoter and intracellular VHb expression increases cytochrome *d* promoter activity. Biotechnol. Prog. 11, 288–293.

[9] Khosla, C., Curtis, J.E., Bydalek, P., Swartz, J.R. and Bailey, J.E. (1990) Expression of recombinant proteins in *Escherichia coli* using an oxygen-responsive promoter. Bio/Technology 8, 554–558.

[10] Kallio, P.T., Frey, A.D. and Bailey, J.E. (2001) From *Vitreoscilla* hemoglobin (VHb) to a novel class of growth stimulating hemoglobin proteins. In: Recombinant Protein Production with Prokaryotic and Eukaryotic Cells. A Comparative View on Host Physiology (Merten, O.-W., Mattanovich, D., Larsson, G., Neubauer, P., Lang, C., Porro, D., Teixeira de Mattos, J., Postma, P. and Cole, J., Eds.), pp. 75–87. Kluwer Academic, Dordrecht.

[11] Frey, A.D., Farrés, J., Bollinger, C.J.T. and Kallio, P.T. (2002) Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli*. Appl. Environ. Microbiol. 68, 4835–4840.

[12] Kaur, R., Pathania, R., Sharma, V., Mande, S.C. and Dikshit, K.L. (2002) Chimeric *Vitreoscilla* hemoglobin (VHb) carrying a flavoreductase domain relieves nitrosative stress in *Escherichia coli*: New insight into the functional role of VHb. Appl. Environ. Microbiol. 68, 152–160.

[13] LaCelle, M., Kumano, M., Kurita, K., Yamane, K., Zuber, P. and Nakano, M.M. (1996) Oxygen-controlled regulation of the flavohemoglobin gene in *Bacillus subtilis*. J. Bacteriol. 178, 3803–3808.

[14] Hu, Y.M., Butcher, P.D., Mangan, J.A., Rajandream, M.A. and Coates, A.R.M. (1999) Regulation of *hmp* gene transcription in *Mycobacterium tuberculosis*: Effects of oxygen limitation and nitrosative and oxidative stress. J. Bacteriol. 181, 3486–3493.

[15] Poole, R.K. and Hughes, M.N. (2000) New functions for the ancient globin family: Bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. 36, 775–783.

[16] Khosla, C. and Bailey, J.E. (1988) The *Vitreoscilla* hemoglobin gene: Molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. Mol. Gen. Genet. 214, 158–161.

[17] Hughes, D.E., Curtis, J.E., Khosla, C. and Bailey, J.E. (1989) A new oxygen-regulated promoter for the expression of proteins in *Escherichia coli*. BioTechniques 7, 1026–1028.

[18] Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage-T4. Nature 227, 680–685.
[19] Farrés, J. and Kallio, P.T. (2002) Improved cell growth in tobacco suspension cultures expressing *Vitreoscilla* hemoglobin. Biotechnol. Prog. 18, 229–233.
[20] Minas, W., Bailey, J.E. and Duetz, W. (2000) *Streptomycetes* in micro-cultures: Growth, production of secondary metabolites, and storage and retrieval in the 96-well format. Antonie Van Leeuwenhoek 78, 297–305.
[21] Duetz, W.A., Ruedi, L., Hermann, R., O'Connor, K., Buchs, J. and Witholt, B. (2000) Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Appl. Environ. Microbiol. 66, 2641–2646.
[22] Poole, R.K., Anjum, M.F., Membrillo-Hernández, J., Kim, S.O., Hughes, M.N. and Stewart, V. (1996) Nitric oxide, nitrite, and Fnr regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. 178, 5487–5492.
[23] Membrillo-Hernández, J., Kim, S.O., Cook, G.M. and Poole, R.K. (1997) Paraquat regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12 is SoxRS independent but modulated by σ^S. J. Bacteriol. 179, 3164–3170.
[24] Crawford, M.J. and Goldberg, D.E. (1998) Regulation of the *Salmonella typhimurium* flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide. J. Biol. Chem. 273, 34028–34032.
[25] Membrillo-Hernández, J., Coopamah, M.D., Channa, A., Hughes, M.N. and Poole, R.K. (1998) A novel mechanism for upregulation of the *Escherichia coli* K-12 *hmp* (flavohaemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: Nitrosation of homocysteine and modulation of MetR binding to the *glyA-hmp* intergenic region. Mol. Microbiol. 29, 1101–1112.
[26] Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M.N., Scott, C., Thomson, A.J., Green, J. and Poole, R.K. (2002) NO sensing by FNR: Regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. EMBO J. 21, 3235–3244.
[27] Kim, S.O., Orii, Y., Lloyd, D., Hughes, M.N. and Poole, R.K. (1999) Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): Reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett. 445, 389–394.
[28] Gardner, A.M., Helmick, R.A. and Gardner, P.R. (2002) Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. J. Biol. Chem. 277, 8172–8177.
[29] Gomes, C.M., Giuffre, A., Forte, E., Vicente, J.B., Saraiva, L.M., Brunori, M. and Teixeira, M. (2002) A novel type of nitric-oxide reductase. *Escherichia coli* flavorubredoxin. J. Biol. Chem. 277, 25273–25276.
[30] Anjum, M.F., Ioannidis, N. and Poole, R.K. (1998) Response of the NAD(P)H-oxidising flavohaemoglobin (Hmp) to prolonged oxidative stress and implications for its physiological role in *Escherichia coli*. FEMS Microbiol. Lett. 166, 219–223.
[31] Probst, I., Wolf, G. and Schlegel, H.G. (1979) An oxygen-binding flavohemoprotein from *Alcaligenes eutrophus*. Biochim. Biophys. Acta 576, 471–478.
[32] Cramm, R., Siddiqui, R.A. and Friedrich, B. (1994) Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*. J. Biol. Chem. 269, 7349–7354.
[33] Gardner, P.R., Gardner, A.M., Martin, L.A., Dou, Y., Li, T., Olson, J.S., Zhu, H. and Riggs, A.F. (2000) Nitric-oxide dioxygenase activity and function of flavohemoglobins. Sensitivity to nitric oxide and carbon monoxide inhibition. J. Biol. Chem. 275, 31581–31587.
[34] Goretski, J., Zafiriou, O.C. and Hollocher, T.C. (1990) Steady-state nitric oxide concentrations during denitrification. J. Biol. Chem. 265, 11535–11538.