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From *Vitreoscilla* hemoglobin (VHb) to a novel class of protective and growth promoting bacterial hemoglobin proteins

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Summary

Vitreoscilla hemoglobin (VHb) has been successfully expressed in various biotechnological relevant organisms, which resulted in the enhanced growth and productivity of these heterologous hosts from prokaryotic and eukaryotic origin. However, it has been argued that the properties of this protein are not ideally suited to relieve growth limitation from all host cells. Therefore, novel proteins were screened to test their ability to promote growth under oxygen limited conditions as encountered in large-scale production bioprocesses.

Flavohemoglobin protein of *Ralstonia eutropha* (*Alcaligenes eutrophus*) consists of an N-terminal hemoglobin and a C-terminal reductase domain. The redox-active protein tail can be divided into two functionally separate protein modules: a FAD-binding (FAD) and NAD(P)H binding (NAD) domain. In contrast to flavohemoglobins, VHb is lacking an intramolecular reductase domain. However, such a non-linked NADH-dependent methemoglobin reductase has been identified in its native host *Vitreoscilla*. VHb is highly homologous to the hemoglobin domain of FHP (FHPg). Therefore, several chimeric protein fusions of VHb and the reductase domain of FHP and truncated protein structures of FHP were engineered. Expression of either the artificial flavohemoglobin VHb-Red or of FHP resulted in significant growth improvements relative to the native VHb protein in microaerobic growing *Escherichia coli* cells. Furthermore, production of a recombinant reporter protein was increased.

To understand the physiological basis of the observed effects we have performed an in-depth analysis of the metabolism of *E. coli* cells expressing VHb, VHb-Red, FHPg and FHP using fractional ^{13}C-labeling and a 2-dimensional NMR technique. Interestingly, cells expressing VHb or VHb-Red revealed a metabolic configuration typical for anaerobiosis, showing a branched TCA cycle. In contrast, FHP or FHPg expressing cells had a functional TCA cycle. Furthermore, glucose consumption was lower in these cells indicative of more efficient carbon utilization. All studied

strains showed a higher ATP content and an increase ATP/ADP ratio relative to control cells.

Due to the findings that flavohemoglobins are key players in the detoxification of $NO^{\bullet}$ and their expression results in the protection of cells from nitrosative stress, we have analyzed the possibility if hemoglobins and flavohemoglobins in general are also able to mediate this stress relieving effect. Interestingly, hemoglobins, such as VHb, were also able to retain normal cell growth under nitrosative stress conditions. However, *in vitro* assays revealed that the $NO^{\bullet}$ degrading capacity of hemoglobins is limited. By engineering a reductase to the C-terminus of hemoglobins the $NO^{\bullet}$ degrading activity was significantly enhanced, showing the importance of this protein domain in $NO^{\bullet}$ detoxification.

Furthermore, VHb-expressing hybrid aspen trees have been generated to analyze the effect of VHb-expression on a complex, perennial plant species. Growth of these trees was followed under various growth conditions, such as different temperature and UV-B illumination. However, the effect of VHb-expression on growth was dependent on the genetic background of plant material as well as on the line. Only few statistically significant differences in growth and other phenotypical characteristics were observed. However, ultrastructural analysis of cells revealed that VHb expressing cells contain a higher amount of starch, demonstrating that these lines are indeed able to either photosynthesize more efficiently or to utilize the carbon more efficiently.

Zusammenfassung

Vitreoscilla Hämoglobin (VHb) wurde in verschiedenen biotechnologisch wichtigen Organismen exprimiert. Die Expression resultierte in verbesserten Wachstumscharakteristika und erhöhter Produktivität in prokaryotischen und eukaryotischen Zellen. Es ist jedoch nicht bekannt ob die biochemischen Eigenschaften von VHb in allen heterologen Organismen geeignet sind, um Wachstumslimitierungen aufzuheben. Daher wurden neue Proteine evaluiert um ihre Fähigkeit, Wachstum unter Sauerstoff limitierten Bedingungen zu fördern, zu testen.

Das Flavohämoglobinprotein aus *Ralstonia eutropha* (*Alcaligenes eutrophus*) besteht aus einem N-terminalen Hämoglobin und einer C-terminalen Reduktase. Die Reduktasedomäne besteht aus zwei funktionell unabhängigen Modulen: ein FAD bindendes (FAD) und ein NAD(P)H bindendes (NAD) Modul. Im Gegensatz zu den Flavohämoglobinen, fehlt VHb eine intramolekulare redox-aktive Proteindomäne. Eine dazugehörende Methämoglobin Reduktase wurde jedoch in *Vitreoscilla* identifiziert. VHb and die Hämoglobindomäne von FHP (FHPg) sind strukturell stark verwandt. Daher wurden verschiedene Proteinfusionen zwischen VHb und den Komponenten der Reduktase von FHP als auch verkleinerte Formen von FHP hergestellt. Das künstliche Flavohämoglobin VHb-Red und FHP konnten im Vergleich zu VHb das Wachstum von *Escherichia coli* am stärksten stimulieren. Zusätzlich wurde die Produktion eines rekombinanten Reporterproteins in diesen Zellen stark erhöht.

Um die beobachteten Effekte besser verstehen zu können, wurde der Stoffwechsel von *E. coli* Zellen, die entweder VHb, VHb-Red, FHP oder FHPg exprimierten, mittels ^{13}C-Markierung und einer 2-dimensionalen NMR Methode genauer analysiert. In Zellen, die VHb oder VHb-Red exprimieren, konnten festgestellt werden, dass der Zitronensäurezyklus unterbrochen ist, d.h. eine für anaerobe Bedingungen typische Konstellation auftritt. Im Gegensatz dazu war der Zitronensäurezyklus aktiv in FHP oder FHPg exprimierenden Zellen. Zusätzlich

war die Glukoseaufnahme in diesen Zellen tiefer, was auf einen effizienteren Stoffwechsel hindeutet. Alle rekombinante Hämoproteine exprimierenden Zellen wiesen im Vergleich zu Kontrollen einen höheren ATP Gehalt und ein besseres ATP/ADP Verhältnis auf.

Flavohämoglobine sind sehr wichtig für den Abbau von NO• und ihre Expression schützt Zellen vor der schädlichen Wirkung nitrosativen Stresses. Wir haben untersucht, ob Flavohämoglobine im generellen und auch Hämoglobine diese schützende Eigenschaften besitzen. VHb und andere Hämoglobine konnten ein normales Zellwachstum unter nitrosativem Stress erhalten. *In vitro* Experimente hingegen zeigten, dass die Fähigkeit NO• abzubauen bei Hämoglobinen eingeschränkt ist. Durch das Anfügen einer Reduktase an den C-Terminus eines Hämoglobins kann der NO•-Abbau jedoch signifikant erhöht werden, was die Wichtigkeit dieser Proteindomäne für die Entgiftung von NO• demonstriert.

VHb exprimierende Pappeln wurden generiert, um den Effekt von VHb in einer komplexen, mehrjährigen Pflanze zu analysieren. Das Wachstum dieser Bäume wurde unter verschiedenen Wachstumsbedingungen, d.h. bei verschiedenen Temperaturen, normaler und erhöhter UV-B Strahlung, verfolgt. Der Effekt der VHb-Expression auf das Wachstum war vom genetischen Hintergrund als auch von der Zellline abhängig. Nur wenige signifikante Unterschiede im Wachstum und in anderen phänotypischen Eigenschaften konnten festgestellt werden. Die Analyse der Zellstruktur zeigte hingegen, dass VHb exprimierende Zellen einen höheren Stärkegehalt besitzen als nichttransformierte Zellen. Dies ist ein Hinweis, dass die transgenen Pappeln in der Lage sind, Kohlenstoff effizienter zu fixieren oder den Kohlenstoff effizienter zu benutzen.

General introduction and literature overview

Significance of oxygen for life

The importance of oxygen relates to both its primary use as a substrate and its secondary effects on metabolism and physiology. Its use as a substrate offers the cellular metabolism to work at its optimal level regarding substrate utilization and to gain energy at its optimal yield. The latter effect of oxygen includes the regulation of a variety of cellular functions, which are expressed in response to the availability or absence of oxygen or the presence of toxic by-products, which are obligate consequences of aerobic lifestyle. Oxygen or free radicals produced from it can directly or indirectly affect bacterial cells in at least two ways. First, they can affect the biosynthesis of specific sets of proteins. Second, they can affect protein function.

In *Escherichia coli*, two groups of global regulatory proteins are generally involved in these two different set of physiological conditions: switches from aerobic to anaerobic conditions or *vice versa* are regulated by FNR and ArcAB, whereas SoxRS and OxyR are involved in the global regulation of genes, expressed in the response to oxidative stress conditions.

The molecule most intensely associated with utilization of oxygen by mankind is hemoglobin. Hemoglobin is the most studied and best characterized oxygen-binding protein, both at functional and molecular level. The presence of this oxygen-binding molecule has for long been thought to be restricted to mammals. But recent findings prove the all most ubiquitous existence in mammals, nonvertebrates, plants, and bacteria. The state of knowledge on bacterial hemoglobins and its implications on cellular metabolism is discussed below.

Oxygen sensing and molecular adaptation to hypoxia in *E. coli*

Oxygen is the electron acceptor of choice when *E. coli* cells are grown in the presence of oxygen. The use of oxygen allows the cellular metabolism to generate

energy in the most efficient way. During respiratory growth on oxygen, energy is produced by coupling substrate oxidation to the reduction of oxygen. Although *E. coli* possess several types of respiratory chains, in the presence of non-limiting amounts of oxygen it uses exclusively one of two aerobic respiratory chains, namely cytochrome *o*, to oxidize reduced foodstuffs. Substrates, such as glucose, pyruvate, lactate and succinate, are completely oxidized via tricarboxylic acid (TCA) cycle with concomitant transfer of electrons to the cellular quinone pool (43). The electrons are transferred further via cytochrome *b* to one of the two terminal oxidases (cytochrome *o* or cytochrome *d*). These two oxidases differ in their affinity for oxygen and in their turnover rates. Cytochrome *o* has a low affinity for oxygen (K_m = 0.35 μM) but a rather high V_{max}. In contrast, cytochrome *d* has a high oxygen affinity (K_m = 3-5 nM), but shows a substantially lower V_{max} relative to cytochrome *o*, resulting in the generation of a smaller proton motive force. Cytochrome *o* is the terminal oxidase of choice at atmospheric oxygen levels, whereas cytochrome *d* is active under microaerobic growth (119, 120, 123).

In the absence of oxygen but in the presence of an appropriate alternative electron acceptor, *E. coli* cells can produce a set of alternative respiratory oxidoreductases, which can catalyze the transfer of electrons to fumarate, nitrate, dimethyl sulfoxide and trimethylamine-N-oxide (87, 133).

In the complete lack of oxygen and in the absence of alternative electron acceptors the cellular metabolism of *E. coli* is switched to utilize fermentative pathways (17). These metabolic changes involve redox-balanced dismutation of the carbon source, substrate level phosphorylation and the formation of fermentation products: acetate, formate (H_2 and CO_2), ethanol, lactate, and succinate (17).

The switch between different metabolic modes is accompanied by fairly dramatic changes at the enzyme synthesis levels. These regulatory mechanisms ensure that the most energetically favorable metabolic pathway is used. Thus, oxygen is used in preference to nitrate, nitrate to fumarate, and fumarate to the endogenously generated electron acceptors, which are produced in the fermentative pathways

(145). Two sets of global transcriptional regulatory proteins ensure the expression of the appropriate enzymatic equipment: FNR and ArcAB, which can function both synergistically and antagonistically on the expression of certain genes, are involved in the cellular adaptation to the specific growth conditions. Furthermore, the regulatory action of FNR and ArcAB on adaptation to changing oxygen levels is assisted by other regulatory molecules, such as NarLP (Figure 1) (43).

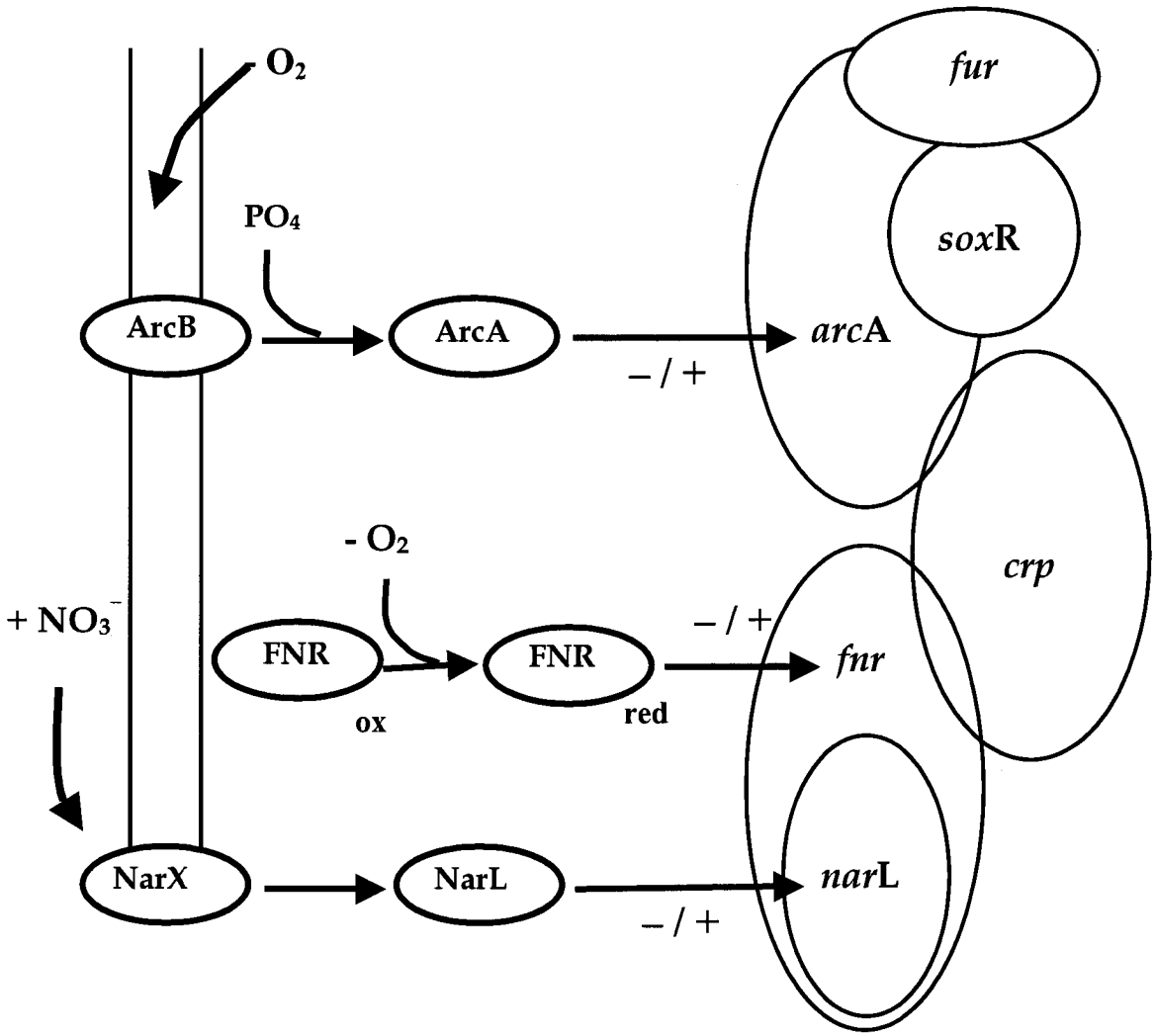

Figure 1. Network of overlapping oxygen related transcriptional regulators. Regulators are given in capitals and their respective modulons are given as ovals with the name of the regulatory protein in italics. All regulators are able to mediate induction and repression of gene expression.

The global transcriptional regulator FNR

The *fnr* gene was initially characterized by the isolation of some pleiotropic *E. coli* mutants, which characteristically lack the ability to use fumarate and nitrate as terminal electron acceptors for supporting anaerobic growth, thus FNR is designating defects in **f**umarate and **n**itrate **r**eduction (125-127).

FNR is a global regulator of a wide variety of *E. coli* genes. These include genes that are involved in respiration as well as in carbon metabolism. FNR elicits its regulatory function under anaerobic conditions and may act either as a transcriptional activator or as a repressor. FNR activates transcription of various operons such as *nar*GHJI, *frd*ABCD, *dms*ABC and *cyd*AB and represses transcription of *cyo*ABCDE, and its own expression (131). Coding sequences of FNR activated operons include genes encoding the expression of enzymes for alternative respiratory pathways and the microaerobic terminal oxidase cytochrome *d*. Repressed operons comprise the enzymes for cytochrome *o* complexes (Table 1). It is worth to notice that FNR and ArcAB are able to modulate gene expression in response to changes in oxygen levels either synergistically or antagonistically.

FNR accounts normally for 0.003 - 0.01% of the total bacterial protein, corresponding to approximately 1000 molecules per cell. Interestingly, FNR content is approximately at the same level in aerobically and anaerobically grown *E. coli* cells (146).

Structurally, FNR, a protein of 28 kDa, is similar to a different global regulatory protein, the catabolite repressor protein (CRP) of *E. coli* (128). Major differences between FNR and CRP include the lack of amino acids, which are involved in liganding of cAMP in CRP, an additional cysteine-rich N-terminal domain in FNR, which includes an [Fe-S] center and a slightly altered specificity for DNA binding, allowing the protein to discriminate between FNR and CRP binding sites within their cognate DNA binding motifs in the regulatory sequences (128). The FNR protein consists of a regulatory and a DNA binding domain, which is composed of a typical helix-turn-helix motif, and in which helix E is thought to lie across the

major groove of the DNA locking helix F in the major groove, where some amino acid side-chains specifically interact with bases in the DNA. Using site directed mutagenesis two residues have been shown to be essential for FNR-DNA interaction, E209 and S212 (130). The DNA binding site includes two inverted repeats TTGAT-N_4-ATCAA, forming the core element and differs from the CRP binding site by a single base in each repeat only, which allows the simple conversion of a FNR into a CRP binding site (7, 160) Where studied, promoters of various genes that are activated by FNR have no -35 sequence but have an FNR binding site centered at -39 to -49 relative to the transcriptional initiation point. Contrastingly, the genes that are repressed by FNR have a site, which either overlaps the -10 sequence and the transcriptional startpoint (+1) or is just upstream of the -35 sequence (50).

The regulatory domain of FNR as mentioned above comprises one Fe-S center. Active FNR, which is in a dimeric form, possess two [4Fe-4S] centers. These [4Fe-4S] centers are essential for sensing changes in oxygen level. Upon exposure to oxygen, the [4Fe-4S] center is converted to a [2Fe-2S] leading to the dissociation of the FNR dimer and to the concomitant inactivation of FNR (49, 73, 81).

It has been hypothesized before that oxygen *per se* is not the key regulator but a change in cellular redox state might be the effector in controlling the activity state of FNR in *E. coli*. Thus, the cysteine rich N-terminus forms part of a redox-sensing domain, the iron of which is in ferrous state under active anaerobic conditions (147). However, most recent results suggest a direct regulation of FNR activity by oxygen (6, 67).

FNR-like proteins have been identified both in gram$^-$ and gram$^+$ bacteria, where they have been implicated in the regulation of diverse anaerobic processes, such as nitrogen fixation, photosynthesis, and denitrification (129).

Table 1. Oxygen dependent gene expression mediated by FNR and Arc

Enzyme	Gene	FNR	Arc
TCA cycle related enzymes			
Aconitase (Stat.)	*acn*A	-[a)]	-
Aconitase	*acn*B		-
FumaraseA	*fum*A	-	-
FumaraseB	*fum*B	+[a)]	+
FumaraseC	*fum*C		-
Citrate synthase	*glt*A		-
Isocitrate DH	*icd*		-
Malate DH	*mdh*		-
Succinate DH	*sdh*CDAB	-	-
α-ketoglutarate DH	*suc*AB	-	-
Succinyl CoA synthetase	*suc*CD	-	-
Respiratory chain dehydrogenases			
Formate DH	*fdn*GHI	+	-
Glycerol-3-phosphate DH (anaerobic)	*glp*ACB	+	
Glycerol-3-phosphate DH (aerbic)	*glp*D		-
Hydrogenase I	*hya*A-F		+
L-lactate DH	*ild*		-
NADH DH I	*nuo*	-	-
NADH DH II	*ndh*	-	
Cytochrome *d* oxides	*cyd*	-	+
Terminal oxidases			
Cytochrome *o* oxidase	*cyoABCDE*	-	-
Cytochrome *d* oxidase	*cyd*AB	-	+
DMSO reductase	*dmsABC*	+	+
Fumarate reductase	*frd*ABCD	+	
Nitrate reductase	*nar*GHJI	+	
Nitrate reductase (periplasmic)	*anp*F	+	
NADH-dependent nitrate reductase	*nirBDC*	+	
Formate linked nitrate reductase	*nrf*A-G	+	
Others			
Formate transport and pyruvate-formate lyase	*foc*A-*pfl*	+	+
Pyruvate DH complex and regulator	*pdhR-ace*EF-*lpd*	-	-
VHb	*vhb*	+	
FHP	*fhp*	+	

[a)] + denotes transcriptional activation, whereas – denotes repression of the respective genes.

The expression of various bacterial hemoglobin and flavohemoglobin genes, such as the *hmp* genes from *Ralstonia eutropha, E. coli, Vitreoscilla* and *Bacillus subtilis* have

been shown to be controlled by changes in oxygen levels via FNR mediated regulation, although other regulatory proteins are involved in the regulation of expression of these diversely regulated genes (18, 74, 85, 111).

ArcAB modulon

The ArcAB system was characterized by Lin and coworkers (64, 66, 86). They isolated *E. coli* mutants, which were unable to repress a whole set of aerobic functions, including TCA cycle, the glyoxylate cycle, fatty acid oxidation enzymes and cytochrome *o*. The corresponding mutant genes were named *arc*A and *arc*B, designating their lack of aerobic respiration control.

The DNA binding site of ArcA has been defined from multiple alignments of promoter sequences that are regulated by ArcA and by DNA footprinting assays. The consensus sequence for ArcA binding includes 10 base pairs (5'-[A/T]GTTAAGGA[A/T]-3') (91).

ArcAB is a two component regulatory system, which consists of a membrane bound sensor ArcB, detecting a switch from aerobic to anaerobic metabolism and the cognate regulatory protein ArcA. ArcB has both kinase and phosphatase activities. The kinase activity is able to auto-phosphorylate ArcB as well as ArcA. When stimulated, ArcB undergoes auto-phosphorylation at His292. The phosphoryl group is concomitantly and intramolecularly transferred to Asp576, causing probably a conformational change. This change allows ArcB to transfer the phosphoryl group from His292 to ArcA instead to the already phosphorylated Asp576 residue during the next round of auto-phosphorylation. When phosphorylated ArcA may function either as a transcriptional activator or repressor (62, 65).

The stimulus for ArcB phosphorylation does not appear to be oxygen itself but instead it has been suggested that either the state of respiration or the redox state of the cytosol regulate the rate of phoshorylation (63, 86). It has been suggested that

the phosphatase activity of ArcB is a key player in the regulation of the ArcAB activity. Metabolites, such as lactate, pyruvate, acetate and NADH, which accumulate during anaerobic growth, inhibit the intrinsic phosphatase activity of ArcB. The lack of the phosphatase activity leads to the irreversible phosphorylation of Asp576 of AcrB and therefore, to the intermolecular transfer of phosphoryl groups to ArcA (62).

ArcB has been proposed to be member of the PAS domain superfamily. PAS domains are signaling domains that are widely distributed in sensory proteins from prokaryotes and eukaryotes, where they monitor changes in light, redox state and oxygen (139). This suggests that the PAS domain of ArcB might respond to a reduced intermediate that accumulates when electron flow to O_2 is interrupted following a decrease in O_2 tension. It is therefore likely that ArcB may monitor the electron flux trough the respiratory chain rather than sensing oxygen itself (91).

Oxidative and nitrosative stress in *E. coli*

Oxidative and nitrosative stress are a common phenomenon and are caused by the exposure of the cell to reactive oxygen and nitrogen intermediates, which can both damage biomolecules such as proteins, nucleic acids and cell membranes. Organisms face both endogenously and exogenously produced stress during their lifetime. Endogenously, common metabolic processes, such as respiratory activity or denitrifying processes, produce these stress factors; exogenous sources include mechanisms such as attack of the immune system or plant pathogen response against invading cells.

It is now well established that the radical nitrogen monoxide (NO) is generated endogenously as an obligate intermediate in the reduction of nitrate to dinitrogen in the denitrifying bacteria. The concentration of NO is estimated to vary between 10^{-9} M and 10^{-7} M (47). The formation of NO is catalyzed by nitrite reductases, such

as copper, cytochrome *cd*1 reductases, where nitrite is reduced to NO; the produced NO is subsequently reduced to nitrous oxide by nitric oxide reductase (47).

In exponentially growing bacteria both H_2O_2 and superoxide are generated by the autooxidation of components of the respiratory chain (46, 59). The flavin of NADH dehydrogenase II is the primary site of electron transfer to oxygen in the aerobic respiratory chain. Steady-state concentration of O_2^- and H_2O_2 are 10^{-10} M and 10^{-7}-10^{-6} M, respectively (46, 48, 59). Both levels are still below the level of toxicity (134).

The constitutive defense mechanisms are inadequate, if the rates of intracellular formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are enhanced. For example, pathogenic bacteria are bombarded by a collection of various ROS and RNS when they are captured by the phagocytes. The arsenal of toxic products utilized by phagocytes to disarm invading bacteria include O_2^-, NO, HOCl, H_2O_2, HO^-, HOONO and RSNO (27). Each of these reactive species is bacteriostatic or even bacteriocidal, but their impact is strongly dependent on their ultimate concentrations inside of the bacteria (134).

To overcome these threatening radicals bacterial cells possess constitutively expressed enzyme machinery that detoxifies the reactive species and repairs the damage. Under normal growth conditions a balance between the amount of produced radicals and the cellular defense mechanisms exists. In addition, exposure of cells to a boost of radicals results in the rapid induction of adaptive defense enzymes, which are under the common global control of SoxRS and OxyR regulatory proteins in *E. coli*.

SoxRS modulon

The SoxRS regulon comprises a variety of defense related enzymes, which have in common the induction by superoxide radicals and NO. SoxRS is a two-component regulatory system. SoxR is a latent transcription factor, which controls the activity of SoxS. SoxR is a homodimer of 17 kDa per polypeptide, which contain a helix-

turn-helix DNA binding motif in the N-terminal domain. At its C-terminus, a redox-sensitive $[2Fe\text{-}2S]^{1+}$ cluster is located (55, 159), which controls the activity of the protein. It is not yet clear if the SoxR protein directly or indirectly responds to superoxide and NO. Indirect means of activation include depletion of the cellular NADPH pool; the size of it in turn would be regulated by the SoxS responsive *zwf* (glucose-6-phophate dehydrogenase). The latter mechanism would represent an attractive auto-regulation mechanism of SoxRS activity (162).

Upon oxidation of the cluster to $[2Fe\text{-}2S]^{2+}$, the SoxR protein undergoes a conformational change, which enables the protein to activate the regulatory sequence of *sox*S gene. The up to 100-fold increase in SoxS level in turn mediates thereafter the expression of the various defense proteins, such as manganese superoxide dismutase (*sod*A), the DNA repair enzyme endonuclease IV (*nfo*), and the superoxide radical resistant isoenzymes of fumarase (*fum*C) and aconitase (*acn*A) in *E. coli*. SoxS also activates glucose-6-phosphate dehydrogenase (*zwf*), which increases the reducing power of the cell. SoxRS regulon has been found to confer resistance against many different drugs as well as organic solvents and reactive nitrogen species (99, 104).

The oxyR modulon

The expression of many H_2O_2 inducible activities is regulated by the OxyR transcription factor in *E. coli*. OxyR-mutants are hypersensitive to hydrogen peroxide (15). Active OxyR forms a tetramer *in vivo* with subunits of 34 kDa. The helix-turn-helix DNA binding motif is located at the N-terminus. The binding sequence includes four sets of 4 base pair sequences, which are separated by 7 non-conserved bases. The binding sequence of OxyR is spanning between -35 and -80 region of various *E. coli* promoters (140).

OxyR is activated by H_2O_2 through oxidation. Upon oxidation of OxyR, disulfide bridges are formed and the protein is in its active conformation. Mutation of either

of the two conserved cysteine residues involved in disulfide bond formation abolishes the ability to sense hydrogen peroxide. OxyR remains oxidized and activated only for a finite period of time *in vivo*. It has been proposed that the inactivation of OxyR occurs after the reduction of the disulfide bridges by glutaredoxin 1 that is itself part of the OxyR response regulon (117, 161). Thus, the inactivation of OxyR is auto-mediated. The minimal hydrogen peroxide concentration required to completely oxidize OxyR *in vivo* is 5 μM (4).

Furthermore, OxyR is activated by nitrosative stress (53). Activation of OxyR by nitrosative stress occurs by S-nitrosylation of cysteine residue and not by oxidation (53). Hausladen et al. (53) suggest that the activation of OxyR by nitrosative stress effects is reasonable inasmuch as the effects of ROS and RNS are synergistic and the effects of ROS and RNS exposure are similar on the cellular metabolism, for example the depletion of the cellular GSH pool.

The members of OxyR regulon include catalase (*kat*G), glutaredoxin 1 (*grx*A), and glutathione reductase (*gor*A). In *oxyR*$^-$ strains 20 to 30 proteins are still inducible by hydrogen peroxide, but theses strains have increased spontaneous mutation rates.

OxyR regulated activities mediate resistance against HOCl, organic solvents and reactive nitrogen species (134).

OxyR and SoxRS independent functions against nitrosative and oxidative stress

Besides genes that are regulated in response to nitrosative and oxidative stress by the above-described transcriptional regulatory proteins, there exists accumulated evidence that other cellular components are also involved in the defense response towards ROS and RNS, which are regulated independently of SoxRS and OxyR.

Role of microbial flavohemoglobins in the defense against nitrosative and oxidative stress

Flavohemoglobin proteins from several bacteria and yeasts have been shown to be major contributors to the detoxification of NO under aerobic conditions and to be essential for resistance against nitrosative stress (20, 40, 112). The expression of flavohemoglobin of *E. coli* has been shown to be independent of SoxRS regulation but to be controlled by MetR (95, 111). The role of flavohemoglobins in the resistance to nitrosative stress is discussed below.

The knowledge on the interaction of flavohemoglobins and oxidative stress is still in its infancy. Although the involvement of these proteins in response to oxidative stress has been reported, it is not yet clear what role these proteins play in bacteria. A potential role as amplifier of oxidative stress has been proposed for the flavohemoglobin of *E. coli* (112).

NO reduction by *nor*RVW modulon

Recent findings by Gardner and coworkers (36, 37) showed the existence of an additional $NO^{\bullet}$ detoxification pathway in *E. coli*, which is active under anaerobic conditions. The operon includes three genes *yga*AKD. *Yga*A encodes a transcription regulator; *yga*K and *yga*D encode a flavorubredoxin (RB) and a NADH:flavoRB oxidoreductase, respectively (36, 37). Gardner et al. (37) proposed to designate the *yga*AKD gene cluster as the *nor*RVW modulon for NO reduction and detoxification. YgaA encodes a protein bearing 42% identity with the NO-modulated *Ralstonia euthropha* transcription regulators NorR1 and NorR2 (110). NorR homologous are also encountered in *Vibrio cholera* and *Pseudomonas aeruginosa* (54, 135).

Hemoglobins: an overview

Hemoglobins are encountered in all five kingdoms of organisms. Generally, Hb's are classified into vertebrate and nonvertebrate Hb's. The latter group also includes hemoglobins encountered in plants, fungi, and protozoa. The common characteristic of all hemoglobins is their ability to reversibly bind oxygen. Although the alignment of the protein sequences of hemoglobins from various sources reveals a highly variable or even almost random primary amino acid sequence, two key residues are conserved among all proteins encountered so far: Phe at position CD1 and His at position F8. Analysis of protein structures of hemoglobins reveals a typical tertiary structure, the classical Hb fold. This highly conserved structure consists of six to eight α–helical structures, which are connected by short intervening loops. The 3-on-3 helix structure forms a sandwich-like assembly, which is able to bind the heme moiety within a cavity surrounded by hydrophobic residues.

Phylogenetic trees based on the known present-day globin sequences point to a common and quite ancient globin ancestor, which possibly is a primitive archaebacterium that developed approximately 3.5×10^9 years ago.

Vertebrate hemoglobins are encountered circulating in body fluids, where they serve to deliver oxygen or are expressed in muscles tissues (myoglobin) where they donate oxygen to the respiring cells. Very recently, an alternative Hb has been discovered in nerve tissues of man and mouse. This neuroglobin has been proposed to deliver oxygen to brain tissue. Neuroglobin has a unique exon-intron structure and therefore it is proposed to represent a distinct protein family (13).

Nonvertebrate Hb's occur from monomeric to multisubunit structures and are encountered in widely different anatomical sites either in cytoplasm of specific tissues or freely dissolved in various body fluids. Nonvertebrate Hb's display a much higher variation in their primary and tertiary structures. This high degree of variability may indicate their much broader defined adaptations to specific

functions compared to their vertebrate homologs. The knowledge about non-vertebrate Hb is not as complete as in the case of vertebrate Hb.

Over the last dozen years, monomeric single domain globins have been found in symbiont-containing leguminous and nonleguminous plants, algae, and a number of prokaryotes, ranging from bacteria to cyanobacteria. Chimeric Hb's consisting of an N-terminal globin domain and an adjacent C-terminal redox-active protein domain (collectively called flavohemoglobins) have been found in bacteria and yeast. Furthermore, truncated Hb's have been identified in plants, bacteria and cyanobacteria (98, 116, 150).

Bacterial hemoglobins and flavohemoglobins

Identification of hemoglobins and flavohemoglobins and their primary sequences

Bacterial hemoglobins and flavohemoglobin proteins have been identified in a variety of microorganisms. A summary of hemoglobins and flavohemoglobins genes that are isolated and characterized or of genes whose existence is predicted due to high amino acid homologies to known proteins is given in Table 2. Besides these two groups of proteins, a third group, designated as truncated hemoglobins exists, and it is characterized by a shortened protein sequence of approximately 110-120 amino acids. Truncated hemoglobins are suggested not to originate from ancestral hemoglobin but to have evolved from a different gene, namely an indoleamine 2,3-dioxygenase (136).

Vitreoscilla hemoglobin (VHb) is the best-characterized member of the group of bacterial Hb proteins. VHb is expressed under oxygen-limited conditions in *Vitreoscilla*, which lives in stagnant ponds and decaying plant material (153). The primary protein sequence and the DNA sequence of VHb have been reported (25, 77, 149). The gene encoding VHb is 441 bp long, which results in a protein of a molecular weight of 15.7 kDa as determined experimentally and calculated from the amino acid composition (149). Initially called cytochrome *o*, VHb was believed

to be a soluble terminal oxidase in *Vitreoscilla* (153, 154). Interestingly, when purifying VHb, an NADH metHb reductase was also copurified (45, 154).

Table 2. Compilation of bacterial hemoglobin and flavohemoglobin genes, which are currently deposited at the Gene bank, or of genomic sequences which allow the identification of potential globin genes

Organism	Gene	Gene bank	Length (bp)	Reference
Campylobacter jejuni NCTC11168	chb	AL139079	423	(8)
Clostridium perfringens hyp27	Cp-*hb*	AB028630	435	(33)
Vitreoscilla sp.	*vhb*	M30794	441	(25, 77)
Aquifex aeolicus		AE000678.1 NC_000918.1	420	
Bacillus halodurans C-125	*hmp*Bh	AB024563	1236	(33)
Bacillus subtilis 168trpC2	*hmp*Bs	D78189	1200	(85)
Deinococcus. radiodurans	*hmp*Dr	AE001863	1212	(8)
Escherichia coli MG1655	*hmp*	X58872	1191	(148)
Erwinia chrysanthemi	*hmp*X	X75893	1188	(84)
Pseudomonas. aeruginosa PAO1	*hmp*Pa	AE004695	1182	(8)
Ralstonia eutropha	*fhp*	X74334	1212	(18)
Staphylococcus. aureus MU50		NC_002758.1	1146	
Staphylococcus aureus N315		AP003129.2	1146	
Streptomyces coelicolor A3	*hmp*A2	AL354616.1	1212	(33)
Streptomyces coelicolor A3	*hmp*A	AL158061	1197	(33)
Streptomyces coelicolor A3 cosmid J11		AL109949.1	1308	
Salmonella enterica subsp. Typhi	*hmp*St	AL627275	1191	(8)
Salmonella typhimurium	*hmp*Stm	AF020388	1191	(19, 20)
Salmonella typhimurium LT2		AE008816.1	1191	(19, 20)
Sinorhizobium meliloti	*hmp*Sm	NC_003037	1212	(33)
Vibrio cholerae		AE004358.1	1185	
Vibrio parahämolyticus		U09005.1	1185	
Xylella fastidiosa 9a5c		AE003859.1 NC_002488.1	1194	
Yersinia pestis OC92		AJ414154.1	1191	

VHb is encountered *in vivo* as a dimer, but it is not known if other hemoglobins share this property of VHb. Early work on VHb has been reviewed and is not summarized in this work (152). Further hemoglobin proteins, which include

hemoglobins from *Campylobacter jejuni* and *Clostridium perfringens*, have been isolated and characterized in our laboratory (8, 33).

The strictly respiratory, denitrifying, Gram-negative bacterium *R. eutropha*, formerly *Alcaligenes eutrophus*, synthesizes an alternative form of bacterial hemoglobins - a flavohemoprotein or flavohemoglobin called FHP (118). The coding sequence of this protein is plasmid determined (158) and was isolated from a megaplasmid library by using FHP-specific antibodies and oligonucleotide probes based on the amino-terminal polypeptide sequence of a yeast flavohemoglobin, determined previously by Zhu and Riggs (163). The *fhp* gene encodes a soluble monomeric polypeptide of 403 amino acids having a molecular weight of 44 kDa (18).

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FHP      M----LTQKTKDIVKATAPVLAEHGYDIIKCFYQRMFEAHPELK-NVFNMAHQEQGQQQQALARAVYAYAENIEDPNSLM 75
HmpEc    M----LDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPELK-EIFNMSNQRNGDQREALFNAIAAYASNIENLPALL 75
HmpStm   M----LDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPELK-EIFNMSNQRNGDQREALFNAIAAYASNIENLPALL 75
HmpSt    M----LDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPELK-EIFNMSNQRNGDQREALFNAIAAYASNIENLPALL 75
HmpX     M----LDQQTIATIKSTIPLLAETGPALTAHFYQRMFHHNPELK-DIFNMSNQRNGDQREALFNAICAYATHIENLPALL 75
HmpBs    M----LDNKTIEIIKSTVPVLQQHGETITGRFYDRMFQDHPELL-NIFNQTNQKKKTQRTALANAVIAAAANIDQLGNII 75
HmpBh    MSTATLSQETKQIVKATVPILAEHGEAITKHFYKRMFSHHPELL-NIFNQTHQKQGRQPQALANSIYAAAEHIDNLEAIL 79
HmpDr    M----LTPEQKAIVKATVPALEAHGETITRTFYASMFAAHPELL-NIFNPANQQTGKQARSLAASVLAYAAHIDHPEALG 75
HmpPa    M----LSNAQRALIKATVPLLETGGEALITHFYRTMLGEYPEVR-PLFNQAHQASGDQPRALANGVLMYARHIDQLQELG 75
HmpSc    M----LSSQSAQIVRDTLPTVGASLGTITDLFYRRMFEERPELLRDLFNRANQASGVQREALAGAVAAFATALVKHPDER 76
CHb      M-----TKEQIQIIKDCVPILQKNGEDLTNEFYKIMFNDYPEVK-PMFNMEKQISGEQPKALAMAILMAAKNIENLENMR 74
Cp-Hb    M----LDQKTIDIIKSTVPVLKSNGLEITKTFYKNMFEQNPEVK-PLFNMNKQESEEQPKALAMAILAVAQNIDNLEAIK 75
VHb      M----LDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVR-PLFDMGRQESLEQPKALAMTVLAAAQNIENLPAIL 75

FHP      A--VLKNIANKHASLGVKPEQYPIVGEHLLAAIKEVLGN-AATDDIISAWAQAYGNLADVLMGMESELYERSAEQPGGWK 152
HmpEc    P--AVEKIAQKHTSFQIKPEQYNIVGEHLLATLDEMFSP---GQEVLDAWGKAYGVLANVFINREAEIYNENASKAGGWE 150
HmpStm   P--AVEKIAQKHTSFQIKPEQYNIVGTHLLATLDEMFNP---GQEVLDAWGKAYGVLANVFIHREAEIYHENASKDGGWE 150
HmpSt    P--AVEKIAQKHTSFQIKPEQYNIVGTHLLATLDEMFNP---GQEVLDAWGKAYGVLANVFIHREAEIYHENASKDGGWE 150
HmpX     P--AVERIAQKHASFNIQPEQYQIVGTHLLATLEEMFQP---GQAVLDAWGKRYGVLANVFIQRESDIYQQSAGQNGGWH 150
HmpBs    P--VVKQIGHKHRSIGIKPEHYPIVGKYLLIAIKDVLGD-AATPDIMQAWEKAYGVIADAFIGIEKDMYEQAEEQAGGWK 152
HmpBh    P--VVSRIAHKHRSLNIKPEQYPIVGENLLAAMREVLGD-AASDDVLEAWREAYELIADVFIQVEKKMYEEASQAPGGWR 156
HmpDr    G--MVGRIAHKHVSLEVLPEHYPIVGQYLLGAIAGVLGD-AAKPEILDAWAAAYGELADLMIGIEKGMYDAGAGQPGGWR 152
HmpPa    P--LVAKVVNKHVSLQVLPEHYPIVGTCLLRAIREVLGEQIATDEVLEAWGAAYQQLADLLIEAEESVYAASAQADGGWR 153
HmpSc    PDAVLGRIANKHASLGITSDQYTLVGRHLLAAVAEVLGD-AVTPAVAAAWDEVYWLMANALIAMEARLYARSDVEDG--S 153
CHb      S--FVDKVAITHVNLGVKEEHYPIVGACLLKAIKNLLN---PDEATLKAWEVAYGKIAKFYIDIEKKLYDK          140
Cp-Hb    P--VVNRIGVIHCNAKVQPEHYPIVGKHLLGAIKEVLGD-GATEDIINAWAKTYGVIAEVFINNEKEMYASR          144
VHb      P--AVKKIAVKHCQAGVAAAHYPIVGQELLGAIKEVLGD-AATDDILDAWGKAYGVIADVFIQVEADLYAQAVE        146

FHP      GWRTFVIREKRPESDVITSFILEPADGGPVVNFEPGQYTSVAIDVPALGLQQIRQYSLSDMPNGRTYRISVKREGG-G-P 230
HmpEc    GTRDFRIVAKTPRSALITSFELEPVDGGAVAEYRPGQYLGVWLKPEGFPHQEIRQYSLTRKPDGKGYRIAVKREEG---- 226
HmpStm   GTRPFRIVAKTPRSALITSFEFEPVDGGTVAEYRPGQYLGVWLKPEGFAHQVFRQYSLTRKPDGKGYRIAVKREDG---- 226
HmpSt    GTRPFRIVAKTPRSALITSFEFEPVDGGTVAEYRPGQYLGVWLKPEGFAHQEIRQYSLTRKPDGKGYRIAVKREDG---- 226
HmpX     GIRPFRIVAKQPQSSLITSFTLEPVDGGPIAAFRPGQYLAVYIRDKRFEYQEIRQYSLTNEPNGRYYRIAVKRETM---- 226
HmpBs    EYKPFVIAKKERESKEITSFYLKPEDSKPLPEFQAGQYISIKVRIPDSEYTHIRQYSLSDMPGKDYYRISVKKD------ 226
HmpBh    EFRSFVVEKKQRESATITSFYLKPEDGKALASYKPGQYITVKVTIPGHEHTHMRQYSLSDAPEKGYYRITVKREEGDG-D 235
HmpDr    DFRPFRVARKVAESRVITSFVLEPVGGGALPAYQPGQYLSLKVKVPGQERWQIRQYSLSDAPSPDHYRISVKREGG---- 228
HmpPa    GVRRFRVARKQAESEEITSFYLEPVDGQPLLAFQPGQYIGLRLDIDGEE--VRRNYSLSAASNGREYRISVKREAG---- 227
HmpSc    VWQSMEIVERHEETPDTASFVLRPADGSPTRPFVPGQYVSVRAELPDGAH-QIRQYSLSSAPGGGTWRFTVKRERSLDGQ 232
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FHP      QPPGYVSNLLHDHVNVGDQVKLAAPYGSFHIDVDAKTPIVLISGGVGLTPMVSMLK-VALQAPPRQVVFVHGARNSAVHA 309
HmpEc    ---GQVSNWLHNHANVGDVVKLVAPAGDFFMAVADDTPVTLISAGVGQTPMLAMLDTLAKAGHTAQVNWFHAAENGDVHA 303
HmpStm   ---GQVSNWLHHHASVGDVVHLAAPAGDFFMNVAADTPVSLISAGVGQTPMLAMLDTLAKEQHTAQVNWFHAAENGDVHA 303
HmpSt    ---GQVSNWLHHHANVGDVVHLAAPAGDFFMNVAADTPVSLISAGVGQTPMLAMLDTLAKEQHTAQVNWFHAAENGDVHA 303
HmpX     ---GSVSGYLHDVAREGDVIELAAPHGDFYLEVTPETPVALISAGVGQTPMLSMLHSLKNQQHQADIFWLHAAENTEVHA 303
HmpBs    ---GVVSSYLHDGLQEGDSIEISAPAGDFVLDHASQKDLVLISAGVGITPMISMLKTSVSKQPERQILFIHAAKNSEYHA 303
HmpBh    LPPGIVSNYLHQHIHEGDVLEITAPAGDFTLQEEGERPIVFISGGVGITPLMSMFNTLMQRGVKREVIFIHAAINGFYHA 315
HmpDr    ---GLVSEYLHGAVQEGDELLVHVPAGDFVLQQ-SERPVVLISAGVGITPMLAMVQTLAQAGSQRPVTFIHAAQNGSVHA 304
HmpPa    ---GRVSNYLHDRVAEGDELDLFPPAGDFVLRD-SDKPLVLITAGVGITPALAMLQEALPQA--RPIRFIHCARHGGVHA 301
HmpSc    VPDGEVSTWLHTHARPGDVLRVSLPFGDLLLPE-GDGPLLLASAGIGVTPMLAMLEHLATAAPDRPVTVVHADRSPALHA 311

FHP      MRDRLREAAKTYENLDLFVFYDQPL-PEDVQGRDYDYPGLVDVKQIEKSILLP-DADYYICGPIPFMRMQHDALKNLGIH 387
HmpEc    FADEVKELGQSLPRFTAHTWYRQPS-EADRAKGQFDSEGLMDLSKLEGAFSDP-TMQFYLCGPVGFMQFTAKQLVDLGVK 381
HmpStm   FADEVSELGRTLPRFTAHTWYREPT-EADRAQRLFDSEGLMDLSKLEAAISDP-AMQFYLCGPVGFMQFAAKQLVSLGVN 381
HmpSt    FADEVSELGRTLPRFTAHTWYREPT-EADRAQRVFDSEGLMDLSKLEAAISDP-AMQFYLCGPVGFMQFAAKQLVSLGVN 381
HmpX     FADEIADVAATLPQLQSYVWYREASSEAARSAHAFH--GLMALKDLPTPLPMT-NLHCYLCGPVAFMQFAARQLLELGIT 380
HmpBs    LRHEVEEAAKHS-AVKTAFVYREPT-EEDRAGDLHFHEGQIDQQFLKELIANT-DADYYICGSPSFITAMHKLVSELGSA 380
HmpBh    MHDHLAQTASQQENVHYAVCYERPT-PGDRMNPFMKKEGFIDESFLRSILHDR-EADFYFCGPVPFMKTIAQILKDWDVP 393
HmpDr    FRDDVARLTHEYPHFRKVVFYDEAG-PDDQLGTHHDVAGRLSLDAVRGALPAG-EAEFYYCGPAGFAGAVEAILDDLQVP 382
HmpPa    FRDWIEDVSAQHEQVEHFFCYSEP-----RAGDSADAEGLLSREKLADWLPQERDLDAYFLGPRPFMAQVKRHLADLGVP 376
HmpSc    HRLELTALVERLPHASLHLWYEDTADHPDASADHVN-EGWADLTGLSPVPGTT----AFLCGPLSFMKAVRTDLLAHGLS 386

FHP      EARIHYEVFGPDLFAE----- 403
HmpEc    QENIHYECFGPHKVL------ 396
HmpStm   NENIHYECFGPHKVL------ 396
HmpSt    NENIHYECFGPHKVL------ 396
HmpX     ESQIHYECFGPHKVI------ 395
HmpBs    PESIHYELFGPQLSLAQSV-- 399
HmpBh    EQQVHYEFFGPAGTLASS--- 411
HmpDr    AERRFTETFGPSQSFAPVILG 403
HmpPa    SQQCHYEFFGPAAALDA---- 393
HmpSc    PRAIHYEVFGPDLWLSK---- 403
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Figure 2. Multiple amino acid alignment of flavohemoproteins and hemoglobins, which have recently been studied. VHb (*Vitreoscilla,* (25, 77), HmpEc (*E. coli,* (148), FHP (*R. eutropha,* (18)), HmpX (*E. chrysanthemi,* (35)), HmpBs (*B. subtilis,* (85)), HmpStm (*S. typhimurium,* (19, 20)) CHb, HmpPa, HmpDr and HmpSt (*C. jejuni, D. radiodurans, S. typhi,* (8)), HmpSc, HmpBh, and Cp-Hb (*S. coeolicolor, B. halodurans, C. perfringens,* (33)). Throughout all sequences conserved amino acids are highlighted.

Flavohemoprotein from *E. coli* (HMP) has been isolated in an attempt to identify dihydropteridine activities and it is up to date the best characterized member of flavohemoglobin protein family (148). The molecular weight of HMP is 44 kDa and shows high amino acid similarity with FHP. Both HMP and FHP share the same structural characteristics typically encountered in flavohemoproteins; a globin domain at the N-terminus with a characteristic ability to abduct CO and a redox-active domain at the C-terminus. Previously, flavohemoglobins from *Erwinia chrysanthemi, B. subtilis, Salmonella typhimurium* and *Mycobacterium tuberculosis* have been isolated and characterized at the level of DNA and protein (19, 20, 35, 57, 85). Further flavohemoglobin proteins have been recently isolated from *Deinococcus*

radiodurans, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus halodurans, Streptomyces coelicolor, Salmonella typhi (Table 2) (8, 33). The length of the coding sequences of flavohemoglobins is approximately 1200 basepairs long, resulting in proteins of approximately 400 amino acids and a molecular weight of approximately 44kDa.

The alignment of the protein sequences reveals the occurrence of several conserved residues in both the hemoglobin domain and in the redox-active domain (Figure 2). The role of these conserved residues is discussed below.

Structure and biochemical properties

Structure of VHb and FHP

The three dimensional (3-D) structures of FHP and VHb have been resolved by X-ray crystallography (30, 31, 137, 138). The 3-D structures of the hemoglobin domain of FHP and VHb conform to the well-known globin fold. The heme molecule is embedded in a hydrophobic crevice formed by the helices B, C, E, F, G and H. A rigid binding between the polypeptide chain and the porphyrin ring is provided predominantly by hydrophobic side chains towards the proximal side. His85 binds covalently the heme in both FHP and VHb. No protein-heme interactions are detected at the distal side of the heme molecule. A clear difference of these two structures relative to mammalian hemoglobins is the lack of helix D, i.e. the residues connecting helices C and E do not adopt the usual α-helical conformation. The observed disorder in the CE region might indicate a potential site of interaction of the globin domain of FHP with the FAD of its reductase domain. In analogy, this would provide a potential contact site of VHb with the NADH methemoglobin reductase. (9, 30, 138). Overall the structures of VHb and the globin domain of FHP are highly similar (9). A striking difference between VHb and the globin domain of FHP is the displacement of helix E in FHP, opening the angle between helix E and F. The heme-binding crevice is therefore enlarged thus providing more space at the

ligand-binding site (30). Indeed, a phospholipid has been found to bind the hydrophobic crevice, but the function of this ligand is not known (105).

The quaternary assembly of homodimeric VHb, which has not been observed before within the globin family, is based on a molecular interface defined by helices F and H of both subunits and essentially involves van der Waals contacts among the two subunits. The two heme-iron atoms approach to a distance of 34 Å (138).

In contrast to the classical hemoglobins, FHP contains a C-terminal-located redox-active domain with potential binding sites for NAD(P)H and FAD, which adopts a fold similar to ferredoxin NADP+ reductase (FNR)-like proteins (72). FNR-like proteins form a group of structurally related proteins, which do not share any functional and structural properties with the global regulatory protein FNR. The reductase domain can be divided into a FAD binding (residues153-258) and a NADH binding (266-403) domain. The former consists of a six-stranded antiparallel β-barrel, whereas the latter is built up of a five-stranded parallel β-sheet flanked by two respectively one helix on each side. The FAD group binds non-covalently in an extended conformation to all three domains of FHP. The heme and FAD molecules approach each other to a minimal distance of 6.3 Å enabling electron transfer from FAD to heme moiety in a predominantly polar environment provided by several amino acid side chains (30). FHP displays a large interdomain cleft. The relative spatial arrangement of FAD and NADH binding domains within FNR-like proteins allows variance. A clear characterization of NADH binding to FHP has not been shown.

In general, hemoglobins and flavohemoglobins share a number of residues, which are invariant in all known sequences. The role of some of the conserved residues is known for some characterized proteins or can be derived from the homologies of the sequences (Figure 2). Around Phe43(CD1) (topological position according to the standard globin fold) a hydrophobic cluster is formed. A further hydrophobic cluster is centered around Trp122(H7). These two clusters are essential for folding and stability. In all known globins Phe43(CD1) and His85(F8) are invariant.

His85(F8), Tyr95(G5) and Glu137(H23) form a hydrogen bond network. The interaction of Fe-His-Glu-Tyr can modulate the redox properties of the heme iron atom and the structural arrangement resembles the catalytic triad observed in cytochrome *c* peroxidase. These interesting structural differences around His85(F8) may transform the bacterial hemoglobin and flavohemoglobin proteins form oxygen storage and transport proteins to constituents of an electron-transferring enzyme (30). Tyr29(B10) is thought to stabilize the Fe-O_2 complex in the HMP protein and is present in all known bacterial hemoproteins (38).

In the FAD binding domain of flavohemoglobin proteins several residues are highly conserved: Tyr209, which contacts Lys84(F7) and Glu394 and the flavin ring of FAD, Gln207, which stabilizes a water molecule, this water bridges FAD and heme, Gln207 is replaced by Asn in HmpPa, Arg206, Tyr208 and Ser209, which side chains contact the FMN moiety. In the NAD binding domain Glu384 is highly conserved and its side chain contacts Lys84(F7) forming a salt bridge. Lys84(F7) and Gln384 are part of a number of polar residues whose predominantly large side chains originate from all three domains. These residues may influence the electrochemical potential of the prosthetic heme and FAD groups. Lys84(F7) is replaced by Thr and Ile in the Hb sequences of *C. jejuni* and *C. perfringens*, respectively, but is conserved in VHb.

Biochemical properties of hemoglobins and flavohemoglobins

Hemoglobins are generally associated with oxygen binding and transport. Indeed bacterial Hb and flavohemoglobin proteins are able to reversibly bind molecular oxygen. However, due to their structural properties, i.e. the occurrence of a intermolecular redox-domain in flavohemoglobins and the presence of the catalytic triade at the distal heme side in both hemoglobins and flavohemoglobins, Ermler et al. (30) proposed an electron transferring rather than an oxygen delivering role for FHP. Thus, this suggestion may question the role of bacterial hemoglobins and flavohemoglobins in simple oxygen storage and delivery.

Ligand binding of O_2, CO and NO

Bacterial hemoglobins and flavohemoglobins are able to bind various heme ligands, such as O_2, CO, NO and CN^-, and the binding of those is dependent on the oxidation state of the heme iron. The absorption spectra of bacterial hemoglobins and flavohemoglobins with various ligands have been shown to be characteristic for hemoglobins (11, 61, 106, 144, 149, 155). Whereas oxygen and CO binds only to the Fe(II), NO in contrary binds to Fe(II) and Fe(III), although the reactivity and binding kinetics are strongly dependent on the oxidation state of the heme. *E. coli* HMP is isolated in its ferric form, and upon aerobic incubation with NAD(P)H, it is reduced to the oxygenated ferrous state. This oxy-complex is not stable and on exhaustion of NADH with residual oxygen decays into a form in which heme and flavin are oxidized. The reversibility of the oxygen reaction and the rapid reassociation kinetics after photodissociation confirm the hemoglobin-like features of HMP protein (106). Addition of dithionite and nitrite to the ferric protein results in the formation of a nitrosyl complex (61, 106).

An overview of known ligand binding kinetics and affinities of hemoglobins and flavohemoglobins is given in Table 3. Interestingly, for VHb two different values for oxygen release have been published. Orii and Webster (107) reported a very fast release of oxygen from VHb (k_{off} = 5600 s^{-1}), which is several fold higher than the value for any other known hemoglobin. More recent data, however, indicate a more moderate rate of oxygen release from VHb (biphasic release with k_{off} = 4.2 s^{-1} (fast phase) and 0.15 s^{-1} (slow phase)). The kinetic parameters for oxygen release from VHb reported by Giangiacomo et al. (44) are in good agreement with data obtained in our laboratory (33). The biphasic behavior in oxygen release may be due to conformational changes with the presence or absence of hydrogen bonding of iron-O_2 to TyrB10 (44).

Replacement of distal GluE7 by HisE7 has deleterious effects on oxygen binding properties of VHb (24). Binding of O_2 to the mutated protein is impaired, which became obvious from the absence of a stable oxy-form, thus suggesting that the distal His is liganding the heme iron itself. However, the mutation had little effect

on CO binding properties of VHb. Interestingly, a change of GluE7 to Leu had little effect on oxygen binding characteristics, indicating that the distal glutamine does not stabilize the bound oxygen with a hydrogen bond (24).

Table 3. Binding of oxygen and nitric oxide to various hemoglobin and flavohemoglobin proteins.

Ligand pair[a)]	Association [$\mu M^{-1} s^{-1}$]	Dissociation [s^{-1}]	Reference
O_2			
HMP	38	0.44	(38)
VHb	78	5000	(107)
	200	4.2 (1.5)	(44)
	117		(33)
VHb-Red	70	0.24	(33)
FHP	12	0.53	(33)
	50	0.2	(40)
FHPg	225		(33)
Cj-Hb	132	1	(33)
Cp-Hb	197	2	(33)
HmpBs	39	7	(33)
HmpSt	9	2	(33)
NO			
HMP	26	0.0002	(38)
FHP	10-20		(40)
Cj-Hb	2	0.002	(33)
Cp-Hb	4	0.001	(33)
HmpBs	366	0.002	(33)
HmpSt	41	0.0003	(33)

a) Ligand binding to the protein in its ferrous form.

Enzymatic activities of flavohemoglobins and hemoglobins

HMP can mediate electron transfer either from the NADH via FAD to a heme bound ligand such as oxygen or NO or in the absence of a ligand from NADH via FAD to an external electron acceptor (115). In the presence of oxygen and NADH, HMP has a NADH oxidase activity, which leads to the rapid consumption of NADH and to the generation of superoxide and H_2O_2 *in vitro* and *in vivo* (96, 100). On exhaustion of oxygen, the oxygenated species disappear to generate the deoxy Fe(II) heme, whereupon the flavin becomes reduced (113). Various external electron acceptors are reduced by HMP *in vitro*, including dihydropteridine (148), ferrisiderphores (2), ferric citrate (32), cytochrome *c*, and Fe(III)-hydroxamate (115). However, it can be questioned, if these reactions are of biological significance *in*

vivo; the dihydropteridine and ferric reductase activities have been regarded as negligible since more potent enzymatic activities for these reactions exist in *E. coli* (32, 148).

Despite of the lack of a reductase domain, VHb is also able to perform catalytic reactions. Purified VHb is able to generate hydrogen peroxide in the presence of NADH in a 1:1 stoichiometry (151).

Nitric oxide dioxygenase activity of flavohemoglobins

Gardner et al. (39) identified in *E. coli* an inducible, O_2 dependent, cyanide sensitive and protective enzymatic activity, which is able to shield the NO sensitive aconitase. Exposure of aerobically growing cells to NO elevated this activity. Gardner et al. (41) and Hausladen et al. (52) attributed the observed detoxification mechanism to HMP expression, which was shown to play a central role in the inducible response to nitrosative stress. The authors stated that NO detoxification is consuming O_2 and NADH and is converting NO and O_2 in equistoichiometric amounts to nitrate, designating the activity nitric oxide dioxygenase (NOD) (Figure 3).

The reaction kinetics and mechanism of HMP mediated NO degrading activity have been analyzed in details and the results showed an NOD activity of 94 s^{-1} (20°C) and 240 s^{-1} (37°C) at oxygen concentration of 200 μM and NO concentration of 1 μM (38, 40). NOD activity of *R. eutropha* FHP was determined to be 7.4 s^{-1} (20°C) and 120 s^{-1} (37°C), respectively, thus showing that NOD activity of FHP is more temperature dependent than that of HMP (40). Substitution of Tyr29(B10), a residue known to stabilize Fe^{2+}-O_2 complex, by Phe, Glu or His diminished NOD of HMP activity approximately 15-35 fold. NOD activity is suggested to be hampered by NO when the ratio of NO/O_2 is exceeding 1:100 (38). Furthermore, Mills et al. (100) reported that 35 - 100 μM of O_2 are required for maximal oxygenase activity. These two findings raise the question of whether HMP provides effective NO

removal under microaerobic conditions, since the natural environment of most pathogenic bacteria lacks or is low in oxygen. Furthermore, bacteriostatic NO concentrations are in the high micromolar to millimolar range - concentrations, which have previously been proposed to be highly inhibitory to HMP activity.

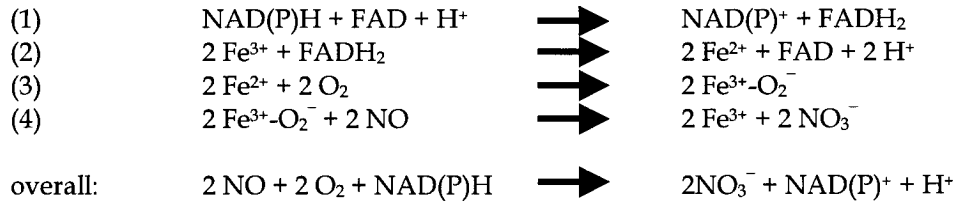

Figure 3. The reaction mechanism of HMP catalyzed NO detoxification as outlined by Gardner et al. (41).

Anaerobic conversion of NO and denitrosylase activity of *E. coli* HMP

Under anaerobic conditions the reaction of NO with HMP leads to the generation of nitrite under consumption of NAD(P)H. NO binds to the active site of HMP and is reduced to the nitroxyl anion (NO^-). This species then reacts forming N_2O via a dimeric intermediate (82). This reaction proceeds at a rate of 0.14 s^{-1} (1 μM NO, 37°C), which is several folds slower than the rate of the NOD activity determined for HMP (40). Latest results showed a novel enzymatic activity, which was shown to efficiently remove NO under anoxic conditions and which was identified to be a rubredoxin reductase (36, 37).

Recently, Hausladen et al. (51) reported a third reaction of HMP with NO, a denitrosylase activity (O_2 nitroxylase). They pointed out that, due to the higher affinity of ferrous HMP to NO relative to O_2 and the inability to bind O_2 in the ferric form, HMP preferentially binds NO at biologically relevant O_2 concentrations (38). During steady-state turnover, the enzyme can be found in the ferric (Fe(III)) state. The formation of a heme-bound nitroxyl equivalent and its subsequent oxidation is possibly a novel enzymatic function of HMP. Despite of the recently suggested NO inhibition of HMP activity (38), the denitrosylase reaction proceeds at a rate (at 40 μM NO and 10 μM O_2), which is approximately 50% slower than the rate of the NOD activity of HMP.

Intracellular localization of VHb and HMP

Studies on the localization of VHb in transgenic *E. coli* showed first an allocation of the protein into cytoplasmic and periplasmic space, and up to 30% of the protein is exported into the periplasm. The export of VHb into the periplasmic space is explained by the presence of a 16 amino acids long sequence at the N-terminus of VHb, which may direct the protein partially into the periplasmic space. This sequence is not cleaved during or after the translocation process and is an intrinsic part of the hemoglobin protein (75).

Analysis of the primary structure of this sequence reveals several unusual features, which are not typically found in bacterial signal sequences. Instead of having positively charged residues at its amino terminus, it has a negative charge. The overall hydrophobicity of the central region of this putative signal sequence is significantly lower than in typical leader peptides due to the presence of a charged residue (60, 75). Soluble fractions from periplasm and cytoplasm were found to contain functional VHb, as they were able to abduct carbon monoxide *in vitro*. Furthermore, similar results were obtained in *Vitreoscilla* using Western blotting. In addition, fusions of the N-terminal sequence of VHb (16-23 amino acids) to *pho*A allowed the export of alkaline phosphatase into the periplasm of *E. coli* (75).

E. coli HMP has been found to also allocate into periplasmic and cytoplasmic space, and approximately 30% of HMP were recovered from the periplasmic fraction of *E. coli*. The allocation in both cytoplasm and periplasm were found in a HMP overproducing strain as well as in strain bearing only a single chromosomal copy of *hmp* using Western blotting. However, spectral analysis of both fractions revealed that the CO-binding hemoproteins are exclusively found in the cytoplasmic fraction. The N-terminal sequence of HMP has been shown to be highly similar to that of VHb (148).

Recently, Webster and coworkers (121) analyzed VHb localization using immunogold labeling both in *Vitreoscilla* and recombinant *E. coli* expressing VHb. The results clearly indicate that VHb has mainly a cytoplasmic and not periplasmic

localization in both organisms (121). The authors suggest that the observed VHb export by Khosla and Bailey (75) may be due to the overexpression and extrusion of VHb. In addition, it cannot be ruled out that the osmotic shock procedure used for the periplasmic sample preparation generated these putatively false results.

Regulation and function of bacterial flavohemoglobins and hemoglobins

Vitreoscilla hemoglobin VHb

The oxygen-dependent promoter of *vhb* (P_{vhb}), which is induced under oxygen-limited conditions in *Vitreoscilla* (153), controls the expression of the *vhb* gene. The P_{vhb} has been characterized in *E. coli* (74), and has also been shown to be completely functional in various heterologous hosts, such as *E. coli* (74), *Pseudomonas*, *Azotobacter* and *Rhizobium* (23), *Streptomyces* sp. (92), *Serratia marcescens* (156, 157), *Rhizobium etli* (122) *Burkholderia* sp., (108). P_{vhb} is maximally induced under microaerobic conditions, when dissolved oxygen level is less than 2% of air saturation in both *Vitreoscilla* and *E. coli* (74, 76). Activity of P_{vhb} in *E. coli* has been shown to be positively modulated by CRP and FNR, i. e. expression from P_{vhb} was activated by FNR and by CRP. In the promoter sequence a DNA motif is encountered, which reveals high similarities to the consensus sequences of either *E. coli* FNR or *E. coli* CRP (74, 141). Expression from P_{vhb} was substantially reduced in strains that were unable to synthesize CRP or cAMP (74). Despite the influence of catabolite repression, glucose and glycerol-containing media gave comparable expression levels of reporter enzymes from P_{vhb} under carbon-limited conditions such as those encountered in typical fed-batch fermentations (78). In an *fnr* negative *E. coli* strain the expression from P_{vhb} was reduced 2-fold under microaerobic conditions (141). The DNA binding sequences of these two global *E. coli* regulatory molecules are highly identical and differ only in 1 base pairing. It has been shown that switching of the noncongruent base pair in the half-site motifs of target promoters can result in their *in vivo* regulation by the corresponding noncognate

regulator (130). Thus it would seem possible that *E. coli* FNR and CRP are able to bind to the identical site in P_{vhb}. The FNR binding site within the promoter structure has been divergently allocated, Tsai et al. (141) located this binding site around position -41.5, whereas Joshi and Dikshit (68) located it around -23.5. The latter binding site is not sharing any sequence homology to the known *E. coli* and non-*E. coli* FNR consensus sequences (129). 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} (78).

In bioreactor cultivations under conditions of full induction, the level of expressed reporter protein accounted up to 10% of total cellular protein. High level expression as well as the manifold regulatory mechanisms have rendered P_{vhb} extremely interesting for applications in biotechnological production processes since the induction is readily achieved by lowering dissolved oxygen concentrations in the bioreactor. Thus, expensive chemical inducers can be omitted (78).

Upon expression of VHb in *E. coli*, recombinant cells grew to higher final cell densities relative to plasmid bearing controls under oxygen limited conditions (76). Further experiments showed that the effect of VHb was not restricted to growth improvements but also resulted in enhanced protein synthesis (78). Therefore, it has been proposed by Khosla et al. (78) that VHb expression could facilitate oxygen diffusion and improve aerobic metabolism in *E. coli*.

Based on a mathematical model, Kallio et al. (70) formulated a hypothesis stating that the expression of VHb increases the intracellular effective oxygen concentration under microaerobic conditions. This increase thereby would shift the relative activity of terminal oxidases and the energetically more favorable cytochrome *o* will predominantly be used in microaerobic *E. coli*. Cytochrome *o* complex (aerobic terminal oxidase) has higher proton translocation activity, H/O^+ ratio, and is able to generate a larger proton gradient across the cell membrane relative to cytochrome *d* complexes (microaerobic terminal oxidase) (119, 120).

This hypothesis has been studied in details and expression of VHb in cells lacking either cytochrome *o* or cytochrome *d* complexes revealed a 5-fold increase in cytochrome *o* and a 1.5-fold increase in cytochrome *d* complexes in VHb-positive *E. coli* relative to hemoglobin-free controls (142). VHb-expressing cells are indeed able to generate a 50% higher proton flux per reduced oxygen molecule relative to a VHb-negative control. The higher proton flux thereby leads to a 30% higher ATP synthase activity and a 65% higher ATP turnover rate, thus supporting the model of Kallio et al. (14, 70, 142). Furthermore, Dikshit et al. (26) showed that an *E. coli* mutant lacking both terminal oxidases could grow on the aerobic substrate succinate and lactate when the cells were engineered to express VHb. These results suggest therefore that VHb itself may contain a terminal oxidase function. However, this potential function of VHb has not been investigated further and we have not been able to verify these results using a different *E. coli* strain lacking the terminal oxidases (Kallio P., personal communication).

Additionally, the steady state level of cellular NAD(P)H is 1.8-fold lower in VHb-expressing strains relative to control cells near anoxic conditions (143). Anoxia is known to reduce electron flow through the respiratory chain, so that NAD(P)H is consumed more slowly. Therefore, one may hypothesize that under low oxygen tension the expression of the oxygen delivering VHb may increase the electron flux through the respiratory chain in *E. coli*, thus reducing the level of cellular NAD(P)H.

Furthermore, VHb-positive cells display increased amounts of both tRNA's and active 70S ribosome complexes under microaerobic conditions in *E. coli*. This was also accompanied by a corresponding increase of a marker enzyme activity (103). Thus, the upregulation of VHb expression and the observed positive effects of VHb expression in cells grown under oxygen limited conditions clearly indicate that VHb is able to stimulate the energy production and thereby increase the metabolic efficiency of VHb expressing cells.

***R. eutropha* FHP**

FHP expression is 20-fold upregulated under oxygen-limited conditions in *R. eutropha* (118). Two potential binding motifs for NarL and FNR in the upstream noncoding region of *fhp* have been identified and the regulation of FHP by these two global regulatory molecules suggests a role of FHP in the anaerobic metabolism of *R. eutropha*. However, isogenic *fhp*$^-$ mutants show no significant differences in aerobic or anaerobic growth. Compared with the wild type, however, the mutant did not accumulate nitrous oxide during denitrification with nitrite as electron acceptor. Recently, the NOD activity of FHP has been demonstrated (40). Altogether, these results suggest that FHP interacts directly or indirectly with the gas metabolism during denitrification in *R. eutropha* as proposed earlier by Cramm et al. (18).

***E. coli* flavohemoglobin HMP**

To elucidate the possible roles of *E. coli* HMP protein Poole et al. (111) studied the response of the *hmp* promoter (P_{hmp}) under various growth conditions. P_{hmp} was found to be responsive to iron depletion. Anaerobic expression is enhanced in an *fnr*$^-$ mutant and a putative FNR-binding site occurs at positions -2 to +11 in P_{hmp} region (93). In addition, HMP expression is stimulated directly by the presence of nitrate and nitrite; nitric oxide is also an inducer under aerobic conditions. The effects of these nitrogen compounds were independent of NarLP and SoxRS regulatory proteins (111). Furthermore, a growth state dependent induction of HMP expression was observed when cells were entering the stationary growth phase. This induction is mediated by the stationary phase-specific sigma subunit (σ^S) of RNA polymerase (93).

The upregulation of *hmp* expression by NO was studied in more details and shown to be dependent on the MetR, which is the global regulator of the methionine biosynthetic pathway in *E. coli*. Interestingly, two MetR binding sites are located in the intergenic region between *hmp* and *gly*A, which are divergently transcribed (95).

Homocysteine is a co-regulator for MetR and mutations in the methionine pathway (*met*L), the function of which is also needed for homocysteine synthesis, made these mutants sensitive to various NO-compounds. Upon feeding of homocysteine to the growing cultures resistance against nitrosative stress was restored (21). MetR has been shown to be necessary for induction of *hmp-lac*Z by SNP and GSNO but only the downstream MetR binding site is essential for *hmp* induction (95). Induction occurs upon depletion of homocysteine, which allows MetR to bind to the promoter region. Inactivation of homocysteine may occur via a nitrosation reaction (95).

Membrillo-Hernández et al. (97) also reported the induction of an *hmp-lac*Z fusion in the presence of paraquat. The induction of *sod*A-*lac*Z followed a subtler pattern, as did the *hmp-lac*Z, the expression of which was induced only by paraquat concentrations above 200 mM. The independence of HMP upregulation by SoxRS was shown using *E. coli* mutants of these two transcriptional activators. Furthermore, *hmp* regulation by paraquat is dependent on σ^s (97). Anjum et al. (3) showed even a 28-fold induction of an *hmp-lac*Z fusion after a prolonged incubation in the presence of paraquat, a treatment that induced HMP expression to spectrally detectable levels.

The observation that HMP is a NADH oxidase and is thereby able to generate superoxide radicals and H_2O_2 led to the hypothesis that HMP might be involved in the oxidative stress response in *E. coli*. Interestingly, HMP is not only generating ROS, but is also needed for full expression of SodA and SoxS. Furthermore, a *hmp*$^-$ mutant was markedly more sensitive towards paraquat, particularly at lower paraquat concentrations, as shown by a viability assay (94) Therefore, Poole and Hughes (112) suggested that HMP might act as an amplifier of superoxide stress leading to the full expression of SoxRS stress response.

Several lines of evidence clearly prove the implications of HMP in the response to and resistance against nitrosative stress. First, it is well established that HMP is able to degrade NO under aerobic conditions, although it has to be analyzed in details, which catalytic activity, dioxygenase or denitrosylase, is more relevant *in vivo*.

Second, *in vivo* experiments revealed the important role of HMP in the resistance against various agents producing nitrosative stress. Various experiments showed that *hmp*$^-$ cells are more sensitive towards the thread provided by these compounds. Third, the expression of HMP is induced by NO in *E. coli*.

Various classes of proteins are considered to be targets of nitrosative stress, such as Fe-S containing proteins, heme-containing proteins, and various Cu/Fe proteins. The protective activity of HMP was already demonstrated for the Fe-S containing enzyme aconitase (39), whereas the protection of heme and Cu containing proteins was revealed very recently. Terminal oxidases are main targets of NO inhibition. These proteins contain both heme and Cu. Stevanin et al. (132) reported a protective effect of HMP on both terminal oxidases (cytochrome *o* and *d*) in a mutant strain lacking either terminal oxidase. Additionally, respiration of an *hmp*$^-$ mutant was extremely sensitive to NO. However, they had to admit that due to the reduced activity of HMP under microaerobic conditions an enhanced NO$^\bullet$ toxicity is to be expected.

Alternatively, Poole et al. (114) proposed that HMP could act as an oxygen sensor in *E. coli*, since the binding of oxygen modulates the reduction level of FAD, thus limiting flavin reduction in the aerobic steady state. Lowering of the oxygen concentration causes dissociation of the oxygenated HMP species and sustained flavin reduction. Due to the ability of HMP to reduce Fe(III) in a flavin mediated fashion, such a mechanism might control Fe(III) reduction, which is required for activation of the anaerobic gene regulator FNR in *E. coli*.

Flavohemoglobins from various bacterial organisms

The expression of many hemoproteins is induced by environmental stress conditions such as oxygen deprivation or oxidative and nitrosative stress. Several regulatory proteins have been implicated in the control of hemoprotein gene expression in various bacteria, such as the global anaerobic response regulator

FNR, RedD/E, Fur, NAR, MetR and stationary phase responsive σ^S (19, 57, 85). Furthermore, expression of these genes is increased in the presence of RNS and expression levels are either positively or negatively modulated by ROS. Interestingly, in general, induction of *hmp* expression by ROS and RNS was mainly independently mediated by of the oxidative stress responsive SoxRS and OxyR transcription factors (19, 57, 85).

Early support to the hypothesis that flavohemoproteins function as an integral component of a stress response was reported by Favey et al. (35). They showed that upon inactivation of *hmp*X gene in the plant pathogen *E. chrysanthemi*, the bacteria became unable to infect plant species. This behavior could be attributed to the reduction in the synthesis of pectate lyases (PL), which are pathogenicity determinants of *E. chrysanthemi* in conditions of low oxygen tension. The synthesis of PL was abolished in the *hmp*X$^-$ mutant when infecting plants. The regulation of *hmp*X was shown to be dependent on plant host factors as shown by the upregulation of an *hmp*X-*gus* fusion by cocultivation of *E. chrysanthemi* in the presence of a tobacco suspension culture. Microaerobic conditions *per se*, as encountered upon infection, were not sufficient to start transcription of *hmp*X-*gus* fusion (35). In the meantime the involvement of NO$^\bullet$ -compounds in the plant defense has been shown (10); and therefore it is very likely that *hmp*X expression contributes to the resistance against those radicals.

A growing body of evidence has accumulated in the last few years that showed a role of flavohemoglobins in the response to the thread with NO radicals and nitrosative stress in general. This general role of flavohemoprotein in NO defense might be of great interest for the clinical microbiology. As shown above flavohemoproteins of clinically relevant microorganisms, such as *M. tuberculosis* and *S. typhimurium* take the advantage of this resistance mechanism upon infection of a potential host. Bearing in mind the steadily increasing number of antibiotics resistant pathogens, flavohemoproteins would offer a novel drug target for therapy.

Hemoglobins and flavohemoglobins in biotechnology

The commercial production of a variety of desirable metabolites and important pharmaceuticals employ the overexpression capacity of oxygen-requiring bacteria, fungi and mammalian cells. Oxygen has very low solubility in water and various microorganisms and cultured cell types have high nutritional demand for oxygen especially during large-scale and high cell density production processes. The high demand for oxygen can be partially satisfied by improving process parameters and bioreactor configurations, e.g. improved mixing rates, high-efficiency dispersion systems and modifications of the medium (83).

Use of VHb to improve cell growth and productivity

The metabolic engineering approach seeks genetic strategies to alleviate adverse effects of oxygen limitation in microorganisms, higher cells, and plants. The approach was based on previous observation that under oxygen deprivation *Vitreoscilla* expresses hemoglobin. This gene was successfully transferred to *E. coli* and upon expression of the bacterial hemoglobin VHb, growth and protein production of recombinant *E. coli* was enhanced under microaerobic conditions (76, 79). The positive effects of VHb-expression on promoting the efficiency of oxygen-limited growth and protein production in numerous organisms are well documented (Table 4). The use of VHb expression technology has successfully extended in collaboration with biotechnological industry to an industrially significant *Saccharopolyspora erythraea* strain. This strain has been optimized before for erythromycin production. However, since the mycelium-forming bacterium is growing a highly viscous media, it has been argued that oxygen transfer to the mycelial pellets will be limited. The *vhb* gene was therefore stably integrated into the chromosome (12). VHb expression significantly increased erythromycin production resulting in an enhanced space-time yield of approximately 100% relative to the original strain (101).

Furthermore, the beneficial effects of VHb expression technology were shown not to be restricted to bacterial organisms. The use of plants and plants cells for the production of natural or recombinant compounds has gained momentum (28). Oxygen supply to plant cells is known to affect both cell growth and the production of metabolites (58). Therefore, Farrés and Kallio (34) have expressed VHb in suspension cultured tobacco cells. VHb expression resulted in a shortened lag-phase and increased final cell densities in batch cultures relative to control cultures.

Table 4. Summary of beneficial effects of VHb expression in various organisms

Organism	Effects	References
Acremonium chrysogenum	3.2-fold increase of cephalosporin C production	(22)
Bacillus subtilis	30% increase of neutral protease, 7-15% increase of α-amylase activity, and 1.5-fold increase of protein secretion	(69)
Burkholderia sp	15% increase of biomass, and 2-fold strain DNT increase in the rate of DNT degradation	(102, 108)
Chinese hamster ovary cells	40%-100% increase of tissue plasminogen activator production	(109)
Corynebacterium glutamicum	30% increase of L-lysine titer, and 24% increase of L-lysine yield	(124)
Escherichia coli	2.1-fold increase of total cell protein	(76)
Escherichia coli	2.2-fold increase of total cell protein	(141)
Escherichia coli	30% increase of total cell protein	(79)
Escherichia coli	80% increase of CAT activity	(79)
Escherichia coli	40% increase of β-galactosidase activity	(79)
Escherichia coli	61% increase of β-lactamase activity	(103)
Escherichia coli	3.3-fold increase of α-amylase activity	(29, 80, 88)
Escherichia coli	1.8-fold increase in ferritin production	(16)
Nicotiana tabaccum	50% faster germination rate, enhanced growth , 80-100% more dry weight, 30-40% more chlorophyll, 34% more nicotine	(56)
Nicotiana tabaccum, suspension culture	Enhanced growth, shortened lag phase	(34)
Pseudomonas aeruginosa	11% increase of viable cell number, increased oxygen uptake	(42, 89)
Rhizobium etli	68% higher nitrogenase activity in nodules of bean plants, and 53% higher total nitrogen content	(122)
Saccharomyces cerevisiae	3-fold increase of final cell density	(14)
Saccharopolyspora erythraea	70% increase of final erythromycin titer	(12, 101)
Serratia marcescens	Enhancement of acetoin, and 2,3-butanediol production, increase in cell size	(156, 157)
Streptomyces coelicolor	10-fold increase of actinorhodin production	(92)
Streptomyces lividans	50% increase of final cell density	(92)
Streptomyces rimosus	2.2-fold increase of oxytetracycline production	(5)
Xantomonas maltophila	15% increase of viable cell number, enhanced degradation of benzoic acid	(90)

The above-cited results show that heterologous VHb expression is able to cure oxygen limitation in a wide range of distinct organisms. Thus, the inverse metabolic

engineering approach, i. e. the genetic transfer of useful phenotypes to heterologous organisms, represents a fast means to alleviate the adverse effects of oxygen limitation (5).

Novel hemoglobin proteins for improved performance in heterologous hosts

It has been hypothesized that the properties of VHb are not optimized for foreign hosts cells, screening of novel globin genes and generation of VHb mutants with improved properties have been performed to ameliorate the VHb-technology for biotechnological applications. Andersson et al. (1) used error-prone PCR to generate a number of randomly mutated *vhb* genes. VHb mutants were screened for improved growth properties under microaerobic conditions and several clones were analyzed to elucidate the physiological effects of novel VHb proteins The expression of four VHb mutants, carried by pVM20, pVM50, pVM104, and pVM134, were able to enhance microaerobic growth of *E. coli* by approximately 22% (mutations: Glu19(A17) and Glu137(H23) to Gly), 155% (His36(C1) to Arg36 and Gln66(E20) to Arg66), 50% (Ala56(E10) to Gly), and 90% (Ile24(B5) to Thr), respectively, with a concomitant decrease of acetate excretion into the culture medium. Due to the cloning strategy the mutated VHb proteins also contained an extension of eight residues (MTMITPSF) at their N-termini. The experiments also indicate that the positive effects elicited by mutant VHb-expression from pVM20 and pVM50 are linked to the N-terminal peptide tail. Removal of the N-terminal sequence reduced cell growth approximately 23% and 53%, respectively, relative to native VHb controls (1).

Bollinger et al. (8) screened various microbial genomes for the existence of uncharacterized and unidentified hemoglobins and flavohemoglobins, which could be used in biotechnological production processes and to enhance cell growth under oxygen-limited conditions. Growth behavior and byproduct formation of *E. coli* cells expressing various hemoproteins were analyzed in microaerobic fed-batch

cultivations. *E. coli* cells expressing flavohemoglobins from *P. aeruginosa, S. typhi,* and *D. radiodurans* grew to similar final cell densities, as did the strain expressing VHb. Although, the clones expressing flavohemoglobins from *E. coli, B. subtilis,* and *K. pneumoniae* or the hemoglobin from *C. jejuni,* outperformed the plasmid bearing control strain, these clones did not reach the final cell densities measured for VHb. Additionally, increased yield of biomass on glucose was measured for all recombinant strains, and an approximately 2-fold yield enhancement was obtained with *D. radiodurans* flavohemoglobin-expressing *E. coli* relative to the *E. coli* control carrying the parental plasmid only. Thus, showing that these recombinant hemoprotein expressing strains are able to utilize the carbon substrate more efficiently (8). The expression of horse heart myoglobin did not result in any growth improvements during microaerobic growth. Interestingly, an *E. coli* strain engineered to express yeast flavohemoglobin (Yfb) was not able to reach similar final cell densities as the plasmid bearing control strain (71).

References

1. **Andersson, C. I. J., N. Holmberg, J. Farrés, J. E. Bailey, L. Bülow, and P. T. Kallio.** 2000. Error-prone PCR of *Vitreoscilla* hemoglobin (VHb) to support the growth of microaerobic *Escherichia coli.* Biotechnol Bioeng. **70:**446-55.
2. **Andrews, S. C., D. Shipley, J. N. Keen, J. B. C. Findlay, P. M. Harrison, and J. R. Guest.** 1992. The hemoglobin-like protein (Hmp) of *Escherichia coli* has ferrisiderophore reductase-activity and its C-terminal domain shares homology with ferredoxin $NADP^+$ reductases. Febs Lett. **302:**247-252.
3. **Anjum, M. F., N. Ioannidis, and R. K. Poole.** 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.
4. **Aslund, F., M. Zheng, J. Beckwith, and G. Storz.** 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. Proc. Natl. Acad. Sci. U S A. **96:**6161-5.
5. **Bailey, J. E., A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner, and P. S. Tsai.** 1996. Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes Biotechnol. Bioeng. **52:**109-121.
6. **Becker, S., G. Holighaus, T. Gabrielczyk, and G. Unden.** 1996. O_2 as the regulatory signal for FNR-dependent gene regulation in *Escherichia coli.* J. Bacteriol. **178:**4515-4521.
7. **Bell, A. I., K. L. Gaston, J. A. Cole, and S. J. Busby.** 1989. Cloning of binding sequences for the *Escherichia coli* transcription activators, FNR and CRP: location of bases involved in discrimination between FNR and CRP. Nucleic Acids Res. **17:**3865-74.
8. **Bollinger, C. J. T., J. E. Bailey, and P. T. Kallio.** 2001. Novel hemoglobins to enhance microaerobic growth and substrate utilization in *Escherichia coli.* Biotechnol. Prog. **17:**798-808.

9. **Bolognesi, M., D. Bordo, M. Rizzi, C. Tarricone, and P. Ascenzi.** 1997. Nonvertebrate hemoglobins: structural bases for reactivity. Prog. Biophys. Mol. Biol. **68:**29-68.
10. **Bolwell, G. P.** 1999. Role of active oxygen species and NO in plant defence responses. Curr. Op. Plant Biol. **2:**287-294.
11. **Bowien, B., and H. G. Schlegel.** 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Annu. Rev. Microbiol. **35:**405-52.
12. **Brünker, P., W. Minas, P. T. Kallio, and J. E. Bailey.** 1998. Genetic engineering of an industrial strain of *Saccharopolyspora erythraea* for stable expression of the Vitreoscilla haemoglobin gene (*vhb*). Microbiology. **144:**2441-8.
13. **Burmester, T., B. Weich, S. Reinhardt, and T. Hankein.** 2000. A vertebrate globin expressed in the brain. Nature **407:**520-523.
14. **Chen, W., D. E. Hughes, and J. E. Bailey.** 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters the aerobic metabolism of *Saccharomyces* cerevisiae. Biotechnol. Prog. **10:**308-313.
15. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753-62.
16. **Chung, Y. J., K. S. Kim, E. S. Jeon, K. I. Park, and C. U. Park.** 1998. Effects of the *Vitreoscilla* hemoglobin gene on the expression of the ferritin gene in *Escherichia coli*. J. Biochem Mol. Biology. **31:**503-507.
17. **Clark, D. P.** 1989. The fermentation pathways of *Escherichia coli*. Fems Microbiol. Reviews **63:**223-234.
18. **Cramm, R., R. A. Siddiqui, and B. Friedrich.** 1994. Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*. J. Biol. Chem. **269:**7349-54.
19. **Crawford, M. J., and D. E. Goldberg.** 1998. Regulation of the *Salmonella typhimurium* flavohemoglobin gene. A new pathway for bacterial gene expression in response to nitric oxide. J. Biol. Chem. **273:**34028-32.
20. **Crawford, M. J., and D. E. Goldberg.** 1998. Role for the *Salmonella flavohemoglobin* in protection from nitric oxide. J. Biol. Chem. **273:**12543-12547.
21. **De Groote, M. A., T. Testerman, Y. Xu, G. V. Stauffer, and F. C. Fang.** 1996. Homocysteine antagonism of nitric oxide-related cytostasis in *Salmonella typhimurium*. Science **272:**414-416.
22. **DeModena, J. A., S. Gutierrez, J. Velasco, F. J. Fernandez, R. A. Fachini, J. L. Galazzo, D. E. Hughes, and J. F. Martin.** 1993. The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. Bio/Technology **11:**926-9.
23. **Dikshit, K. L., R. P. Dikshit, and D. A. Webster.** 1990. Study of *Vitreoscilla* globin (*vgb*) gene expression and promoter activity in *E. coli* through transcriptional fusion. Nucleic Acids Res. **18:**4149-55.
24. **Dikshit, K. L., Y. Orii, N. Navani, S. Patel, H. Y. Huang, B. C. Stark, and D. A. Webster.** 1998. Site-directed mutagenesis of bacterial hemoglobin: The role of glutamine (E7) in oxygen-binding in the distal heme pocket. Arch. Biochem. Biophys. **349:**161-166.
25. **Dikshit, K. L., and D. A. Webster.** 1988. Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene. **70:**377-86.
26. **Dikshit, R. P., K. L. Dikshit, Y. X. Liu, and D. A. Webster.** 1992. The bacterial hemoglobin from *Vitreoscilla* can support the aerobic growth of *Escherichia coli* lacking terminal oxidases. Arch Biochem Biophys. **293:**241-5.
27. **Ding, A. H., C. F. Nathan, and D. J. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J. Immunol. **141:**2407-12.
28. **Doran, P. M.** 2000. Foreign protein production in plant tissue cultures. Curr. Opin. Biotechnol. **11:**199-204.
29. **Enayati, N., C. Tari, S. J. Parulekar, B. C. Stark, and D. A. Webster.** 1999. Production of alpha-amylase in fed batch cultures of *vgb*$^+$ and *vgb*$^-$ recombinant *Escherichia coli*: Some observations. Biotechnol. Progress. **15:**640-645.

30. **Ermler, U., R. A. Siddiqui, R. Cramm, and B. Friedrich.** 1995. Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 A resolution. Embo J. **14:**6067-77.
31. **Ermler, U., R. A. Siddiqui, R. Cramm, D. Schroder, and B. Friedrich.** 1995. Crystallization and Preliminary-X-Ray Diffraction Studies of a Bacterial Flavohemoglobin Protein. Proteins-Structure. Function and Genetics. **21:**351-353.
32. **Eschenbrenner, M., J. Coves, and M. Fontecave.** 1994. Ferric reductases in *Escherichia coli*: the contribution of the haemoglobin-like protein. Biochem. Biophys. Res. Commun. **198:**127-31.
33. **Farrés, J., A. D. Frey, and P. T. Kallio.** manuscript in preparation.
34. **Farrés, J., and P. T. Kallio.** 2002. Improved growth in tobacco suspension cultures expressing *Vitreoscilla* hemoglobin. Biotechnol Prog. in press.
35. **Favey, S., G. Labesse, V. Vouille, and M. Boccara.** 1995. Flavohaemoglobin HmpX - a new pathogenicity determinant in *Erwinia chrysanthemi* Strain-3937. Microbiology **141:**863-871.
36. **Gardner, A. M., and P. R. Gardner.** 2002. Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, *Escherichia coli*. Evidence for a novel inducible anaerobic nitric oxide scavanging activity. J. Biol. Chem. **277:**8166-71.
37. **Gardner, A. M., R. A. Helmick, and P. R. Gardner.** 2002. Flavorubredoxin, an inducible catalyst for nitric oxide reduction and detoxification in *Escherichia coli*. J. Biol. Chem. **277:**8172-7.
38. **Gardner, A. M., L. A. Martin, P. R. Gardner, Y. Dou, and J. S. Olson.** 2000. Steady-state and transient kinetics of *Escherichia coli* nitric- oxide dioxygenase (flavohemoglobin) - The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. J. Biol. Chem. **275:**12581-12589.
39. **Gardner, P. R., G. Costantino, and A. L. Salzman.** 1998. Constitutive and adaptive detoxification of nitric oxide in *Escherichia coli* - Role of nitric-oxide dioxygenase in the protection of aconitase. J. Biol. Chem. **273:**26528-26533.
40. **Gardner, P. R., A. M. Gardner, L. A. Martin, Y. Dou, T. Li, J. S. Olson, H. Zhu, and A. F. Riggs.** 2000. Nitric-oxide dioxygenase activity and function of flavohemoglobins. Sensitivity to nitric oxide and carbon monoxide inhibition. J. Biol. Chem. **275:**31581-7.
41. **Gardner, P. R., A. M. Gardner, L. A. Martin, and A. L. Salzman.** 1998. Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. Proc. Natl. Acad. Sci. USA. **95:**10378-10383.
42. **Geckil, H., B. C. Stark, and D. A. Webster.** 2001. Cell growth and oxygen uptake of *Escherichia coli* and *Pseudomonas aeruginosa* are differently effected by the genetically engineered *Vitreoscilla* hemoglobin gene. J. Biotechnol. **85:**57-66.
43. **Gennis, R. B., and V. Stewart.** 1996. Respiration, In: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology. American Society for Microbiology, Washington, DC. pp. 217-261.
44. **Giangiacomo, L., M. Mattu, A. Arcovito, G. Bellenchi, M. Bolognesi, P. Ascenzi, and A. Boffi.** 2001. Monomer-dimer equilibrium and oxygen binding properties of ferrous *Vitreoscilla* hemoglobin. Biochemistry **40:**9311-9316.
45. **Gonzales-Prevatt, V., and D. A. Webster.** 1980. Purification and properties of NADH-cytochrome *o* Reductase from *Vitreoscilla*. J. Biol. Chem. **255:**1478-82.
46. **Gonzalez-Flecha, B., and B. Demple.** 1995. Metabolic sources of hydrogen peroxide in aerobically growing *Escherichia coli*. J. Biol. Chem. **270:**13681-7.
47. **Goretski, J., O. C. Zafiriou, and T. C. Hollocher.** 1990. Steady-state nitric oxide concentrations during denitrification. J. Biol. Chem. **265:**11535-8.
48. **Gort, A. S., and J. A. Imlay.** 1998. Balance between endogenous superoxide stress and antioxidant defenses. J. Bacteriol. **180:**1402-10.
49. **Green, J., B. Bennett, P. Jordan, E. T. Ralph, A. J. Thomson, and J. R. Guest.** 1996. Reconstitution of the [4Fe-4S] cluster in FNR and demonstration of the aerobic-anaerobic transcription switch *in vitro*. Biochem. J. **316:**887-92.
50. **Guest, J. R.** 1992. Oxygen-regulated gene expression in *Escherichia coli*. The 1992 Marjory Stephenson Prize Lecture. J. Gen. Microbiol. **138:**2253-63.

51. **Hausladen, A., A. Gow, and J. S. Stamler.** 2001. Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. Proc. Natl. Acad. Sci. USA **98:**10108-12.

52. **Hausladen, A., A. J. Gow, and J. S. Stamler.** 1998. Nitrosative stress: Metabolic pathway involving the flavohemoglobin. Proc.Natl. Acad. Sci. USA **95:**14100-14105.

53. **Hausladen, A., C. T. Privalle, T. Keng, J. DeAngelo, and J. S. Stamler.** 1996. Nitrosative stress: activation of the transcription factor OxyR. Cell **86:**719-29.

54. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, and O. White.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477-83.

55. **Hidalgo, E., J. M. Bollinger, Jr., T. M. Bradley, C. T. Walsh, and B. Demple.** 1995. Binuclear [2Fe-2S] clusters in the *Escherichia coli* SoxR protein and role of the metal centers in transcription. J. Biol. Chem. **270:**20908-14.

56. **Holmberg, N., G. Lilius, J. E. Bailey, and L. Bülow.** 1997. Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. Nature Biotechnology **15:**244-247.

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

58. **Huang, S. Y., and C. J. Chou.** 2000. Effect of gaseous composition on cell growth and secondary metabolite production in suspension culture of *Stizolobium hassjoo* cells. Bioproc. Engin. **23:**585-593.

59. **Imlay, J. A., and I. Fridovich.** 1991. Assay of metabolic superoxide production in *Escherichia coli* J. Biol. Chem. **266:**6957-6965.

60. **Inouye, S., G. Duffaud, and M. Inouye.** 1986. Structural requirement at the cleavage site for efficient processing of the lipoprotein secretory precursor of *Escherichia coli*. J. Biol. Chem. **261:**10970-5.

61. **Ioannidis, N., C. E. Cooper, and R. K. Poole.** 1992. Spectroscopic studies on an oxygen-binding hemoglobin-like flavohaemoprotein from *Escherichia coli*. Biochem. J. **288:**649-655.

62. **Iuchi, S.** 1993. Phosphorylation/dephosphorylation of the receiver module at the conserved aspartate residue controls transphosphorylation activity of histidine kinase in sensor protein ArcB of *Escherichia coli*. J. Biol. Chem. **268:**23972-80.

63. **Iuchi, S., V. Chepuri, H.-A. Fu, R. B. Gennis, and E. C. C. Lin.** 1990. Requirement for terminal cytochromes in generation of the aerobic signal for the *arc* regulatory system in *Escherichia coli*: study utilizing deletions and *lac* fusions of *cyo* and *cyd*. J. Bacteriol. **172:**6020-6025.

64. **Iuchi, S., and E. C. C. Lin.** 1988. *Arc*A, a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. USA **85:**1888-92.

65. **Iuchi, S., and E. C. C. Lin.** 1992. Purification and phosphorylation of the Arc regulatory components of *Escherichia coli*. J. Bacteriol. **174:**5617-23.

66. **Iuchi, S., Z. Matsuda, T. Fujiwara, and E. C. C. Lin.** 1990. The *arc*B gene of *Escherichia coli* encodes a sensor-regulator protein for anaerobic repression of the arc modulon. Mol. Microbiol. **4:**715-27.

67. **Jordan, P. A., A. J. Thomson, E. T. Ralph, J. R. Guest, and J. Green.** 1997. FNR is a direct oxygen sensor having a biphasic response curve. FEBS Lett. **416:**349-52.

68. **Joshi, M., and K. L. Dikshit.** 1994. Oxygen dependent regulation of *Vitreoscilla* globin gene: evidence for positive regulation by FNR. Biochem. Biophys. Res. Commun. **202:**535-42.

69. **Kallio, P. T., and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin (VHb) enhances total protein secretion and improves the production of α-amylase and neutral protease in *Bacillus subtilis*. Biotechnol. Prog. **12:**31-39.

70. **Kallio, P. T., D. J. Kim, P. S. Tsai, and J. E. Bailey.** 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur. J. Biochem. **219:**201-208.

71. **Kallio, P. T., P. S. Tsai, and J. E. Bailey.** 1996. Expression of *Vitreoscilla* hemoglobin is superior to horse heart myoglobin or yeast flavohemoglobin expression for enhancing *Escherichia coli* growth in a microaerobic bioreactor. Biotechnol. Prog. **12:**751-757.

72. **Karplus, P. A., M. J. Daniels, and J. R. Herriott.** 1991. Atomic-structure of ferredoxin-$NADP^+$ reductase - prototype for a structurally novel flavoenzyme family. Science **251:**60-66.

73. **Khoroshilova, N., C. Popescu, E. Munck, H. Beinert, and P. J. Kiley.** 1997. Iron-sulfur cluster disassembly in the FNR protein of *Escherichia coli* by O_2: [4Fe-4S] to [2Fe-2S] conversion with loss of biological activity. Proc. Natl. Acad. Sci. USA **94:**6087-92.

74. **Khosla, C., and J. E. Bailey.** 1989. Characterization of the oxygen-dependent promoter of the *Vitreoscilla* hemoglobin gene in *Escherichia coli*. J. Bacteriol. **171:**5990-6004.

75. **Khosla, C., and J. E. Bailey.** 1989. Evidence for partial export of *Vitreoscilla* hemoglobin into the periplasmic space in *Escherichia coli*. Implications for protein function. J. Mol. Biol. **210:**79-89.

76. **Khosla, C., and J. E. Bailey.** 1988. Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant *Escherichia coli*. Nature **331:**633-5.

77. **Khosla, C., and J. E. Bailey.** 1988. The *Vitreoscilla* hemoglobin gene: molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. Mol. Gen. Genet. **214:**158-61.

78. **Khosla, C., J. E. Curtis, P. Bydalek, J. R. Swartz, and J. E. Bailey.** 1990. Expression of recombinant proteins in *Escherichia coli* using an oxygen- responsive promoter. Bio/Technology **8:**554-8.

79. **Khosla, C., J. E. Curtis, J. DeModena, U. Rinas, and J. E. Bailey.** 1990. Expression of intracellular hemoglobin improves protein synthesis in oxygen-limited *Escherichia coli*. Bio/Technology **8:**849-53.

80. **Khosravi, M., D. A. Webster, and B. C. Stark.** 1990. Presence of the bacterial hemoglobin gene improves α-amylase production of a recombinant *Escherichia coli* strain. Plasmid **24:**190-4.

81. **Kiley, P. J., and H. Beinert.** 1998. Oxygen sensing by the global regulator, FNR: the role of the iron- sulfur cluster. FEMS Microbiol. Rev. **22:**341-52.

82. **Kim, S. O., Y. Orii, D. Lloyd, M. N. Hughes, and R. K. Poole.** 1999. Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett. **445:**389-394.

83. **Konz, J. O., J. King, and C. L. Cooney.** 1998. Effects of oxygen on recombinant protein expression. Biotechnol. Prog. **14:**393-409.

84. **Labesse, G., C. T. Craescu, J. Mispelter, G. Chottard, M. C. Marden, S. Pin, E. Forest, J. P. Mornon, and M. Boccara.** 1998. Engineering, expression and biochemical characterization of the hemoglobin domain of a *Erwinia chrysanthemi* flavohemoprotein. E. J. Biochem. **253:**751-759.

85. **LaCelle, M., M. Kumano, K. Kurita, K. Yamane, P. Zuber, and M. M. Nakano.** 1996. Oxygen-controlled regulation of the flavohemoglobin gene in *Bacillus subtilis*. J. Bacteriol. **178:**3803-3808.

86. **Lin, E. C. C., and S. Iuchi.** 1991. Regulation of gene expression in fermentative and respiratory systems in *Escherichia coli* and related bacteria. Annu. Rev. Genet. **25:**361-87.

87. **Lin, E. C. C.** 1996. Dissimilatory pathways for Sugars, Polyols, and Carboxylates. In: *Escherichia coli* and *Salmonella*. Cellular and Molecular Biology. American Society for Microbiology, Washington, DC. pp. 307-342.

88. **Liu, S. C., B. Ogretmen, Y. Y. Chuang, and B. C. Stark.** 1992. Selection and characterization of α-amylase-overproducing recombinant *Escherichia coli* containing the bacterial hemoglobin gene. Appl. Microbiol. Biotechnol. **38:**239-42.

89. **Liu, S. C., D. A. Webster, and B. C. Stark.** 1995. Cloning and expression of the *Vitreoscilla* hemoglobin gene in *pseudomonads*: Effects on cell growth. Appl. Microbiol. Biotechnol. **44:**419-424.

90. **Liu, S. C., D. A. Webster, M. L. Wei, and B. C. Stark.** 1996. Genetic engineering to contain the *Vitreoscilla* hemoglobin gene enhances degradation of benzoic acid by *Xanthomonas maltophilia* Biotechnol. Bioeng. **49:**101-105.

91. **Lynch, A. S., and E. C. C. Lin.** 1996. Responses to Molecular Oxygen. In: *Escherichia coli* and *Salmonella.* Cellular and Molecular Biology. American Society for Microbiology, Washington, DC. pp. 1526-1538.

92. **Magnolo, S. K., D. L. Leenutaphong, J. A. DeModena, J. E. Curtis, J. E. Bailey, J. L. Galazzo, and D. E. Hughes.** 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.

93. **Membrillo-Hernández, J., G. M. Cook, and R. K. Poole.** 1997. Roles of RpoS (sigmaS), IHF and ppGpp in the expression of the *hmp* gene encoding the flavohemoglobin (Hmp) of *Escherichia coli* K-12. Mol. Gen. Genet. **254:**599-603.

94. **Membrillo-Hernández, J., M. D. Coopamah, M. F. Anjum, T. M. Stevanin, A. Kelly, M. N. Hughes, and R. K. Poole.** 1999. The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. **274:**748-754.

95. **Membrillo-Hernández, J., M. D. Coopamah, A. Channa, M. N. Hughes, and R. K. Poole.** 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.

96. **Membrillo-Hernández, J., N. Ioannidis, and R. K. Poole.** 1996. The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide *in vitro* and causes oxidative stress *in vivo.* FEBS Lett. **382:**141-4.

97. **Membrillo-Hernández, J., S. O. Kim, G. M. Cook, and R. K. Poole.** 1997. Paraquat regulation of *hmp* (flavobemoglobin) gene expression in *Escherichia coli* K-12 is SoxRS independent but modulated by sigma(s). J. Bacteriol. **179:**3164-3170.

98. **Milani, M., A. Pesce, Y. Ouellet, P. Ascenzi, M. Guertin, and M. Bolognesi.** 2001. *Mycobacterium tuberculosis* hemoglobin N displays a protein tunnel suited for O_2 diffusion to the heme. EMBO J. **20:**3902-9.

99. **Miller, P. F., and M. C. Sulavik.** 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli.* Mol. Microbiol. **21:**441-8.

100. **Mills, C. E., S. Sedelnikova, B. Soballe, M. N. Hughes, and R. K. Poole.** 2001. *Escherichia coli* flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of nitric oxide. Biochem. J. **353:**207-13.

101. **Minas, W., P. Brünker, P. T. Kallio, and J. E. Bailey.** 1998. Improved erythromycin production in a genetically engineered industrial strain of *Saccharopolyspora erythraea.* Biotechnol. Prog. **14:**561-566.

102. **Nasr, M. A., K. W. Hwang, M. Akbas, D. A. Webster, and B. C. Stark.** 2001. Effects of culture conditions on enhancement of 2,4-dinitrotoluene degradation by *Burkholderia* engineered with the *Vitreoscilla* hemoglobin gene. Biotechnol. Prog. **17:**359-61.

103. **Nilsson, M., P. T. Kallio, J. E. Bailey, L. Bülow, and K. G. Wahlund.** 1999. Expression of *Vitreoscilla* hemoglobin in *Escherichia coli* enhances ribosome and tRNA levels: A flow field-flow fractionation study. Biotechnol. Prog. **15:**158-163.

104. **Nunoshiba, T., T. deRojas-Walker, J. S. Wishnok, S. R. Tannenbaum, and B. Demple.** 1993. Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. Proc. Natl. Acad. Sci. USA **90:**9993-7.

105. **Ollesch, G., A. Kaunzinger, D. Juchelka, M. Schubert-Zsilavecz, and U. Ermler.** 1999. Phospholipid bound to the flavohemoprotein from *Alcaligenes eutrophus.* Eur. J. Biochem. **262:**396-405.

106. **Orii, Y., N. Ioannidis, and R. K. Poole.** 1992. The oxygenated flavohaemoglobin from *Escherichia coli*: evidence from photodissociation and rapid-scan studies for two kinetic and spectral forms. Biochem. Biophys. Res. Commun. **187:**94-100.

107. **Orii, Y., and D. A. Webster.** 1986. Photodissociation of oxygenated cytochrome *o* (*Vitreoscilla*) and kinetic studies of reassociation. J. Biol. Chem. **261:**3544-7.

108. **Patel, S. M., B. C. Stark, K. W. Hwang, K. L. Dikshit, and D. A. Webster.** 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.

109. **Pendse, G. J., and J. E. Bailey.** 1994. Effect of *Vitreoscilla* hemoglobin expression on growth and specific tissue-plasminogen activator productivity in recombinant Chinese Hamster Ovary cells. Biotechnol. Bioeng. **44:**1367-1370.

110. **Pohlmann, A., R. Cramm, K. Schmelz, and B. Friedrich.** 2000. A novel NO-responding regulator controls the reduction of nitric oxide in *Ralstonia eutropha.* Mol. Microbiol. **38:**626-38.

111. **Poole, R. K., M. F. Anjum, J. Membrillo-Hernández, S. O. Kim, M. N. Hughes, and V. Stewart.** 1996. Nitric oxide, nitrite, and FNR regulation of *hmp* (flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. **178:**5487-92.

112. **Poole, R. K., and M. N. Hughes.** 2000. New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. **36:**775-783.

113. **Poole, R. K., N. Ioannidis, and Y. Orii.** 1996. Reactions of the *Escherichia coli* flavohaemoglobin (Hmp) with NADH and near-micromolar oxygen: Oxygen affinity of NADH oxidase activity. Microbiology. **142:**1141-1148.

114. **Poole, R. K., N. Ioannidis, and Y. Orii.** 1994. Reactions of the *Escherichia coli* flavohaemoglobin (Hmp) with oxygen and reduced nicotinamide adenine dinucleotide: evidence for oxygen switching of flavin oxidoreduction and a mechanism for oxygen sensing. Proc. R. Soc. Lond. B Biol. Sci. **255:**251-8.

115. **Poole, R. K., N. J. Rogers, R. A. M. Dmello, M. N. Hughes, and Y. Orii.** 1997. *Escherichia coli* flavohaemoglobin (Hmp) reduces cytochrome *c* and Fe(III)-hydroxamate K by electron transfer from NADH via FAD: Sensitivity of oxidoreductase activity to haem-bound dioxygen. Microbiology **143:**1557-1565.

116. **Potts, M., S. V. Angeloni, R. E. Ebel, and D. Bassam.** 1992. Myoglobin in a *Cyanobacterium.* Science **256:**1690-1692.

117. **Prinz, W. A., F. Aslund, A. Holmgren, and J. Beckwith.** 1997. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. J. Biol. Chem. **272:**15661-7.

118. **Probst, I., G. Wolf, and H. G. Schlegel.** 1979. An oxygen-binding flavohemoprotein from *Alcaligenes eutrophus.* Biochim. Biophys. Acta **576:**471-8.

119. **Puustinen, A., M. Finel, T. Haltia, R. B. Gennis, and M. Wikstrom.** 1991. Properties of the 2 Terminal Oxidases of *Escherichia coli.* Biochemistry. **30:**3936-3942.

120. **Puustinen, A., and M. Wikström.** 1991. The heme groups of cytochrome *o* from *Escherichia coli* Proc. Natl. Acad. Sci. USA **88:**6122-6126.

121. **Ramandeep, K. W. Hwang, M. Raje, K. J. Kim, B. C. Stark, K. L. Dikshit, and D. A. Webster.** 2001. *Vitreoscilla* hemoglobin. Intracellular localization and binding to membranes. J. Biol. Chem. **276:**24781-9.

122. **Ramirez, M., B. Valderrama, R. Arrendondo-Peter, M. Soberon, J. Mora, and G. Hernandez.** 1999. *Rhizobium etli* genetically engineered for the heterologous expression of *Vitreoscilla* sp. hemoglobin: Effect on free-living and Symbiosis. Mol. Plant-Microbe Interactions **12:**1008-1015.

123. **Rice, C. W., and W. P. Hempfling.** 1978. Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli.* J. Bacteriol. **134:**115-124.

124. **Sander, F. C., R. A. Fachini, D. E. Hughes, J. L. Galazzo, and J. E. Bailey.** 1994. Expression of *Vitreoscilla* hemoglobin in *Corynebacterium glutamicum* increases final concentration and yield of L-Lysine. In: Proceedings of the 6. European Congress on Biotechnology 1993. Elsevier Science B. V. pp. 607-610.

125. **Shaw, D. J., and J. R. Guest.** 1982. Amplification and product identification of the *fnr* gene of *Escherichia coli.* J. Gen. Microbiol. **128:**2221-8.

126. **Shaw, D. J., and J. R. Guest.** 1981. Molecular cloning of the *fnr* gene of *Escherichia coli* K12. Mol. Gen. Genet. **181:**95-100.

127. **Shaw, D. J., and J. R. Guest.** 1982. Nucleotide sequence of the *fnr* gene and primary structure of the FNR protein of *Escherichia coli.* Nucleic Acids Res. **10:**6119-30.
128. **Shaw, D. J., D. W. Rice, and J. R. Guest.** 1983. Homology between CAP and FNR, a regulator of anaerobic respiration in *Escherichia coli.* J. Mol. Biol. **166:**241-7.
129. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Van Leeuwenhoek. **66:**23-36.
130. **Spiro, S., K. L. Gaston, A. I. Bell, R. E. Roberts, S. J. Busby, and J. R. Guest.** 1990. Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. Mol. Microbiol. **4:**1831-8.
131. **Spiro, S., and J. R. Guest.** 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia coli.* FEMS Microbiol. Rev. **6:**399-428.
132. **Stevanin, T. M., N. Ioannidis, C. E. Mills, S. O. Kim, M. N. Hughes, and R. K. Poole.** 2000. Flavohemoglobin hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes *bo* or *bd,* from nitric oxide. J. Biol. Chem. **275:**35868-35875.
133. **Stewart, V.** 1988. Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. **52:**190-232.
134. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188-194.
135. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, and I. T. Paulsen.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959-64.
136. **Suzuki, T., and T. Takagi.** 1992. A myoglobin evolved from indoleamine 2,3-dioxygenase. J. Mol. Biol. **228:**698-700.
137. **Tarricone, C., S. Calogero, A. Galizzi, A. Coda, P. Ascenzi, and M. Bolognesi.** 1997. Expression, purification, crystallization, and preliminary X-ray diffraction analysis of the homodimeric bacterial hemoglobin from *Vitreoscilla stercoraria.* Proteins **27:**154-6.
138. **Tarricone, C., A. Galizzi, A. Coda, P. Ascenzi, and M. Bolognesi.** 1997. Unusual structure of the oxygen-binding site in the dimeric bacterial hemoglobin from *Vitreoscilla* sp. Structure **5:**497-507.
139. **Taylor, B. L., and I. B. Zhulin.** 1999. PAS domains: Internal sensors of oxygen, redox potential, and light. Microbiol. Mol Biol. Reviews. **63:**479-507.
140. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA- binding site: a mechanism for differential promoter selection. Cell **78:**897-909.
141. **Tsai, P. S., P. T. Kallio, and J. E. Bailey.** 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:**288-93.
142. **Tsai, P. S., M. Nageli, and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o.* Biotechnol.Bioeng. **49:**151-160.
143. **Tsai, P. S., G. Rao, and J. E. Bailey.** 1995. Improvement of *Escherichia coli* microaerobic oxygen-metabolism by *Vitreoscilla* hemoglobin - new insights from NAD(P)H fluorescence and culture redox potential. Biotechnol. Bioeng. **47:**347-354.
144. **Tyree, B., and D. A. Webster.** 1979. Intermediates in the reaction of reduced cytochrome *o* (*Vitreoscilla*) with oxygen. J. Biol. Chem. **254:**176-9.
145. **Unden, G., and J. Bongaerts.** 1997. Alternative respiratory pathways of *Escherichia coli*: energetics and transcriptional regulation in response to electron acceptors. Biochim. Biophys. Acta **1320:**217-34.
146. **Unden, G., and J. R. Guest.** 1985. Isolation and characterization of the FNR protein, the transcriptional regulator of anaerobic electron transport in *Escherichia coli.* Eur. J. Biochem. **146:**193-9.
147. **Unden, G., and M. Trageser.** 1991. Oxygen regulated gene expression in *Escherichia coli*: control of anaerobic respiration by the FNR protein. Antonie Van Leeuwenhoek **59:**65-76.

148. **Vasudevan, S. G., W. L. F. Armarego, D. C. Shaw, P. E. Lilley, N. E. Dixon, and R. K. Poole.** 1991. Isolation and nucleotide-sequence of the *hmp* gene that encodes a hemoglobin-like protein in *Escherichia coli* K-12. Mol. Gen. Genetics **226:**49-58.

149. **Wakabayashi, S., H. Matsubara, and D. A. Webster.** 1986. Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla.* Nature **322:**481-3.

150. **Watts, R. A., P. W. Hunt, A. N. Hvitved, M. S. Hargrove, W. J. Peacock, and E. S. Dennis.** 2001. A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. Proc. Natl. Acad. Sci .USA **98:**10119-24.

151. **Webster, D. A.** 1975. The formation of hydrogen peroxide during the oxidation of reduced nicotinamide adenine dinucleotide by cytochrome *o* from *Vitreoscilla.* J. Biol. Chem. **250:**4955-8.

152. **Webster, D. A.** 1988. Structure and Function of Bacterial Hemoglobin and Related Proteins. Adv. Inorg. Biochem. **7:**245-265.

153. **Webster, D. A., and D. P. Hackett.** 1966. The purification and properties of cytochrome *o* from *Vitreoscilla.* J. Biol. Chem. **241:**3308-15.

154. **Webster, D. A., and C. Y. Liu.** 1974. Reduced nicotinamide adenine dinucleotide cytochrome *o* reductase associated with cytochrome *o* purified from *Vitreoscilla.* Evidence for an intermediate oxygenated form of cytochrome *o.* J. Biol. Chem. **249:**4257-60.

155. **Webster, D. A., and Y. Orii.** 1977. Oxygenated Cytochrome *o.* An active intermediate observed in whole cells of *Vitreoscilla.* J. Biol. Chem. **252:**1834-6.

156. **Wei, M. L., D. A. Webster, and B. C. Stark.** 1998. Genetic engineering of *Serratia marcescens* with bacterial hemoglobin gene: Effects on growth, oxygen utilization, and cell size. Biotechnol. Bioeng. **57:**477-483.

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

158. **Weihs, V., K. Schmidt, B. Schneider, and B. Friedrich.** 1989. The formation of an oxygen-binding flavohemoprotein in *Alcaligenes eutrophus* is plasmid determined. Arch. Microbiol. **151:**546-550.

159. **Wu, J., W. R. Dunham, and B. Weiss.** 1995. Overproduction and physical characterization of SoxR, a [2Fe- 2S] protein that governs an oxidative response regulon in *Escherichia coli.* J. Biol. Chem. **270:**10323-10327.

160. **Zhang, X. P., and R. H. Ebright.** 1990. Substitution of 2 base pairs (1 base pair per DNA half-site) within the *Escherichia coli lac* promoter DNA site for catabolite gene activator protein places the *lac* promoter in the FNR regulon. J. Biol. Chem. **265:**12400-3.

161. **Zheng, M., F. Aslund, and G. Storz.** 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279:**1718-21.

162. **Zheng, M., B. Doan, T. D. Schneider, and G. Storz.** 1999. OxyR and SoxRS regulation of *fur* J.Bacteriol. **181:**4639-4643.

163. **Zhu, H., and A. F. Riggs.** 1992. Yeast flavohemoglobin is an ancient protein related to globins and a reductase family. Proc. Natl. Acad. Sci. USA **89:**5015-5019.

Expression of *Alcaligenes eutrophus* Flavohemoprotein and Engineered *Vitreoscilla* Hemoglobin-Reductase Fusion Protein for Improved Hypoxic Growth of *Escherichia coli*

Abstract

Expression of the *vhb* gene encoding hemoglobin (VHb) from *Vitreoscilla* sp. in several organisms has been shown to improve microaerobic cell growth and enhance oxygen-dependent product formation. The amino terminal-hemoglobin domain of the flavohemoprotein (FHP) of the gram-negative hydrogen-oxidizing bacterium *Alcaligenes eutrophus* has 51% sequence homology with VHb. However, like other flavohemoglobins and unlike VHb, FHP possesses a second (carboxy-terminal) domain with NAD(P)H and flavin adenine dinucleotide (FAD) reductase activities. To examine whether the carboxy-terminal redox active site of flavohemoproteins can be used to improve the positive effects which VHb exerts in microaerobic *Escherichia coli* cells, we fused sequences encoding NAD(P)H, FAD, or NAD(P)H-FAD reductase activities of *A. eutrophus* in frame after the *vhb* gene. Similarly, the gene for FHP was modified and expression cassettes were constructed encoding the amino terminal hemoglobin (FHPg), FHPg-FAD, FHPg-NAD or FHP activities. Biochemically active hemeproteins were produced from all these constructions in *E. coli* as indicated by their ability to abduct carbon monoxide. The presence of FHP or of VHb-FAD-NAD-reductase increased the final cell density of transformed wild-type *E. coli* cells approximately 50% and 75%, respectively, for hypoxic fed-batch culture relative to the control synthesizing VHb. Approximately the same final optical densities were achieved with the *E. coli* strains expressing FHPg and VHb. The presence of VHb-FAD or FHPg-FAD increased the final cell density slightly relative to the VHb-expressing control under the same cultivation conditions. The expression of VHb-NAD or FHPg-NAD fusion proteins reduced the final cell densities approximately 20% relative to the VHb-expressing control. The VHb-FAD-NAD-reductase expressing strain was also able to synthesize 2.3-fold more recombinant β-lactamase relative to the VHb-expressing control.

Introduction

One of the foremost examples of inverse metabolic engineering (the genetic transfer of useful phenotypes to heterologous organisms) is expression of *Vitreoscilla* hemoglobin (VHb) in aerobic bacteria, yeast, fungi and plants to enhance their growth and productivity (See articles 2, 6, 14, 25 summarizing the results). Although the exact mechanism by which VHb causes these effects is unknown, it has been hypothesized that, due to its unusual kinetic parameters for oxygen binding and release (K_D = 72 μM) (45), VHb is able to scavenge oxygen molecules from solution and provide them for cellular activities in heterologous organisms (18, 45, 49). More detailed observations of biochemical and physiological changes accompanying VHb-expression in *Escherichia coli* indicate increased overall ATP production and turnover rates, increase in cytochrome *o* expression and specific activity, decreased levels of reduced pyridine nucleotides and changes in central carbon metabolism (7, 18, 27, 38-40). Experiments were conducted using engineered *E. coli* to determine if globins besides VHb can be applied to enhance hypoxic growth and protein production levels. *E. coli* cells expressing either of two different hemoglobin-like proteins, horse heart myoglobin and yeast flavohemoglobin, each having some sequence homology with VHb, grew to lower final cell densities than did VHb-expressing *E. coli* in oxygen-limited fed-batch cultivations (19).

Recently, a family of two-domain globins containing N-terminal oxygen binding and C-terminal reductase activities, termed flavohemoproteins have been identified both in prokaryotic and eukaryotic organisms such as *E. coli* (43), *Erwinia chrysanthemi* (12), *Bacillus subtilis* (23), *Candida norvegensis* (15), *Fusarium oxysporum* (37) and *Saccharomyces cerevisiae* (51). One such protein, a megaplasmid encoded cytoplasmic flavohemoglobin (FHP), was also identified from a facultatively lithoautotrophic, hydrogen oxidizing, gram-negative bacteria *Alcaligenes eutrophus* (4, 30, 48). *A. eutrophus* grows, like *Vitreoscilla,* in oxygen-scarce environments and has respiratory type of metabolism, but *A. eutrophus* is also able to grow without oxygen, if either nitrate or nitrite are present as terminal electron acceptors (4, 30). Limited oxygen supply causes approximately 20-fold increase in FHP content,

suggesting that expression of FHP in *A. eutrophus*, like VHb in *Vitreoscilla* is regulated by oxygen (3, 31).

The N-terminal hemoglobin domain of FHP (termed FHPg in this publication) shares high sequence homology (51%) with VHb (8, 44). FHP is a monomeric polypeptide of 403 amino acids (44.8 kDa) consisting of two different protein modules: a N-terminal hemoglobin- (FHPg; residues 1 - 147), and a C-terminal redox active domain with binding sites for FAD (residues 153 - 258) and for NAD^+ (residues 266 - 403), which share high sequence homology to the FNR-like (**F**erredoxin **N**ADP$^+$ **R**eductase) protein family. The FNR-like proteins are not identical with the well-characterized global transcriptional regulator FNR (Fumarate Nitrate Reduction), which controls the expression of genes required for anaerobic metabolism in *E.* coli (42). proteins. The reductase domain is able to reduce several artificial electron acceptors as well as cytochrome *c*. Furthermore, the *b*-type heme-iron complex of the FHPg domain is capable of reversibly binding oxygen in its reduced state (30, 31). The different domains of the FHP polypeptide are connected by short sequences of 5-7 amino acids Pro148-Gly-Gly-Trp-Lys152 and Phe259-His-Ile-Asp-Val-Asp-Ala265 between FHPg-FAD and FAD-NAD domains, respectively (8). The three-dimensional structure of the FHP has also been resolved by X-ray crystallography (10).

The goal of this research was to study potential biotechnological applications of the expression of FHPg, FHP and fusion parts thereof (NAD^+/FAD domains) in *E. coli* under microaerobic culture conditions in a bioreactor. In addition, the *vhb* gene was fused with sequences encoding NAD^+, FAD and FAD-NAD activities of the C-terminal domain of FHP. Hypoxic growth of different *E. coli* constructs expressing these different heterologous and fusion proteins was compared with growth of VHb- expressing *E. coli*, reaching higher final cell densities relative to plasmid carrying VHb-negative *E. coli* (21), in a controlled hypoxic bioreactor.

Materials and Methods

E. coli strains and plasmids.

E. coli DH5α [F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gryA96 relA1* (ϕ80d*lacZ*ΔM15) (*lacZYA-argF*) U169; Gibco BRL Life Technologies] was used as a host during the subcloning steps. *E. coli* MG1655 [λ^-, F^-; Cold Spring Harbor Laboratory] was used to analyze the effect of VHb or of other globin and globin-reductase constructions on growth of cells in a microaerobic bioreactor. pRED2 carrying the *Vitreoscilla vhb* gene has been described elsewhere (21, 22). pUC19 (50) was used for subcloning and pKQV4 (36) employed as an IPTG-inducible expression vector. pPPC1, a derivative of pKQV4 and containing the *vhb* gene, has been described by Kallio et al. (19). pGE276 (8) contains the *fhp* gene of *A. eutrophus* and was a generous gift from Dr. B. Friedrich (Humboldt-University of Berlin, Berlin, Germany).

DNA manipulations.

All restriction endonucleases were purchased from commercial suppliers and used according to the recommended protocols. DNA manipulations were performed according to standard protocols (33).

PCR technique (32) was used to amplify the genes encoding VHb (GeneBank accession no. X13516) (22) or FHP (GeneBank accession no. X74334) (8) and open reading frames coding for FHPg, NAD, and FAD protein domains of FHP or their combinations. PCR amplifications were performed using a Perkin Elmer Gene Amp 9600 PCR System and *Pwo* Polymerase (Boehringer Mannheim). Oligonucleotides for PCR reactions were synthesized by Microsynth (Balgach, Switzerland) and are shown in Table 1. Translational stop codons (CTA, TTA) were also inserted into the gene structures encoding new fusion proteins if necessary (Table 1). The oligonucleotides were designed in a way that the hinge sequences between the various domains of fusion proteins (globin-reductase) remained highly conserved and that amino acid sequences relative to the original sequence of FHP were

minimally altered (summarized in Figure 1). The amino acid sequence of either FHPg or VHb domain contains 147 and 146 residues, respectively. Thus, Gly150 within the FHP protein sequence was changed to Thr149 in VHb-FAD and VHb-FAD-NAD fusion proteins. This change generates a new restriction site for *Kpn*I. The linkage of NAD domain (LHIDVDA) either with VHb or FHPg domains changed **L**eu to Phe (F) and Pro (P), respectively (Figure 1 B). PCR amplified gene fragments were purified using QIAquick PCR Purification Kit (Qiagen, Basel, Switzerland). DNA fragments were separated by agarose gel electrophoresis and recovered using QIAquick Gel Extraction Kit.

Table 1. Oligonucleotides used for PCR amplifications of the *vhb*, *fhp*, and the open reading frames of *fhp* encoding FHPg, NAD and FAD subunits[a)]

Oligo 1:	CG**GAATTC**CG**ATG***CTGACCCAGAAAACAAAAG*		
	*Eco*RI	start	*fhp*
Oligo 2:	TGCA**CTGCAG**TGCA**CTATTA***TTGTTCCGCAGAGCGCTC*		
	*Pst*I	stop 2x	*fhp*
Oligo 3:	TGCA**CTGCAG**TGCA**TTACTA***CTCAGCAAACAGGTCG*		
	*Pst*I	stop 2x	*nad*
Oligo 4:	CG**GAATTC**CG**ATG***TTAGACCAGCAAACCATT*		
	*Eco*RI	start	*vhb*
Oligo 5:	GG**GGTACC**GGG*TTCAACCGCTTGAGCGTAC*		
	*Kpn*I		*vhb*
Oligo 6:	GG**GGTACC**TGGAAG*GGGTGGCGCACCTTTGTG*		
	*Kpn*I		*fad*
Oligo 7:	AC**GTTGAC**GCC*AAGACACCCATTGTnCCTGATC*		
	*Hinc*II		*nad*
Oligo 8:	GC**GTCAAC**GTCGATATGGAA*TTCAACCGCTTGAGCGTAC*		
	*Hinc*II		*vhb*
Oligo 9:	C**GTCAAC**GTCGATATGGGG*TTGTTCCGCAGAGCGCTC*		
	*Hinc*II		*fhp*
Oligo 10:	TGCA**CTGCAG**TGCA**CTATTA***GCTTCCATAGGGCGCAG*		
	*Pst*I	stop 2x	*fad*

a) Restriction sites, translational start (**ATG**) and stop codons (**CTA/TTA**) are written in bold letters and the coding sequence of the genes are given in italics. All oligonucleotides are written in 5' -> 3' direction.

A PCR screening method was used to identify *E. coli* DH5α transformants carrying the correct gene inserts either in pUC19 or in pKQV4. The reaction mixture for one sample contained 0.4 µl DMSO, 1.0 µl 10X *Taq*-Buffer, 0.1 µl *Taq*-Polymerase (5U/µl; Boehringer Mannheim, Germany), 1 µl of each primer (final concentration of each primer 100 pmol/µl), dNTP's were added to final concentration of 10 mM and H_2O to give total volume of 10 µl in a well of a PCR plate containing 96 wells (Axon Lab, Baden-Dättwil, Switzerland). A single, ampicillin resistant colony was picked aseptically from overnight-incubated LB agar plates (33) supplemented with ampicillin (100 µg/ml) and transferred into a well of the PCR plate. Standard PCR techniques were used for DNA amplification (32). Reaction mixtures were analyzed for amplified gene fragments of expected molecular size by agarose gel electrophoresis (33) and the correct recombinant plasmid was used for further characterization.

Plasmid DNA for DNA sequencing were isolated and purified from overnight grown bacteria cultures using a QIAprep Spin MiniPrep Kit (Qiagen, Switzerland). The Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham International) was used for DNA sequencing reactions (34) and cycle sequencing was performed according to the recommended protocol of the manufacturer. The infrared (IRD-41) labelled sequencing primers pKK_{for} (5' CTC AAG GCG CAC TCC CGT TCT) or pKK_{rev} (5' GAG TTC GGC ATG GGG TCA GGT G) and -40 forward or -40 reverse universal primers for DNA sequencing of pKQV4 and of pUC19 derivatives, respectively, were obtained from MWG-Biotech (Ebersberg, Germany). The sequencing reactions were separated in a LI-COR 4000 L automated DNA sequencer (LI-COR, Inc., Lincoln, Nebraska). IRD-41 labelled DNA fragments were visualized by using a scanning laser microscope assembly. DNA sequence data were collected and analyzed by using the Base ImagIR™ software of LI-COR.

Construction of VHb-reductase, FHP and FHPg-reductase expression vectors.

Plasmids pRED2 and pGE276 were used as templates for *vhb* and *fhp* gene amplifications, respectively. The oligonucleotides were designed so that they changed only one amino acid within the proposed linker region between the protein domains (Figure 1). The gene fragments encoding FHP, or FHPg and FHPg-FAD domains of FHP were PCR amplified using oligonucleotides 1 and 3, 1 and 2, and 1 and 10, respectively (Table 1). Purified DNA fragments were digested with *Eco*RI and *Pst*I and subcloned directly into pKQV4 digested with the same enzymes. Correct plasmids were identified using the PCR screening method and authenticity of reading frames of the expression cassettes in pAX1 (FHPg), pAX5 (FHP), and pAX6 (FHPg-FAD) were verified by DNA sequencing.

Construction of the plasmids pAX4 (VHb-FAD-NAD), pAX9 (VHb-FAD), pAX12 (VHb-NAD) and pAX14 (FHPg-NAD) is summarized below (Figure 1 B). In all cases two subcloning steps using pUC19 were necessary for the construction of the expression cassettes. The gene fragment encoding FHPg subunit of FHP was PCR amplified using oligonucleotides 1 and 9 (Table 1) and a purified 6.5 kb *Sal*I-*Xho*I fragment of pGE276 as a template. The PCR fragments were isolated and digested with *Eco*RI and *Hinc*II. The fragments were subcloned into pUC19 digested with the same enzymes and the new plasmid was named pAX13. The gene fragment encoding NAD-domain was amplified using oligonucleotides 3 and 7 (Table 1) and the same template as mentioned above. The purified PCR fragments were *Hinc*II-*Pst*I digested and subcloned into pAX13 to generate pAX2. The expression cassette synthesizing FHPg-NAD was removed with *Eco*RI-*Pst*I digestion and subcloned into *Eco*RI-*Pst*I digested pKQV4 to generate pAX14. An identical cloning strategy was used to construct the expression cassette synthesizing VHb-NAD using oligonucleotides 4 and 8, and 3 and 7 (Table 1) for the *vhb* and the open reading frame encoding NAD domain of FHP, respectively. The expression vector producing VHb-NAD was named pAX12.

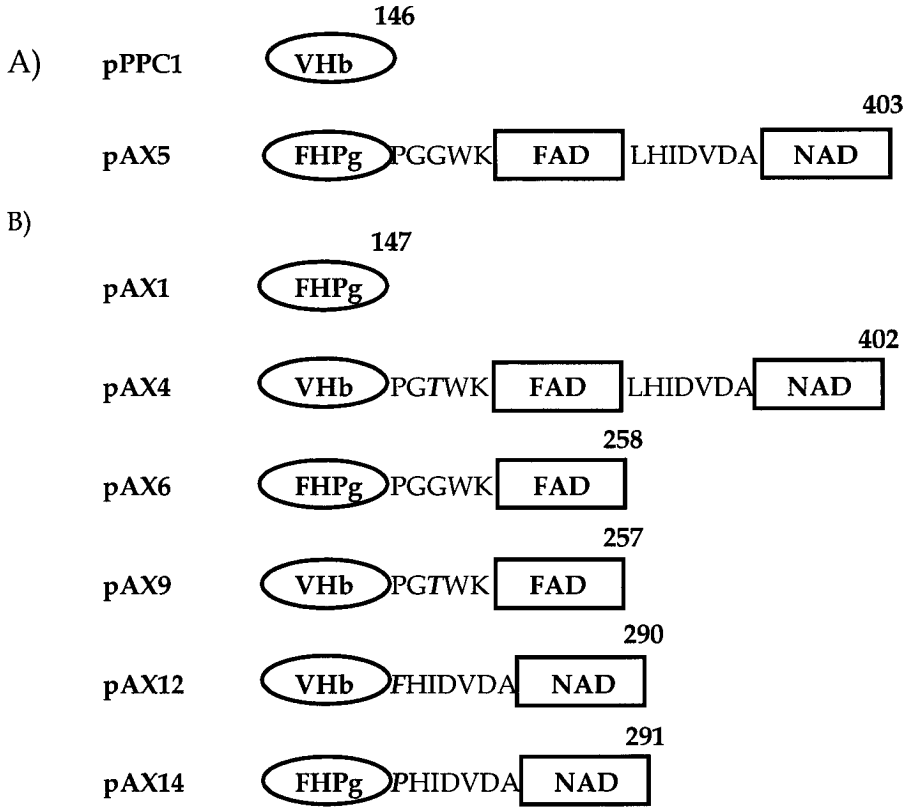

Figure 1. (A) Protein domains of native *Vitreoscilla* hemoglobin (VHb) and *R. eutropha* flavohemoprotein (FHP). Alignment of VHb (1-146) and FHP (1-403) amino acid sequence reveals three different modules in FHP: hemoglobin domain FHPg (1-147), FAD binding domain (153-258) and NAD(P)H binding domain (266-403). The linker regions between the FHPg-FAD (PGGWK) and the FAD-NAD (LHIDVDA) domains are shown. The gene bank accession number for *vhb* and *fhp* is X13516 and X74334, respectively. (B) The protein modules of chimeric proteins are shown with the hinge sequences. Gly150 was changed to Thr149 in pAX4 and pAX9 expressing VHb-FAD-NAD (1-402) or VHb-FAD (1-257) proteins, respectively, and written in bold italics. VHb is one residue shorter (146) relative to FHPg (147) and thus, the renumbering of amino acid residues in VHb fusion proteins was necessary. LHIDVDA linker sequence has changed to ***F***HIDVDA and ***P***HIDVDA sequences in pAX12 (VHb-NAD) and pAX14 (FHPg-NAD), respectively, and the changed amino acid residue is shown in bold italics.

Expression cassettes for VHb-FAD and VHb-FAD-NAD production (Figure 1 B) were constructed using a slightly modified protocol as outlined above. The *vhb* gene was amplified using oligonucleotides 4 and 5 (Table 1). The *vhb* PCR fragment was double digested with *Eco*RI and *Kpn*I and inserted into pUC19 also digested with the same enzymes. The plasmid was named pAX7. The open reading frame of

the *fhp* gene encoding FAD module was amplified using oligonucleotides 6 and 10 (Table 1), double digested with *Kpn*I and *Pst*I and ligated with pAX7, resulting in pAX16. The complete expression cassette for VHb-FAD-expression was excised from pAX16 using *Eco*RI and *Pst*I and ligated with *Eco*RI-*Pst*I digested pKQV4 to form pAX9. An identical cloning strategy was applied for VHb-FAD-NAD-expression cassette. The *vhb* and the *fhp* gene fragment encoding FAD-NAD were amplified using oligonucleotides 4 and 5 and 3 and 6, respectively (Table 1). The final expression plasmid producing VHb-FAD-NAD was named pAX4. The amplified expression cassettes in vectors pAX4 (VHb-FAD-NAD), pAX9 (VHb-FAD), pAX12 (VHb-NAD) and pAX14 (FHPg-NAD) were subjected to DNA sequencing and the sequences of the reading frames verified.

Bioreactor cultivations.

Cultures for both plasmid DNA isolations and for bioreactor inoculations were grown at 37°C, 250 rpm for approximately 14 hours in 50 ml shake flasks containing 10 ml of LB medium (33) supplemented with ampicillin (100 µg/ml).

Fed-batch cultivations of *E. coli* MG1655, carrying various VHb, VHb-reductase, FHPg or FHPg-reductase expression vectors, were performed in a defined glucose batch medium supplemented with 150 mg/l casamino acids (Difco), 30 mg/l yeast extract (Difco) and 100 mg/l ampicillin (19) and using a Sixfors bioreactor unit (Infors, Bottmingen, Switzerland) allowing six controlled cultivations at the same time. To start Sixfors bioreactor fed-batch cultivations, 3.0 ml of seeding culture was used to inoculate 300 ml of glucose batch medium, and process parameters were maintained at 37°C, pH 7 ± 0.2 (adjusted either with 2 M NaOH or with 2 M H_3PO_4), 300 rpm, and 120 ml/min of air. The composition of feed medium has been described previously (19). Expression of hemoglobins and hemoglobin-reductase constructions were induced by IPTG-addition (final concentration of 100 µM) when the optical densities (A_{600}) of cultures were approximately 1. Fed-batch mode was commenced with 1 ml/h of feed medium when the culture reached an A_{600} of

approximately 2, and the feeding rate was increased to 2 ml/h when A_{600} was approximately 4. Thereafter, the feeding rate was maintained constant at 2 ml/h until the end of 30 hours microaerobic fed-batch cultivations.

Dissolved oxygen concentration and exhaust gases (CO_2 and O_2) from bioreactors were monitored as described previously (17, 19).

Analytical techniques

The soluble fractions of both non-globin-producing and globin-expressing *E. coli* MG1655 cells (for VHb, FHPg, FHPg-FAD-NAD, FHPg-FAD, FHPg-NAD, VHb-FAD-NAD, VHb-FAD, and VHb-NAD constructions) were prepared by harvesting 100 ml of bioreactor cultivated cells followed by centrifugation using a Beckman J2-21M centrifuge with rotor JLA 10.500, at 4°C for 10 min and 7.000 g. Supernatants were discarded and cell pellets resuspended in 10 ml lysis buffer (100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA). Cells were disrupted using a French press (SLM-Aminco) at 1200 psi. The cell debris were removed by centrifugation using Beckman GS-6R at 4°C for 15 min, and 3200 g. The supernatants were poured in new tubes and clear soluble fractions were recovered by centrifugation 15 min, 14000 rpm at 4°C in an Eppendorf centrifuge. Globin activities were assayed using reduced + CO minus reduced difference spectroscopy as described previously (13).

Acetate concentrations of bioreactor samples were determined by a Beckman SYNCHRON CX5CE autoanalyzer. Boehringer Mannheim Acetate Kit (#148261) was adapted for the Beckman autoanalyzer system and was used to measure acetate concentrations from the bioreactor samples. Ethanol concentrations were measured enzymatically with a Beckman autoanalyzer and using the alcohol kit of Beckman. The measured concentrations were normalized to the final A_{600} values of the cultures.

β-lactamase activities encoded by the plasmids pKQV4 (control), pAX1 (FHPg), pAX4 (VHb-FAD-NAD), pAX5 (FHP), pAX6 (FHPg-FAD), pAX9 (VHb-FAD), pAX12 (VHb-NAD), pAX14 (FHPg-NAD) and pPPC1 (VHb) were determined using the chromogenic cephalosporin nitrocefin (Becton & Dickinson) as a substrate (28). The samples were withdrawn at the end of hypoxic bioreactor cultivations, cells were disrupted using a French press and the change of absorbance at 482 nm was recorded at room temperature (27). The total soluble protein of each sample was determined using a Protein Assay Kit (Bio-Rad) based on the method of Bradford (5). Activity of β-lactamase is reported in U/mg of soluble protein.

Results

Construction of hemoglobin (VHb and FHPg) and hemoglobin-reductase expression vectors

Most of the known bacterial hemoglobin proteins, such as FHP and HMP, show two different activities, an oxygen-binding and a reductase activity (8, 43). These two different biochemical properties are linked in a single two-domain protein containing N-terminal hemoglobin and C-terminal reductase modules (Figure 1A). VHb and the recently identified new globin of *Vitreoscilla stercoraria* do not have reductase activity in the same polypeptide (16, 22, 44). However, it is well documented that VHb requires reductase activity for physiological function and such an unlinked NADH-methemoglobin reductase has been identified in *Vitreoscilla. E. coli* also contains an unidentified reductase system capable of catalyzing the reduction of VHb *in vivo* (9, 46). However, maintenance of the redox state of heme iron of VHb, catalyzed by a heterologous reductase, may be an activity-limiting factor in a heterologous host and decrease the beneficial effects of VHb-expression such as relieving oxygen stress under hypoxic conditions. In order to study if fusion of a heterologous reductase domain to VHb provides improved benefits, the following three expression vectors were constructed: pAX4 (VHb-FAD-NAD), pAX9 (VHb-FAD), pAX12 (VHb-NAD). The N-terminal domain of

FHP is highly homologous with VHb (8). Thus, it may also be possible that FHP is able to expedite growth of *E. coli* cells under hypoxic conditions. To study this hypothesis we also constructed four new expression vectors: pAX1 (FHPg), pAX5 (FHP), pAX6 (FHPg-FAD), and pAX14 (FHPg-NAD). The constructions of these plasmids are described in Materials and Methods. The VHb-expression plasmid pPPC1 has been described previously (19). The structures of the novel expression modules with hinge sequences, relative to the wild type structures, are summarized in Figure 1B.

Microaerobic bioreactor cultivations

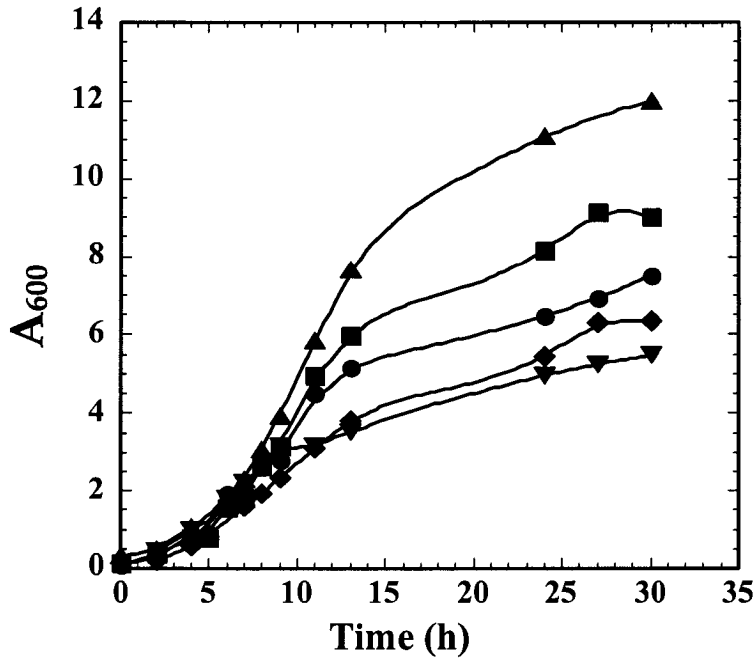

Figure 2. Optical density trajectories of *E. coli* MG1655 expressing proteins showing FHPg hemoglobin domain activity: pAX1 (FHPg;●), pAX5 (FHP; ▲), pAX6 (FHPg-FAD;■), pAX14 (FHPg-NAD;◆) and the control plasmid pKQV4 (▼). The cells were cultivated under hypoxic conditions in a SixFors bioreactor as described in Materials and Methods.

VHb-expressing wild-type *E. coli* MG1655 cells showed improved growth behavior relative to a common VHb-positive cloning host *E. coli* DH5α under oxygen-limited culture conditions (19). Thus, *E. coli* MG1655 cells expressing VHb (pPPC1), FHPg

(pAX1), or separate globin-reductase fusions (pAX5, pAX6, pAX4, pAX9, pAX12 and pAX14) were cultivated at least twice in a microaerobic SixFors bioreactor. The dissolved oxygen concentration readings by polarographic electrodes were zero after approximately 6 hours of various cultivations and remained there until the end of fed-batch processes. Differences in growth behavior between various constructions became apparent after approximately 8 - 9 hours of cultivation, where A_{600} was approximately 3, and the growth of non-VHb-expressing control MG1655:pKQV4 ceased rapidly. Obviously, the supply of oxygen was not sufficient to support effective growth of nonglobin-expressing *E. coli* cells beyond this point (Figure 2).

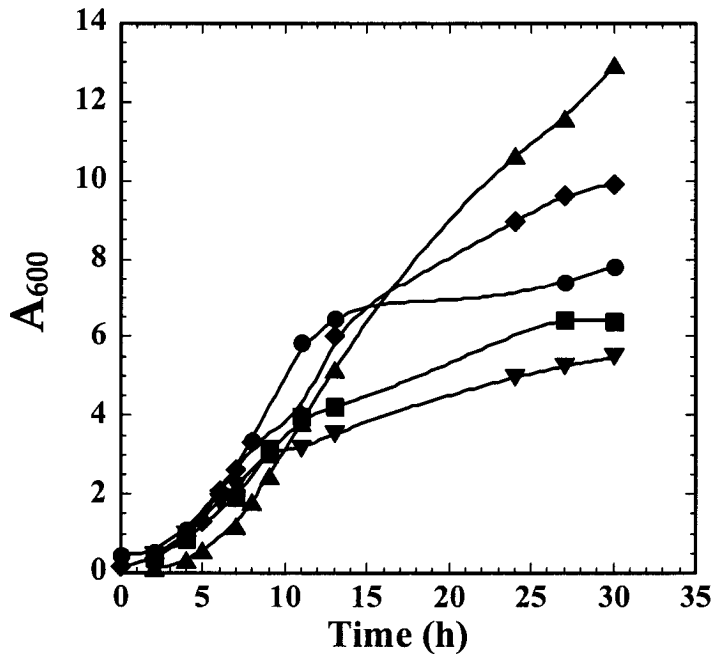

Figure 3. Time course of optical densities of *E. coli* MG1655 with the various VHb hemoglobin constructs: pPPC1 (VHb; •), pAX4 (VHb-FAD-NAD; ▲), pAX9 (VHb-FAD; ◆), and pAX14 (VHb-NAD; ■). Data for cultivations of MG1655 cells carrying the control plasmid pKQV4 are marked with (▼).

The growth curves of MG1655:pAX1, MG1655:pAX5, MG1655:pAX6 and MG1655:pAX14 expressing FHPg or FHP-reductase derivatives of *A. eutrophus* are shown in Figure 2. The growth curves of VHb- (pPPC1) or VHb-reductase-

expressing (pAX4, pAX9, and pAX12) *E. coli* MG1655 cells are shown in Figure 3. Non-globin-expressing MG1655:pKQV4 was used as an internal control between various cultivations and the final A_{600} of the control was 5.5 ± 0.5 at the end of 30 hours cultivation. *E. coli* MG1655:pAX1 (FHPg) expressing cells reached a final A_{600} of 8.3 ± 0.9 which is similar to the final A_{600} of 7.9 ± 0.5 for *Vitreoscilla* VHb-expressing MG1655:pPPC1 cells (Figures 2 and 3).

Coexpression of the NAD part of the reductase together with VHb (MG1655:pAX12) or FHPg (MG1655:pAX14) domains reduced the final A_{600} values of *E. coli* approximately 20 % relative to either MG1655:pPPC1 (VHb) or MG1655:pAX1 (FHPg), but these constructions did eventually attain slightly higher final optical density than the cultures carrying the control plasmid pKQV4. The final A_{600} for MG1655:pAX12 was 6.4 ± 1.0 and for MG1655:pAX14 6.4 ± 0.9. MG1655:pAX6 (FHPg-FAD) and MG1655:pAX9 (VHb-FAD) showed slightly improved final optical densities, 9.0 ± 1.0 and 9.9 ± 0.9, respectively, relative to VHb- or FHPg-expressing cells (Figures 2 and 3). *E. coli* MG1655:pAX5 (12.0 ± 1.5) cells expressing full length *A. eutrophus* FHP flavohemoprotein reached approximately 2.2-fold and 50 % higher final cell densities relative to the control MG1655:pKQV4 (5.5 ± 0.5) and VHb-expressing MG1655:pPPC1 (7.9 ± 0.5), respectively. MG1655:pAX4 (VHb-FAD-NAD) cells reached the highest final optical densities, an A_{600} of 13.9 ± 1.4 at the end of 30 hours hypoxic fed-batch cultivations (Figure 3). This value was on the average approximately 75% and 15% higher relative to the original VHb-expressing clone MG1655:pPPC1 (7.9 ± 0.5) and the FHP-expressing construct MG1655:pAX5 (12.0 ± 1.5), respectively (Figure 2 and 3). These results clearly show that the beneficial effect of VHb can be improved substantially by using protein engineering to combine directly its advantageous oxygen-binding properties with a fused reductase activity.

All expression vectors were also analyzed for insert maintenance by a PCR screening method. The results obtained using different oligonucleotide primer

combinations showed that the expression cassettes were stably maintained during prolonged hypoxic bioreactor cultivations (data not shown).

Byproduct accumulation during hypoxic bioreactor cultivations

The excretion of side products, such as acetate and ethanol, allows *E. coli* cells to relieve a surplus of reduction equivalents, regenerating NAD^+ and $NADP^+$, which are needed to metabolize glucose. Final concentrations of acetate and ethanol were assayed using a Beckman autoanalyzer from samples withdrawn from bioreactors at the end of 30 hours cultivation and normalized to one unit of A_{600}. Our results show reduced excretion of acetate from strains expressing various VHb, FHPg and hemoglobin-reductase constructions relative to the control MG1655:pKQV4. For MG1655:pAX4 (VHb-FAD-NAD) and MG1655:pAX5 (FHP), the smallest specific acetate levels were measured: 4.9 ± 0.3 mM/A_{600} and 4.6 ± 0.1 mM/A_{600}, respectively. These values were approximately 40 % lower relative to specific acetate accumulation of the vector control MG1655:pKQV4 cultivation (8.2 ± 0.7 mM/A_{600}). The expression of the hemoglobin domain alone, either in MG1655:pAX1 (FHPg; 6.4 ± 0.3 mM/A_{600}) or in MG1655:pPPC1 (VHb; 5.9 ± 0.7 mM/A_{600}) resulted in a smaller decrease (approximately 22% and 28%, respectively) in the production of acetate relative to the control (Table 2).

The excretion of ethanol was also measured from the samples at the end of cultivations. The results indicate that FHPg-expressing cells produced 40% less ethanol (1.4 ± 0.4 mM/A_{600}) relative to MG1655:pKQV4 (2.3 ± 1.5 mM/A_{600}). Surprisingly, VHb-expressing MG1655:pPPC1 cells produced 2.9 times more ethanol (4.0 ± 0.1 mM/A_{600}) relative to FHPg-producing strain MG1655:pAX1 under similar culture conditions. In addition, accumulation of ethanol was also reduced in MG1655:pAX6 and MG1655:pAX4, approximately 25% and 38%, respectively, relative to the control MG1655:pKQV4. These results show that

ethanol accumulation is not always decreased in hemoglobin-expressing cells relative to the hemoglobin-negative control.

Table 2. Final acetate concentrations of *E. coli* MG1655 expressing various hemoglobin constructions cultivated in a hypoxic bioreactor[a)]

Plasmid	Hemoglobin	Acetate mM/A_{600} [b)]
pPPC1	VHb	5.94 ± 0.69
pAX1	FHPg	6.43 ± 0.31
pAX4	VHb-FAD-NAD	4.89 ± 0.31
pAX5	FHP	4.63 ± 0.11
pAX9	VHb-FAD	5.63 ± 1.02
pAX6	FHPg-FAD	6.04 ± 0.96
pAX12	VHb-NAD	7.78 ± 1.41
pAX14	FHPg-NAD	6.38 ± 0.30
pQKV4	control	8.20 ± 0.69

[a)] Acetate concentrations were determined at the end of 30 hours of microaerobic cultivation and normalized to A_{600}.

[b)] Mean ± standard error of the mean.

Analysis of biological activities of VHb-, FHPg- and globin-reductase hemoproteins by CO-binding assay

Cells were harvested at the end of hypoxic fed-batch cultivations and samples for CO-binding assays were prepared following the protocol described in Materials and Methods. The biochemical activity of the globins in all constructions can be determined using a standard technique (13). The maximum and minimum values of the recorded spectra of the different constructions are given in Table 3. Due to lack of a reported extinction coefficient either for FHP or for FHPg and to the novel nature of the heme-reductase-fusion proteins, it is impossible to predict the effects of the reductase tails on the extinction coefficients. Thus, it is not possible to compare the specific activities of the different heme domains. Our results are only qualitative, giving no information about the specific activity of globins per mg of soluble protein. However, the results clearly show that biologically active hemoproteins were produced in all *E. coli* strains expressing various recombinant globins (Table 3).

Table 3. CO-binding assay of various *E. coli* MG1655 expressed hemoglobin proteins[a)]

Plasmid	Hemoglobin	minimum [nm]	maximum [nm]
pPPC1	VHb	419	437
pAX1	FHPg	422	437
pAX4	VHb-FAD-NAD	422	443
pAX5	FHP	422	439
pAX9	VHb-FAD	419	436
pAX6	FHPg-FAD	421	438
pAX12	VHb-NAD	416	442
pAX14	FHPg-NAD	419	436

[a)] CO-binding assay performed according to (13).

The CO-difference visible absorption spectrum of MG1655:pKQV4 revealed no hemoglobin activity, as shown previously (19) indicating that the recorded curves represent the activity of overexpressed heterologous hemoproteins and are not artifacts of *E. coli* HMP-expression (data not shown).

The hemoglobin domain (FHPg) of *A. eutrophus* flavohemoprotein has maximal absorption at a slightly longer wavelength (422 nm) relative to the γ-band of VHb (419 nm; Table 3). A similar maximal absorption value is observed in strains expressing FHPg protein fused either with FAD-NAD or FAD domain. A slight shift of the absorption maximum (419 nm) for the FHPg-NAD expressing strain was recorded. In addition, the absorption maximum has changed in VHb-FAD-NAD- (422 nm) and VHb-NAD-expressing (416 nm) strains relative to VHb producing strain (419 nm) (Table 3). This variation may result from a slightly varying three-dimensional structure of the hemoglobin domains of the fusion proteins. These results also verify that FHP contains a CO-binding *b*-type heme cofactor such as VHb and also HMP of *E. coli* (30, 41, 43).

Analysis of β-lactamase activity at the end of hypoxic cultivations.

Production of a model recombinant protein, β-lactamase, was also assayed from samples withdrawn from a microaerobic bioreactor (Table 4). The results showed

that MG1655:pAX1 (FHPg; 109 ± 3 U/mg), MG1655:pAX5 (FHP; 113 ± 10 U/mg), MG1655:pAX9 (VHb-FAD; 106 ± 5 U/mg) and MG1655:pPPC1 (VHb; 119 ± 13 U/mg) produced 24%, 28%, 20% and 35% more β-lactamase, respectively, relative to MG1655:pKQV4 (88 ± 3 U/mg). The production of β-lactamase was 3.1-fold and 2.3-fold higher in the fastest growing strain MG1655:pAX4 (VHb-FAD-NAD; 271 ± 3 U/mg) relative to either MG1655:pKQV4 (control) or MG1655:pPPC1 (VHb; 119 ± 13 U/mg), respectively, at the end of 30 hours hypoxic fed-batch cultivations. The production of β-lactamase was reduced in MG1655:pAX6 (FHPg-FAD; 40 ± 1 U/mg), MG1655:pAX12 (VHb-NAD; 62 ± 15 U/mg), and MG1655:pAX14 (FHPg-NAD; 36 ± 3 U/mg) relative to the control MG1655:pKQV4 (Table 4). This observation was suprising because recombinant *E. coli* MG1655 strains expressing various hemoprotein gene constructions were always able to reach higher final optical densities relative to the non-VHb-expressing control.

Table 4. Final β-lactamase activities of microaerobically grown *E. coli* MG1655 expressing various hemoglobins

Plasmid	Hemoglobin	β-lactamase activity [U/mg][a)]
pPPC1	VHb	119 ± 13
pAX1	FHPg	109 ± 3
pAX4	VHb-FAD-NAD	271 ± 3
pAX5	FHP	113 ± 10
pAX9	VHb-FAD	106 ± 5
pAX6	FHPg-FAD	40 ± 1
pAX12	VHb-NAD	62 ± 15
pAX14	FHPg-NAD	36 ± 3
pQKV4	control	88 ± 3

[a)] Final β-lactamase activity expressed as Units/mg soluble protein is shown. The average and the standard deviation are given.

β-lactamase results reported here do not take into account the various problems and limitations encountered with the optimization of heterologous protein production in E. coli (47). However, each type of experiment conducted here included the same genetical background of the E. coli MG1655 strain and plasmid constructions were derived from the same parental plasmids with the pBR322 origin of replication. Thus, the above results are indicative and suggest that VHb-reductase-expressing cells are able to redirect cellular resources more efficiently towards recombinant protein production relative to FHP-expressing cells.

Discussion

Our results show that the beneficial effect of VHb-expression on microaerobic bacterial growth can be improved substantially by expressing instead a fusion protein (VHb-FAD-NAD) containing the *vhb* and the reductase gene module of *fhp*. The positive effect of fused reductase expression was also observed when FHP protein was expressed in microaerobic *E. coli*. The expression of these two proteins resulted in a 2.2-fold (FHP) and a 2.5-fold (VHb-FAD-NAD) increase in final cell densities relative to the VHb-expressing strain. VHb-expression has been shown to modulate cellular metabolism of *E. coli* (7, 18, 27, 38-40). VHb influences the energy household by interacting with the respiratory chain; thus, leading to increased proton pumping, ATP production rates and to a less reduced contingent of pyridine nucleotide cofactors (7, 18, 38, 39). Recently, it has been shown that HMP-expression elicits different physiological effects relative to VHb in *E. coli*. HMP is able to protect *E. coli* cells against nitrosating agents, NO-related species and oxidative stress (24). The detailed physiological functions of FHP of *A. eutrophus* remain to be investigated in *E. coli* and in other heterologous hosts. No significant changes either in aerobic or anaerobic growth were seen in *A. eutrophus* strains lacking the entire flavohemoprotein gene in the genome (8). However, *A. eutrophus* *fhp*$^-$ mutants did not accumulate nitrous oxide in significant amounts as observed in wild-type cells. This suggests that FHP may interact in an unidentified way with gas metabolism under denitrification conditions in *A. eutrophus* (8).

The reductase domain (FAD-NAD) of *A. eutrophus* FHP is part of the FNR-like protein family and is widely spread among procaryotic and eucaryotic organisms (1). A well-examined example is the flavohemoprotein (HMP) of *E. coli*. HMP has an N-terminal hemoglobin domain and a C-terminal FNR-like module (43). This module consists of two functionally inseparable and evolutionary conserved domains, the FAD- and $NADP^+$-binding domains. The FNR-like module of HMP reduces the heme-iron of the hemoglobin domain by transferring electrons from NAD(P)H to the heme moiety via the protein associated FAD group (1). Poole et al. (29) have shown that HMP is a reductase of broad substrate specificity, and the

prosthetic heme group of the hemoglobin domain is not necessarily involved in the electron transfer in *E. coli*. Thus, the FAD-binding domain is able to donate electrons directly to other acceptors such as cytochrome *c* (29). There are at least two different ways how electrons may be channeled from NAD(P)H; either via FAD to the oxidized heme iron or via FAD to different putative electron acceptors. It may be possible that FHP also functions in a similar way in *A. eutrophus* and even in heterologous prokaryotes.

Normally, the binding of O_2 to the heme iron is a reversible reaction, but the binding of oxygen can lead to a redox reaction, in which the ferroheme [Fe(II)] group of the hemoglobin is oxidized to ferriheme [Fe(III)] (26). The oxidized iron [Fe(III)] is incapable of binding oxygen, therefore limiting the biochemical and physiological effects of the hemoglobins. HMP has been shown to possess flavin reductase activity capable of reducing iron complexes such as ferric citrate with a negligible rate relative to main ferric/flavin reductase activities in *E. coli* (11, 29). Previously, VHb and an associated reductase protein, called NADH-cytochrome *o* reductase, have been shown to constitute an electron-transferring path for the oxidation of NADH and to increase the oxygen uptake several fold in *Vitreoscilla* (46). Recently, it has also been shown that the coexpression of a NADPH cytochrome P450, an alkane inducible monooxygenase, with a NADPH cytochrome P450 oxidoreductase of *Candida tropicalis* led to an elevated level of P450 catalysed monooxygenase activity in *S. cerevisiae*. (35). This experiment shows that the metabolic activity of a heterologous hemeprotein, such as VHb, may be limited by reductase activity in a novel host and suggests that the coexpression of an additional reductase may increase electron flux from NAD(P)H to the heme of the hemoglobin-like domain. The electron flow may concomitantly increase the regeneration rate of the ferriheme to ferroheme of the prosthetic heme of oxygen binding hemoglobin in *E. coli*.

All constructions showed VHb and FHPg activity as judged by CO-binding assay. Our results indirectly suggest that the recombinant reductase system was also expressed in an active form because the fusion constructions expressing either

VHb-FAD, FHPg-FAD, VHb-reductase and FHP were able to increase cell growth relative to either VHb- or FHPg-expressing *E. coli* (Figure 2 and 3). This assumption is supported by results showing that the native enzyme of *E. coli* HMP transfers two H^+ ions from NADH to FAD and consecutively passes the electrons in two steps, via the heme group to a putative substrate (29). Therefore it is likely that either reduction equivalents could not be passed from NADH to the heme due to lack of the electron transfer-mediating FAD moiety or the novel three dimensional structure of fusion proteins is sterically able to hinder the transfer of reduction equivalents from endogenous reductase domain to VHb-NAD and FHPg-NAD constructions. As a consequence, hemoglobin has reduced affinity towards oxygen after the oxidation of the prosthetic heme group of the heme moiety. Thus, the beneficial effect of the heterologous hemoglobin expression is lost in hypoxic *E. coli*.

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References

1. **Andrews, S. C., D. Shipley, J. N. Keen, J. B. C. Findlay, P. M. Harrison, and J. R. Guest.** 1992. The haemoglobin-like protein (HMP) of *Escherichia coli* has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin $NAPD^+$ reductases. FEBS Lett. **302**:247-252.
2. **Bailey, J. E., A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner, and P. S. Tsai.** 1996. Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. Biotechnol. Bioeng. **52**:109-121.
3. **Boerman, S. J. and D. A. Webster.** 1982. Control of heme content in *Vitreoscilla* by oxygen. J. Gen. Appl. Microbiol. **28**:35-43.
4. **Bowien, B., and H. G. Schlegel.** 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Ann. Rev. Microbiol. **35**:405-452.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.
6. **Bülow, L., N. Holmberg, G. Lilius, and J. E. Bailey.** 1999. The metabolic effects of native and transgenic hemoglobins on plants. Trends Biotechnol. **17**:21-24.
7. **Chen, R., and J. E. Bailey.** 1994. Energetic effect of *Vitreoscilla* hemoglobin expression in *Escherichia coli*: An on-line ^{31}P NMR and saturation transfer study. Biotechnol. Prog. **10**:360-364.

8. **Cramm, R., R. A. Siddiqui, and B. Friedrich.** 1994. Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*. J. Biol. Chem. **10:**7349-7354.
9. **Dikshit, K. L., and D. A. Webster.** 1988. Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene **70:**377-386.
10. **Ermler, U., R. A. Siddiqui, R. Cramm, and B. Friedrich.** 1995. Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 Å resolution. EMBO J. **14:**6067-6077.
11. **Eschenbrenner, M., J. Coves, and M. Fontecave.** 1994. Ferric reductases in *Escherichia coli*: the contribution of the haemoglobin-like protein. Biochem. Biophys. Res. Comm. **198:**127-131.
12. **Favey, S., G. Labesse, V. Vouille, and M. Boccara.** 1995. Flavohemoglobin HmpX: a new pathogenecity determinant in *Erwinia chrysanthemi* strain 3937. Microbiology **141:**863-871.
13. **Hart, R. A., and J. E. Bailey.** 1991. Purification and aqueous 2-phase partitioning properties of recombinant *Vitreoscilla* hemoglobin. Enz. Microb. Technol. **13:**788-795.
14. **Holmberg, N., G. Lilius, J. E. Bailey, and L. Bülow.** 1997. Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. Nature Biotechnol. **15:**244-247.
15. **Iwaasa, H., T. Takagi, and K. Shikama.** 1992. Amino acid sequence of yeast hemoglobin. A two-domain structure. J. Mol. Biol. **227:**948-954.
16. **Joshi, M., S. Mande, and K. L. Dikshit.** 1998. Hemoglobin biosynthesis in *Vitreoscilla stercoraria* DW: Cloning, expression, and characterization of a new homolog of a bacterial globin gene. Appl. Environ. Microbiol. **64:**2220-2228.
17. **Kallio, P. T., and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin (VHb) enhances total protein secretion and improves the production of α-amylase and neutral protease in *Bacillus subtilis*. Biotechnol. Prog. **12:**31-39.
18. **Kallio, P. T., D. J. Kim, P. S. Tsai, and J. E. Bailey.** 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur. J. Biochem. **219:**201-208.
19. **Kallio, P. T., P. S. Tsai, and J. E. Bailey.** 1996. Expression of *Vitreoscilla* hemoglobin is superior to horse heart myoglobin and yeast flavohemoglobin expression for enhancing *Escherichia coli* growth in a microaerobic bioreactor. Biotechnol. Prog. **12:**751-757.
20. **Karplus, P. A., M. J. Daniels, and R. J. Herriot.** 1991. Atomic structure of ferredoxin-$NADP^+$ reductase: prototype for a structurally novel flavoenzyme family. Science **25:**60-66.
21. **Khosla, C., and J. E. Bailey.** 1988. Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant *Escherichia coli*. Nature **331:**633-635.
22. **Khosla, C., and J. E. Bailey.** 1988. The *Vitreoscilla* hemoglobin gene: Molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. Mol. Gen. Genet. **214:**158-161.
23. **LaCelle, M., M. Kumano, K. Kurita, K. Yamane, P. Zuber., and M. M. Nakano.** 1996. Oxygen-controlled regulation of the flavohemoglobin gene in *Bacillus subtilis*. J. Bacteriol. **178:**3803-3808.
24. **Membrillo-Hernández, J., M. D. Coopamah, M. F. Anjum, T. M. Stevanin, A. Kelly, M. N. Hughes, and R. K. Poole.** 1999. The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. **274:**748-754.
25. **Minas, W., P. Brünker, P. T. Kallio, and J. E. Bailey.** 1998. Improved erythromycin production in a genetically engineered industrial strain of *Saccharopolyspora erythraea*. Biotechnol. Prog. **14:**561-566.
26. **Naqui, A., and B. Chance.** 1986. Enhanced superoxide dismutase activity of pulsed cytochrome oxidase. Biochem. Biophys. Res. Commun. **136:**433-437.
27. **Nilsson, M., P. T. Kallio, J. E. Bailey, L. Bülow, and K.-G. Wahlund.** 1999. Expression of *Vitreoscilla* hemoglobin in *Escherichia coli* enhances ribosome and tRNA levels: a flow field-flow fractionation study. Biotechnol. Prog. **15**:158-163.
28. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. A novel method for detection β-lactamase by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **4:**283-288.

29. **Poole, R. K., N. J. Rogers, R. A. M. Dímello, M. N. Hughes, and Y. Orii.** 1997. *Escherichia coli* flavohemoglobin (Hmp) reduces cytochrome *c* and Fe(III)-hydroxamate K by electron transfer from NADH via FAD: sensitivity of oxidoreductase activity to haem-bound dioxygen. Microbiology **143:**1557-1565.
30. **Probst, I., and H. G. Schlegel.** 1976. Respiratory components and oxidase activities in *Alcaligenes eutrophus*. Biochim. Biophys. Acta **440:**412-428.
31. **Probst, I., G. Wolf, and H. G. Schlegel.** 1979. An O_2-binding flavohemoprotein from *Alcaligenes eutrophus*. Biochim. Biophys. Acta **576:** 471-478.
32. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Schaarf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487-491.
33. **Sambrook, J., E. F. Frisch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
34. **Sanger, F., S. Nicklen, and A. R. Caulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463-5467.
35. **Sanglard, D., I. Beretta, M. Wagner, O. Käppeli, and A. Fiechter.** 1990. Functional expression of the alkane-inducible monooxygenase system of the yeast *Candida tropicalis* in *Saccharomyces cerevisiae*. Biocatalysis **4:**19-28.
36. **Strauch, M. A., G. B. Spiegelman, M. Perego, W. C. Johnson, D. Burbulys, and J. A. Hoch.** 1989. The transition state transcription regulator *abr*B of *Bacillus subtilis* is a DNA binding protein. EMBO J. **8:**1615-1621.
37. **Takaya, N., S. Suzuki, M. Matsuo, and H. Shoun.** 1997. Purification and characterization of a flavohemoglobin from the denitrifying fungus *Fusarium oxysporum*. FEBS. Lett. **414:**545-548.
38. **Tsai, P. S., V. Hatzimanikatis, and J. E. Bailey.** 1996. Effect of *Vitreoscilla* hemoglobin dosage on microaerobic *Escherichia coli* carbon and energy metabolism. Biotechnol. Bioeng. **49:**139-150.
39. **Tsai, P. S., M. Nägeli, and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o*. Biotechnol. Bioeng. **49:**151-160.
40. **Tsai, P. S., G. Rao, and J. E. Bailey.** 1995. Improvement of *Escherichia coli* microaerobic oxygen metabolism by *Vitreoscilla* hemoglobin: new insights from NAD(P)H fluorescence and culture redox potential. Biotechnol. Bioeng. **47:**347-354.
41. **Tyree, B., and D. A. Webster.** 1978. The binding of cyanide and carbon monoxide to cytochrome *o* purified from *Vitreoscilla*. J. Biol. Chem. **253:**6988-6991.
42. **Unden, G., and J. Schirawski.** 1997. The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions. Mol. Microbiol. **25:**205-210.
43. **Vasudevan, S. G., W. L. F. Armarego, D. C. Shaw, P. E. Lilley, N. E. Dixon, and R. K. Poole.** 1991. Isolation and nucleotide sequence of the *hmp* gene that encodes a haemoglobin-like protein in *Escherichia coli* K-12. Mol. Gen. Genet. **226:**49-58.
44. **Wakabayashi, S., H. Matsubara, and D. A. Webster.** 1986. Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. Nature **322:**481-483.
45. **Webster, D. A.** 1988. Structure and function of bacterial hemoglobin and related proteins, p. 245-265. *In* G. L. Eichhorn and L. G. Marzilli (ed.), Advances in inorganic biochemistry; vol. 7. Elsevier, New York.
46. **Webster, D. A., and C. Y. Liu.** 1974. Reduced nicotinamide adenine dinucleotide cytochrome *o* reductase associated with cytochrome *o* purified in *Vitreoscilla*. J. Biol. Chem. **249:**4257-4260.
47. **Weickert, M. J., D. H. Doherty, E. A. Best, and P. O. Olins.** 1996. Optimization of heterologous protein production in *Escherichia coli*. Curr. Opin. Biotechnol. **7:**494-499.
48. **Weihs V., K. Schmidt, B. Schneider, and B. Friedrich.** 1989. The formation of an oxygen-binding flavohemoprotein in *Alcaligenes eutrophus* is plasmid-determined. Arch. Microbiol. **151:**546-550.
49. **Wittenberg, J. B., and B. A. Wittenberg.** 1990. Mechanisms of cytoplasmic hemoglobin and myoglobin function. Annu. Rev. Biophys. Chem. **19:**217-241.

50. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103-119.
51. **Zhu, H., and A. F. Riggs.** 1992. Yeast flavohemoglobin is an ancient protein related to globins and a reductase family. Proc. Natl. Acad. Sci. USA **89**:5015-5019.

Dissection of the central carbon metabolism of hemoglobin-expressing *Escherichia coli* by ^{13}C NMR flux distribution analysis in microaerobic bioprocesses

Abstract

Escherichia coli MG1655 cells expressing *Vitreoscilla* hemoglobin (VHb), *Ralstonia eutropha* flavohemoprotein (FHP), the N-terminal hemoglobin domain of FHP (FHPg) and a fusion protein of VHb and the *R. eutropha* C-terminal reductase domain (VHb-Red) were grown in a microaerobic bioreactor to study the effects of low oxygen concentrations on the central carbon metabolism, using fractional ^{13}C-labeling of the proteinogenic amino acids and two dimensional [^{13}C, ^{1}H]-correlation NMR spectroscopy. The NMR data revealed differences in the intracellular carbon fluxes between *E. coli* expressing either VHb or VHb-Red and cells expressing *R. eutropha* FHP or the truncated heme-domain (FHPg). *E. coli* MG1655 expressing either VHb or VHb-Red were found to function with a branched TCA cycle. Furthermore, cellular demands for ATP and reduction equivalents in VHb- and VHb-Red-expressing cells were met by an increased flux through glycolysis. In contrast, in *E. coli* cells expressing *R. eutropha* hemeproteins the TCA cycle is running cyclically, indicating a shift towards a more aerobic regulation. Consistently, *E. coli* cells displaying FHP and FHPg activity showed lower production of the typical anaerobic by-products formate, acetate and D-lactate. The implications of these observations for biotechnological applications are discussed.

Introduction

Hemoglobins are a specific group of oxygen-binding proteins that can be found in mammals, plants and microorganisms (15). The homodimeric hemoglobin of *Vitreoscilla* (VHb) is the best-characterized bacterial hemoglobin. The expression of VHb is up regulated by oxygen limitation (hypoxia) in *Vitreoscilla* (50), but its physiological functions have not yet been entirely elucidated. Nonetheless, heterologous expression of VHb has been used to alleviate physiologically unfavorable effects of oxygen limitation and to improve growth properties and productivity of various microorganisms, plants and mammalian cells that yield industrially important metabolites (7, 18, 21, 28-30, 32, 35).

Previous research on the effects arising from heterologous VHb expression has mainly focused on the operation of the respiratory chain. Expression of VHb in *E. coli* cells lacking either cytochrome *o* (aerobic terminal oxidase) or cytochrome *d* complexes (microaerobic terminal oxidase) revealed a 5-fold increase in cytochrome *o* (in *cyd*$^-$, *cyo*$^+$ strain) and a 1.5-fold increase in cytochrome *d* (in *cyd*$^+$, *cyo*$^-$ strain) complexes relative to wild-type cells (48). These results have led to the hypothesis that VHb is able to increase the effective intracellular oxygen concentration with concomitant increase of the amount of cytochrome *o* complexes (20): the proton translocation activity of cytochrome *o* complexes is characterized by a higher H/O ratio relative to cytochrome *d* complexes and is able to generate a larger proton gradient across the cell membrane (25, 36, 38). VHb-expressing cells are indeed able to generate a larger proton flux per reduced oxygen molecule than control cells and have a 30% higher ATPase activity and a 65% higher ATP turnover rate (8, 20).

Furthermore, it has also been reported that the steady state NAD(P)H level is 1.8-fold lower in VHb-expressing strain than in control cells grown under nearly anoxic conditions (47), i. e., cells are in a more reduced state under oxygen limited conditions (16). Anoxia is known to reduce electron flow through the respiratory chain, so that NAD(P)H is consumed more slowly. Therefore, one may hypothesize that under low oxygen tension the presence of VHb in *E. coli* increases the electron

flux through the respiratory chain. Moreover, Tsai et al. (47) postulated that this shift in the $NAD^+/NADH$ concentration ratio might have implications on key steps of the central carbon metabolism in VHb-expressing *E. coli*. Therefore, metabolic flux analysis (MFA) was performed and a metabolic model suggested that VHb-positive cells direct a higher fraction of glucose through the pentose phosphate pathway (PPP) and channel less acetyl-CoA through the tricarboxylic acid cycle (TCA) than wild-type *E. coli* (49). Direction of additional glucose through PPP generates an excess amount of NADPH, and results in a transhydrogenation reaction, which creates an H^+-flux from NADPH to NAD^+. VHb-expressing cells also displayed strongly reduced formate and D-lactate excretion levels relative to controls (49).

Recently, we constructed a novel set of expression systems for native and engineered hemoglobin proteins (12). The native proteins used were VHb and the flavohemoprotein of *R. eutropha* (FHP) (9). FHP is a member of the FNR-like proteins (23) and contains an N-terminal hemoglobin (FHPg) and a C-terminal redox-active (Red) domain. The redox-active domain was fused with VHb to generate the VHb-Red fusion protein. In addition, the hemoglobin domain (FHPg), which shares high sequence homology with VHb, was truncated from the reductase domain and was functionally expressed in *E. coli*. The expression of the hemoglobins showed various interesting features, such as significantly improved growth rates and changes in activity of metabolic pathways under hypoxia (12).

The ratios of intracellular metabolic fluxes in the central carbon metabolism of *E. coli* MG1655 cells grown under oxygen-limited conditions have previously been determined using biosynthetically-directed fractional ^{13}C-labeling of amino acids and 2D [^{13}C, ^1H]-correlation NMR spectroscopy (11). This investigation, which serves as a reference for the presently studied hemoglobin-expressing cells, showed that under microaerobic conditions the topology of the active pathways is characteristic of anaerobic metabolism, as was primarily evidenced by the activity of the pyruvate-formate lyase and the suppression of 2-oxoglutarate dehydrogenase activity. This result is at variance with previously assumed aerobic configurations

and illustrates the importance of experimental characterization of the topology of active pathways.

Further studies of the central carbon metabolism of hemoglobin-expressing cells promise to lead to new insights into the cellular physiology, which in turn may support the design of improved strains for biotechnology. Therefore, we now applied the recently developed approach of biosynthetically-directed fractional labeling of proteinogenic amino acids (43-46) for the assessment of intracellular carbon flux ratios using a variety of hemoglobin-expressing *E. coli* strains.

Materials and Methods

Strains and plasmids

The plasmids pPPC1, carrying the *Vitreoscilla vhb* gene, pAX1, encoding the *R. eutropha* hemoglobin gene domain (*fhpg*), pAX5, containing the native flavohemoglobin gene (*fhp*) of *R. eutropha* and pAX4, carrying the gene fusion between the *vhb* gene and the FHP reductase domain coding sequence of *R. eutropha* (*vhb-red*) were used to study physiological consequences of globin expression in *E. coli* MG1655 [λ^-, F^-; Cold Spring Harbor Laboratory]. The construction of the plasmids has been described previously (12, 22).

Microaerobic bioreactor cultivations

Cultivations of *E. coli* MG1655 were performed under microaerobic conditions (concentration of O_2 = 0.02 mmol/l) in a SixFors bioreactor unit (Infors, Switzerland) in a fed-batch mode using a minimal medium containing glucose as the sole carbon source (4 g/l glucose) (22). Based on previous experiments, which showed smaller glucose consumption for *E. coli* cells expressing the *R. eutropha* hemoglobin genes (*fhpg* and *fhp*), the glucose concentration of the medium was reduced to 2 g/l for these strains. This modification prevents both glucose

accumulation and dilution of the ^{13}C-substrate with unlabeled glucose. The bioreactor parameters were: working volume 300 ml, stirrer speed 300 rpm, temperature 37°C, aeration rate 120 ml/min of air and pH 7.0 ± 0.2, adjusted with addition of either 2 M NaOH or 2 M H_3PO_4.

Inocula were grown for 14 hours in 50 ml shake flasks containing 10 ml of LB-media (39) supplemented with ampicillin (100 μg/ml) at 37°C and 250 rpm. Inoculation of the bioreactors was standardized to obtain a starting absorption (A_{600}) of 0.2. The expression of the various hemoglobins was induced with IPTG to a final concentration of 0.5 mM at $A_{600} \approx 1$. Feeding of the cultures was started with a rate of 1 ml/hour at A_{600} = 2.5, and was increased to 2 ml/hour when the fractional ^{13}C-labeling started at A_{600} = 4.5. The labeling medium contained a mixture of 10% uniformly labeled ^{13}C-glucose (Isotech, Miamisburg, OH) and 90% unlabeled glucose. At A_{600} = 7 the cells were harvested for NMR measurements.

O_2 consumption and CO_2 production of the cells were monitored using an emission monitor (Brüel & Kjaer, Emission Monitor Type 3427) and dissolved oxygen concentration of the cultures was tightly controlled with a polarographic electrode (Mettler-Toledo, Switzerland). Growth of the cultures was monitored with a spectrophotometer (Perkin Elmer) at 600 nm every 30 minutes. Cellular dry weight (CDW) was determined at the beginning (A_{600} = 4.5) and the end of the labeling phase (A_{600} = 7) (40).

Biological activity of the expressed hemoglobin proteins

The biological activity of the expressed hemoglobin proteins was confirmed by a CO-binding activity assay (17).

Quantitative analysis of by-products and intracellular metabolites

By-product concentrations were measured enzymatically with a Beckman SYNCHRON CX5CE autoanalyzer at one-hour intervals. Ethanol and residual glucose concentrations were determined using Beckman alcohol (#445900) and

glucose (#442640) kits, respectively. The acetate kit (#148261) was purchased from Roche Diagnostics. Quantifications of D-lactate, succinate and formate concentrations were performed using enzymatic assays adapted for the Beckman autoanalyzer system (5, 49).

Quantification of the intracellular metabolites pyruvate, ATP and ADP was performed using perchloric acid (PCA) for extraction of the metabolites (2, 10). The concentrations were measured with a Beckman autoanalyzer using enzymatic assays (5, 10). Enzymes were obtained from Roche Molecular Biochemicals. According to the supplier information, unspecific reactions contribute less than 1% to the measured concentrations, which we verified experimentally (data not shown).

Sample preparation and 2D [^{13}C, ^{1}H]-COSY NMR measurements

Fractionally labeled cells were hydrolyzed in 6N HCl at 110°C, and the NMR spectra of the amino acid mixtures were recorded at 40°C and at a proton resonance frequency of 400 MHz, using a Varian Inova 400 spectrometer. Two proton-detected 2D [^{13}C, ^{1}H] heteronuclear single quantum coherence spectra were recorded for each sample (6). Pulsed field gradients were used for coherence pathway rejection (4, 52) and the decoupling scheme WALTZ (42) was applied during detection. The spectra containing the aliphatic resonances were recorded in 12.5 hours (1400x256 complex points; t_{1max} = 412 ms; t_{2max} = 128 ms; 1.5 s relaxation delay between scans) with the carrier frequency set to 42.5 ppm and a spectral width of 33.8 ppm. The spectra for the aromatic resonances were recorded in 7.5 hours (950x512 complex points; t_{1max} = 412ms; t_{2max} = 109 ms; 1.5s relaxation delay between scans) with the ^{13}C-carrier frequency set to 123.8 ppm and a spectral width of 22.8 ppm. Spectra were transformed using the program PROSA (14). The digital resolution after zero-filling was 0.9 Hz/point along ω_1 and 2.0 Hz/point along ω_2 for the spectrum with the aliphatic resonances, and 0.6 Hz/point along ω_1 and 4.6 Hz/point along $_2$ respectively, for the spectra containing the aromatic resonances.

The overall degree of the ^{13}C labeling of the amino acids, P_l, was measured from 1D ^{1}H-NMR spectra (t_{1max} = 1.024s; 8s interscan delay), as shown in Figure 1.

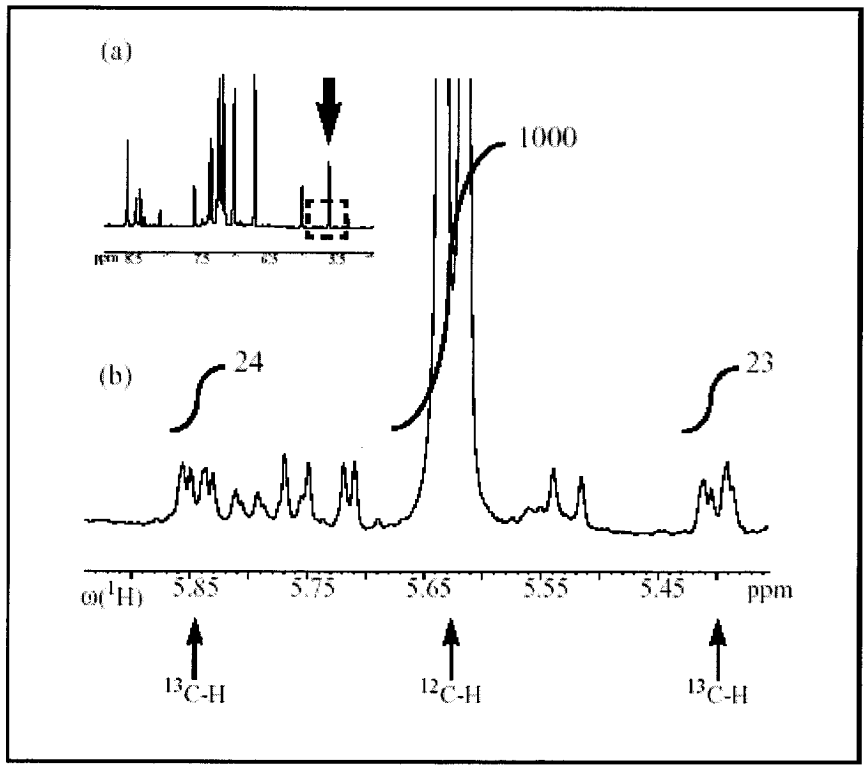

Figure 1. Determination of the degree of overall ^{13}C labeling of the biomass from a 1D ^{1}H-NMR spectrum. (a) Region of the 1D ^{1}H-NMR spectrum recorded for the hydrolyzed biomass comprising the aromatic resonances. Several well-resolved peaks allow observation of the ^{13}C satellites. (b) Expansion showing a well-resolved resonance (unassigned). The satellite doublet arising from protons bound to ^{13}C and the corresponding ^{12}C-H peak are indicated by arrows. The ratio of the sum of the integrals of the two satellites to the total integral of all three peaks yields the overall labeling degree of the biomass (4.5% in this case).

The resulting value of P_l was in good agreement with the value calculated from the fraction of [$^{13}C_6$]-glucose in the minimal medium and the fraction of ^{13}C-labeled biomass assessed via first-order wash-out kinetics (40). The value for P_l was independently confirmed from analysis of the scalar coupling fine structure of Leu-β (43). 42 individual ^{13}C-^{13}C coupling fine structures were analyzed in the correlation spectra, and the relative abundance of intact carbon fragments present in eight principal intermediates of the central carbon metabolism were derived according to Szyperski (43), using the program FCAL (46). Flux ratios through

several key pathways in the cellular central metabolism were then calculated from the abundances of fragments as described previously (40, 43).

Results

Physiological characterization of the hemoglobin-expressing strains

The *E. coli* strains MG1655:pPPC1, MG1655:pAX1, MG1655:pAX4 and MG1655:pAX5 displayed CO-binding activities that are indicative for the expression of biochemically active hemoglobin proteins. The absorption spectra of the various hemoglobins corresponded to the values reported previously (data not shown) (12).

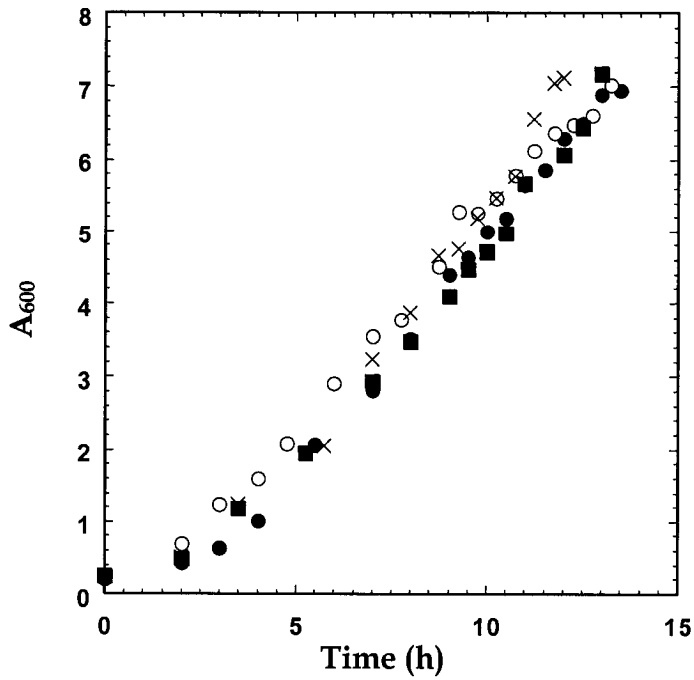

Figure 2. Growth trajectories of the four hemoglobin-expressing *E. coli* MG1655 strains used for this study, when growing in a minimal medium under hypoxic conditions. The expression of the proteins was induced at A_{600} = 1 and the fractional ^{13}C-labeling was started at A_{600} ~ 4.5. The cells were harvested for NMR analysis at the end of the cultivation, with A_{600} = 7. ■ , MG1655:pPPC1, expressing the VHb protein; • , MG1655:pAX5, expressing the FHP protein; ○ , MG1655:pAX1, expressing the FHPg protein; ×, MG1655:pAX4, expressing the VHb-Red protein.

The MG1655 strains that coexpress hemoglobin and reductase activities, FHP and VHb-Red, grew fastest, exhibiting specific growth rates (μ) of 0.168 h^{-1} and 0.180 h^{-1}, respectively. The *E. coli* strains expressing either FHPg or VHb grew with μ = 0.132 h^{-1} and μ = 0.148 h^{-1}, respectively, during the ^{13}C-labeling phase from A_{600} 4.5 to A_{600} 7 (Figure 2).

Glucose uptake and the production of D-lactate, acetate, ethanol and succinate were calculated and normalized to CDW to obtain the specific production rates (q_p; mMol/h/g) (Table 1). Evaluation of q_p for subsequent time intervals during the labeling phase revealed a slight decrease in the specific production rates of all assayed metabolites within the time course of the cultivation. We did not take into account these variations since the values assessed by the NMR analysis represent also a global view over the whole labeling phase.

We observed substantially lower formate production rates for the strains expressing *R. eutropha* FHP (MG1655:pAX5; 0.14 mMol/h/g) or the truncated flavohemoglobin FHPg (MG1655:pAX1; 1.30 mMol/h/g) relative to VHb (MG1655:pPPC1; 2.59 mMol/h/g) and VHb-Red (MG1655:pAX4; 3.31 mMol/h/g). FHP and FHPg showed also decreased D-lactate production relative to the VHb- and VHb-Red-expressing strains, and less pronounced reduction was observed for acetate production. The specific acetate production rates were similar for the MG1655 strains expressing VHb, FHPg and FHP, whereas the strain expressing VHb-Red excreted approximately 2-fold more acetate into the culture media (Table 1). Furthermore, we found an approximately 2-fold higher glucose consumption rate for the strains expressing VHb and VHb-Red (3.97 and 4.60 mMol/h/g) relative to the *R. eutropha* FHP- and FHPg-expressing strains (2.32 and 2.88 mMol/h/g). The control strain showed highest glucose consumption rate being 5.45 mMol/h/g CDW (Table 1).

Table 1. Physiological data of the five *E. coli* strains MG1655:pAX1 (FHPg), MG1655:pAX5 (FHP), MG1655:pPPC1 (VHb), MG1655: pAX4 (VHb-Red) and MG1655 (control) assessed during ^{13}C-labeling phase.

Parameter	FHPg	FHP	VHb	VHb-Red	control[a)]
$q_{glucose}$[b)]	2.88	2.32	3.97	4.60	5.45
$q_{ethanol}$[b)]	0.53	0.09	0.53	1.02	0.32
$q_{acetate}$[b)]	2.68	2.14	2.27	4.57	3.05
$q_{formate}$[b)]	1.30	0.14	2.59	3.31	4.10
$q_{D\text{-}lactate}$[b)]	0.13	0.87	1.43	1.14	0.76
$q_{succinate}$[b)]	0.22	0.25	0.58	1.92	0.51
q_{O2}[b)]	4.18	5.59	2.74	3.19	1.78
q_{CO2}[b)]	3.50	5.19	1.27	2.18	0.78
RQ[c)]	0.84	0.95	0.48	0.69	0.43
μ[d)]	0.132	0.168	0.148	0.180	0.125
$Y_{X/Glc}$[e)]	0.258	0.249	0.176	0.159	0.143

[a)] Non hemoglobin expressing *E. coli* MG1655 strain
[b)] The rates are given in mmol/h/g cdw.
[c)] Respiratory quotient calculated from O_2 uptake and CO_2 production rates.
[d)] Specific growth rate in h^{-1}
[e)] Growth yield on glucose in g biomass / g glucose consumed

Monitoring the exhaust gases yielded specific CO_2 production (qCO_2) and O_2 consumption (qO_2) rates. The strains expressing FHP and FHPg revealed higher respiratory activities than either the VHb- or VHb-Red expressing strains, or the control strain, as evidenced by an at least 2-fold higher qCO_2 and qO_2 values (Table 2). The respiratory quotient, RQ = qCO_2 / qO_2, is an indicator for the metabolic state of the cells. The calculated RQ values for MG1655:pAX1 and for MG1655:pAX5 were 0.84 and 0.95, respectively, whereas those for VHb- or VHb-Red-expressing strains were significantly lower being 0.48 and 0.69, respectively. Thus, the expression of *R. eutropha* FHP or FHPg increased the RQ by 100% (MG1655:pAX5) and 80% (MG1655:pAX1), respectively, relative to MG1655:pPPC1 strain expressing *Vitreoscilla* hemoglobin (Table 1).

Interestingly, we could assess a 2-fold higher intracellular pyruvate concentration for the strains expressing *Vitreoscilla* hemoglobin relative to the strains possessing *R. eutropha* hemoglobin proteins (Table 2). Pyruvate is the endproduct of glucose breakdown in the glycolysis, and is further channeled towards the TCA cycle or is consumed in the anaerobic pathways leading to the formation of the typical by-

products of anaerobic growth. Thus, this difference in intracellular pyruvate concentration can be explained by a higher glucose flow through the glycolytic pathway in MG1655:pPPC1 and MG1655:pAX4 cells. The production of pyruvate was identical with the VHb- or VHb-Red-expressing strains. On the other hand, the analysis of ATP and ADP contents did not reveal any significant differences among the different hemoglobin-expressing strains under study, but the control showed an approximately 2-fold lower ATP/ADP ratio relative to the hemoglobin-expressing strains (Table 2).

Table 2. Concentration of intracellular metabolites pyruvate, ATP and ADP in *E. coli* strains MG1655:pAX1 (FHPg), MG1655:pAX5 (FHP), MG1655:pPPC1 (VHb), MG1655: pAX4 (VHb-Red) and MG1655 (control)

Metabolite[a)]	FHPg	FHP	VHb	VHb-Red	control[b)]
Pyruvate	6.2 ± 2.7	6.7 ± 2.6	11.7 ± 7.3	12.4 ± 3.9	12.2 ± 1.4
ATP	46.7 ± 10.5	33.9 ± 5.3	37.3 ± 9.2	38.5 ± 2.4	22.8 ± 7.6
ADP	12.9 ± 8.8	9.1 ± 3.8	10.4 ± 3.2	15.6 ± 4.7	15.6 ± 1.8
ATP/ADP	3.6 ± 0.2	3.7 ± 0.1	3.6 ± 0.1	2.5 ± 0.1	1.4 ± 0.2

a) For each metabolite four independent samples have been taken and the concentrations have been determined as described in Material and Methods. The mean value and the standard deviation are given in µmol/g CDW.
b) Non hemoglobin expressing *E. coli* MG1655 strain.

Validation of the physiological data

We performed a carbon balance analysis for all hemoglobin-expressing strains, taking into account the glucose uptake, production of biomass, CO_2 evolution and excretion of by-products. The carbon recovery calculated as the ratio of C-output and C-uptake was larger than 93% in all cases, i. e., the carbon balance could be closed to within a few percent (Table 3).

Table 3. Carbon balance for the validation of the physiological data in MG1655:pAX1 (FHPg), MG1655:pAX5 (FHP), MG1655:pPPC1 (VHb), MG1655: pAX4 (VHb-Red) and MG1655 (control)[a)]

Metabolite	FHPg	FHP	VHb	VHb-Red	Control[b)]
Glucose	112.4	88.7	192.7	196.3	166.7
Acetate	35.8	24.6	70.2	40.3	41.5
Ethanol	6.9	1.6	14.2	12.1	13.8
Formate	9.2	0.9	23.8	19.6	26.8
D-lactate	2.7	0.2	24.0	20.7	35.2
Succinate	2.7	6.5	24.0	20.7	20.1
CO_2	12.3	19.5	7.6	4.9	4.0
Biomass	34.6	33.0	33.0	41.4	31.0
$Carbon_{out}$	104.2	86.3	196.0	184.4	172.5
% Recovery	93	97	102	94	103

[a)] Carbon balances were calculated from the concentrations measured in the media over the labeling period. Values are given in mmol carbon. C_{out}: summation over all assayed carbon outputs. For the biomass a standard content of 39.8 mmol carbon per 1 g CDW was assumed.
[b)] Non hemoglobin expressing *E. coli* MG1655 strain.

Analysis of the 2D [^{13}C, ^{1}H]-COSY spectra and derivation of metabolic flux ratios

The ^{13}C-labeling experiments revealed major differences in the central carbon metabolism when comparing VHb-/VHb-Red- and FHPg-/FHP-expressing cells. VHb/VHb-Red-positive cells yielded labeling patterns similar to those previously observed for anaerobically grown wild-type *E. coli* cells (43) and microaerobic MG1655 cells (11), whereas for FHPg- and FHP-expressing cells a more aerobic fluxome was found (Fig. 3 / Table 4).

The labeling pattern of oxaloacetate (OAA) of VHb-/VHb-Red-expressing cells revealed that OAA is solely derived from phosphoenolpyruvate (PEP) by carboxylation (33). Thus, the TCA cycle operates in a branched fashion to generate exclusively building blocks for biosynthesis (Table 4). In contrast, for microaerobically growing strains expressing either FHP or FHPg, only 44% and 88% of the OAA, respectively, were generated through the anaplerotic reaction, indicating that in these cells the TCA cycle also serves for ATP production via respiration. Concomitantly, the activity of the pyruvate-formate lyase (Pfl) (26) is

suppressed as respiration is activated. Further analysis showed increased turnover ratios for the interconversion of pyruvate to AcCoA and formate by Pfl for the strains expressing VHb (0.73) and VHb-Red (0.65) and to a smaller extent for cells expressing FHPg (0.19). In MG1655:pAX5 (FHP) Pfl activity is not detectable, which is compatible with our physiological data displaying a very small specific formate production rate for the FHP-expressing strain (Figure 3, Table 4).

Table 4. Origin of intermediate metabolites in the four cultures of Table 1 and a control cultivation[a)]

Metabolite	FHPg	FHP	VHb	VHb-Red	Control[b)]
Pentose Phosphate Pathway					
PEP from pentoses (ub)[c)]	0.26 ± 0.06	0.35 ± 00.6	0.46 ± 0.05	0.68 ± 0.07	0.46 ± 0.05
R5P From GAP and S7P	0.84 ± 0.02	0.81 ± 0.02	0.92 ± 0.02	0.86 ± 0.02	0.85 ± 0.02
R5P from G6P	0.16 ± 0.02	0.18 ± 0.02	0.08 ± 0.02	0.14 ± 0.02	0.14 ± 0.02
TCA cycle					
OAA from PEP	0.53 ± 0.13	0.88 ± 0.02	0.99 ± 0.02	1.01 ± 0.02	0.99 ± 0.02
PEP from OAA	0.02 ± 0.05	0.03 ± 0.14	n.d.[d)]	n.d.[d)]	n.d.[d)]
Pyr from malate (ub)[c)]	0.00 ± 0.04	0.00 ± 0.16	n.d.[d)]	n.d.[d)]	n.d.[d)]
Pyr from malate (lb)[c)]	0.00 ± 0.02	0.00 ± 0.01	n.d.[d)]	n.d.[d)]	n.d.[d)]
ICT from Suc and GOX (Glyoxylate shunt)	0.01 ± 0.06	0.06 ± 0.03	0.00 ± 0.02	0.00 ± 0.03	0.01 ± 0.02
OAA reversibly converted to FUM and SUCC	0.70 ± 0.09	0.60 ± 0.11	0.19 ± 0.06	0.20 ± 0.09	0.25 ± 0.04
PYR reversibly converted to AcCoA and FOR	0.01 ± 0.03	0.19 ± 0.04	0.65 ± 0.03	0.73 ± 0.04	0.71 ± 0.03
AcCoA from PYR	0.91 ± 0.03	0.88 ± 0.04	0.87 ± 0.03	0.86 ± 0.04	0.96 ± 0.03
C1 metabolism					
Serine from Glycine and C1	0.22 ± 0.04	0.24 ± 0.03	0.22 ± 0.02	0.16 ± 0.02	0.35 ± 0.02
Glycine from CO_2 and C1	0.17 ± 0.03	0.06 ± 0.03	0.04 ± 0.03	0.05 ± 0.03	0.00 ± 0.02

[a)] Analysis of the ^{13}C-labeling patterns of the proteinogenic amino acids allowed calculating the fraction of carbon directed through a particular pathway of the central carbon metabolism. Mean values and standard deviations are listed.
[b)] data as previously published for *E. coli* MG1655:pUC19 (Fiaux et al. 1999)
[c)] ub: upper boundary; lb: lower boundary
[d)] not determined

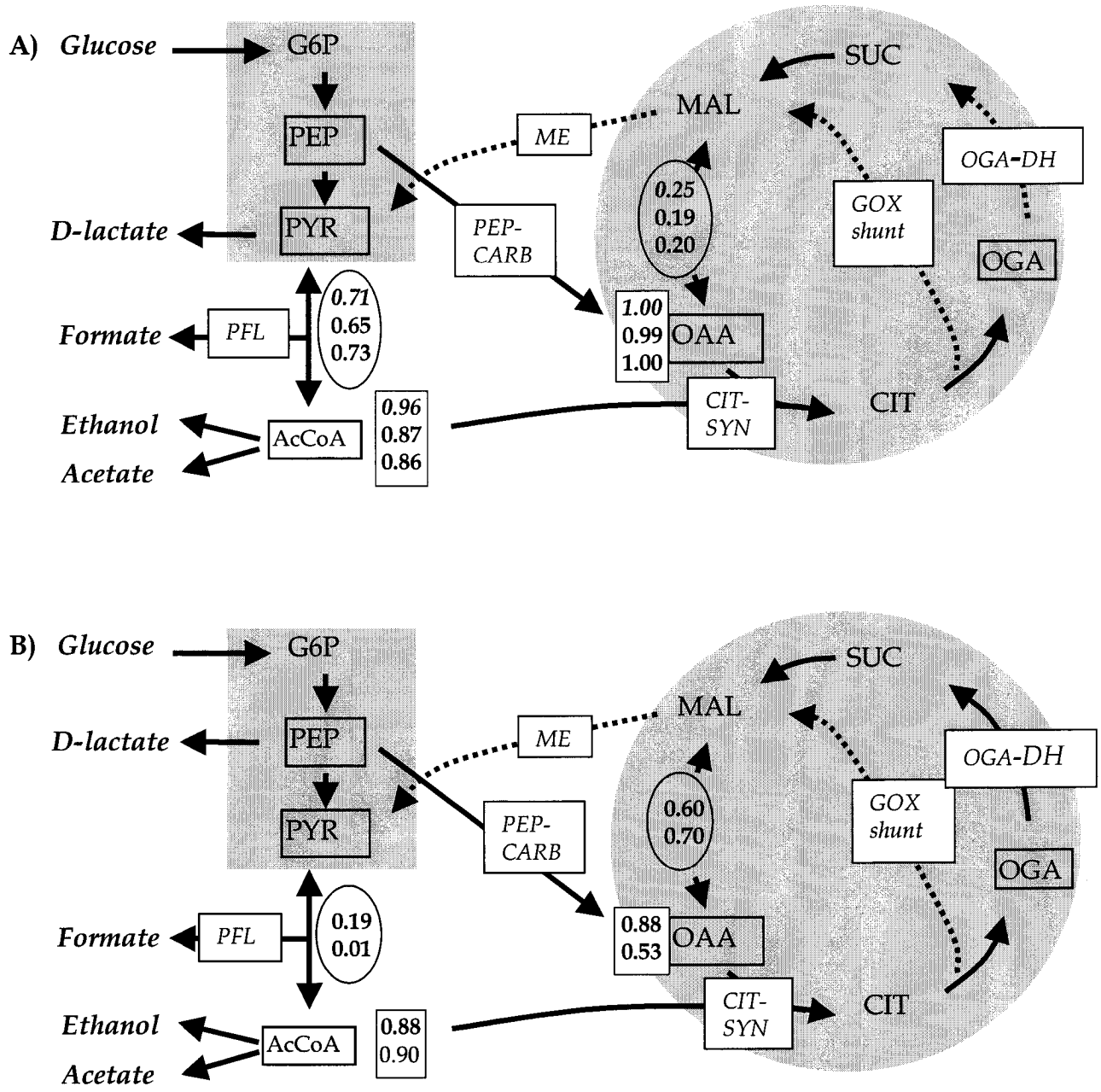

Figure 3. Flux ratios and interaction of glycolysis and the tricarboxylic acid cycle in the central carbon metabolism of hemoglobin expressing microaerobic *E. coli* MG1655 cells. A) Flux ratios of wild-type *E. coli* (Fiaux et al., 1999), VHb-expressing cells and VHb-Red-expressing cells (from top to bottom of the boxes). B) Flux ratios of FHPg- and FHP-expressing *E. coli* (from top to bottom of the boxes). Glycolysis (rectangular shape on the left) and the tricarboxylic acid cycle (circular shape on the right) are highlighted. Enzymatically measured by-products and glucose are given in bold italics. Carbon fragment patterns of the boxed metabolites were directly determined by [^{13}C, ^{1}H]-COSY of proteinogenic amino acids. The fractions of molecules given in square boxes are synthesized via the reactions pointing at them, and numbers in ellipses indicate the amount of reversible interconversion of the molecules. Enzyme names are given in italics. Active pathways are shown with solid arrows. Dashed arrows indicate that the represented enzymatic conversion is inactive (*ME*, *GOX*-shunt and *OGA-DH*). Abbreviations: AcCoA, Acetyl-coenzyme A; CIT, citrate; MAL, malate; OAA, oxaloacetate; OGA, oxoglutarate; PEP, phosphoenolpyruvate; PYR, pyruvate; *ME*, malic enzyme; *PFL*, pyruvate-formate lyase; *PEP-CARB*, phosphoenolpyruvate carboxylase, *CIT-SYN*, citrate synthase, *GOX* shunt, glyoxylate shunt; *OGA-DH*, oxoglutarate dehydrogenase.

An additional notable observation is that the AcCoA pool was diluted with unlabeled molecules, which probably originated from the external acetate pool; the ^{13}C isotopomer abundances found for AcCoA differed slightly from those that one would expect if pyruvate were the sole carbon source for AcCoA synthesis (see Table 4). The ^{13}C-enrichments of AcCoA and corresponding intermediates are therefore not strictly uniform, but the deviations from uniform ^{13}C-enrichment are so small that they were not detectable with the program FCAL; calculation of flux ratios in the TCA cycle based on tracing intact C_3 fragments is in any case hardly affected by such a dilution at the level of AcCoA. The present observation of a decreased isotope enrichment of the AcCoA pool supports the earlier suggestion that exchange of intra- and extracellular acetate in hypoxic *E. coli* cells might be a general metabolic configuration: Sauer et al. (41) postulated the presence of reversible exchange fluxes between intra- and extracellular acetate pools as well as the reversibility of the reactions connecting AcCoA to acetate (Table 4).

Finally, we also observed that the glyoxylate shunt is not active in the presently studied cells (19, 27). Furthermore, the conversion of malate to pyruvate catalyzed by the malic enzyme does not contribute to the labeling pattern observed in FHP- and FHPg-expressing strains (Figure 3). For VHb- and VHb-Red cells these activities could not be assessed (Table 4), since the C^a-C′ fragment, which would be traced from oxaloacetate to pyruvate to determine malic enzyme activity, is not generated when the TCA cycle operates in a branched fashion.

Discussion

The expression of VHb, VHb-Red, FHPg and FHP was previously shown to improve hypoxic growth properties of *E. coli* MG1655 cells in bioreactor cultivations (12). Although the expression of these hemoglobin proteins is able to positively modulate the microaerobic growth of *E. coli*, little is known about the underlying changes of metabolic configurations induced by the expression of these heterologous globins. Therefore, we have studied the microaerobic carbon

metabolism of *E. coli* MG1655 cells expressing VHb, VHb-Red, FHPg or FHP using fractional ^{13}C-labeling and 2D NMR spectroscopy (43-46). In addition, we analyzed the by-product excretion and intracellular metabolite content in order to unravel more accurately the changes of carbon metabolism. Our results revealed major differences in the metabolic states between VHb- and VHb-Red-producing *E. coli* on the one hand and FHPg- and FHP-expressing *E. coli* strains on the other hand.

The genetic approach to alleviate adverse effects of oxygen limitation by VHb-expression has been extensively studied in *E. coli*. Previously, a mathematical model conjectured that VHb is able to increase the intracellular oxygen tension and to activate both terminal oxidases in *E. coli*, albeit to different extents (20, 49). Thus, the model indirectly suggested that VHb-expression is able to shift the global microaerobic regulatory network to a more aerobic regime. This hypothesis was supported by results of Tsai et al. (48) revealing a 5-fold increase in cytochrome *o* and a 1.5-fold increase in cytochrome *d* content in VHb-expressing cells relative to controls. In addition, the specific activity of cytochrome *o* was enhanced by 50% relative to VHb-negative controls. The cytochrome *o* complex is able to extrude two protons per oxygen molecule reduced, whereas the cytochrome *d* complex does not function as a proton pump (38). VHb-expressing cells do actually display a higher transmembrane proton gradient and a higher yield of membrane-translocated protons per oxygen molecule reduced, when compared to controls (20, 48). These findings are in qualitative agreement with previous ^{31}P NMR results showing that VHb-expressing cells have a 65% higher ATP turnover rate than controls. The reentry of the proton flow into the cell via ATPase yields up to 30% higher ATPase activity and ATP production relative to controls (8, 20).

Keeping the above-mentioned findings in mind and taking into account the present data of the VHb-expressing *E. coli*, which showed an interrupted TCA cycle, we tried to integrate all the information into a model for microaerobic growth of VHb-expressing *E. coli*. Interruption of the TCA cycle is normally regarded as a characteristic for anaerobic growth. However, it has also been shown for aerobically growing *E. coli* that the TCA cycle can operate in a branched fashion once the

cellular energy demands are satisfied with energy derived from glycolysis (1). A study of Tsai et al. (49) showed that the carbon fluxes through the TCA cycle are concomitantly decreased with increasing VHb concentration. As a consequence, the expression of increasing amounts of VHb reduces the carbon flux entering the TCA cycle via AcCoA but slightly increases the carbon flux through the anaplerotic reaction connecting glycolysis to TCA. Therefore, we may hypothesize that the TCA cycle is interrupted above a threshold VHb concentration, as shown in this study for VHb- and VHb-Red-expressing *E. coli*. The absence of cyclic TCA activity leads to less efficient oxidation of carbon sources and concomitantly to a decreased generation of reducing equivalents and ATP. Higher glycolytic activity in VHb-expressing cells may then satisfy the ATP and NAD(P)H demands for growth and maintenance. To support this hypothesis we included some previously published NMR data on hemoglobin-negative control cells in Table 4 (11). Comparison of the data on VHb- and VHb-Red-expressing cells with the VHb-negative control reveals similar flux ratios. However, additional data on wild-type *E. coli* MG1655 cells grown with the same experimental conditions reveal a higher production of formate and an increased consumption of glucose but a smaller growth rate relative to the hemoglobin expressing strain (Table 1). These findings may confirm our hypothesis that VHb- and VHb-Red-expression may enable the cells to operate their metabolism more efficiently. Therefore, the lack of functional TCA cycle activity in these strains might not result from anaerobiosis, but simply from the fact the cellular energy demands can be satisfied by a more efficient ATP generation system.

Unfortunately, the physiological role of FHP either in its native host, *R. eutropha*, or in other heterologous expression systems is poorly characterized. FHP expression, like VHb, is induced under microaerobic conditions, and may interact with the gas metabolism during denitrification in *R. eutropha* (9, 37). However, *fhp*$^-$ mutants did not show any difference relative to wild-type cells when grown anaerobically with nitrite as a sole electron acceptor, but did not transiently accumulate nitrous oxide. Preliminary attempts to demonstrate nitric oxide reduction with purified FHP and

NADH as an electron donor were unsuccessful (9), and the role of flavohemoglobins in the detoxification of nitric oxide is still under discussion (24, 31). Since our previous results have shown that FHP can help to support growth of *E. coli* under microaerobic conditions (12), it would nonetheless appear that FHP is able to increase the free intracellular oxygen concentration in *E. coli*. This hypothesis is supported by our data showing that FHP- and FHPg-expression leads to a higher oxygen uptake rate than in VHb-expressing cells and inactivation of the highly O_2-sensitive enzyme Pfl, which was, rather unexpectedly, found to be active in VHb- and VHb-Red-expressing cells.

Recently, Gardner et al. (13) purified FHP and determined the kinetic properties for oxygen binding and release (k_{on} 50 $\mu M^{-1}s^{-1}$, k_{off} 0.2 s^{-1}), and found that the dissociation constant differs strongly from the values reported for VHb (k_{on} = 78 $\mu M^{-1}s^{-1}$, k_{off} = 5600 s^{-1}) (34, 51). The association equilibrium constants (K) determined for FHP and VHb attribute high oxygen affinity to FHP (K = 250 μM^{-1}) and low oxygen affinity to VHb, (K = 0.014 μM^{-1}). Due to the very high values for k_{off}, VHb-expressing cells have previously been proposed to scavenge oxygen and possibly provide O_2 rapidly to respiratory complexes in *E. coli* (20, 47-49). The mechanism of biochemical action of FHP globin on the cellular metabolism of *E. coli* is still unknown.

In this study we have shown that inverse metabolic engineering (3) can be used to improve metabolic efficiency of microaerobic cells by expression of heterologous globin genes. Through introducing FHP and FHPg into *E. coli* cells we were able to reduce glucose consumption by approximately 50% relative to VHb- or VHb-Red-expressing cells without affecting cell growth. This has direct implications for economical aspects of potential biotechnological applications, promising lowered production costs.

Acknowledgments

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References

1. **Amarasingham, C. R., and B. C. Davis.** 1965. Regulation of α-ketoglutarate dehydrogenase formation in *Escherichia coli*. J. Biol. Chem. **240:**3664-3668.
2. **Babul, J., D. Clifton, M. Kretschmer, and D. G. Fraenkel.** 1993. Glucose metabolism in *Escherichia coli* and the effect of increased amount of aldolase. Biochemistry **32:**4685-4692.
3. **Bailey, J. E., A. Sburlati, V. Hatzimanikatis, K. Lee, W. A. Renner, and P. S. Tsai.** 1996. Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. Biotechnol. Bioeng. **52:**109-121.
4. **Bax, A., and S. Pochapsky.** 1992. Optimized recording of heteronuclear multidimensional NMR spectra using pulsed field gradients. J. Magn. Reson. **99:**638-643.
5. **Bergmeyer, J., and M. Gassl.** 1984. Metabolites 1: Carbohydrates. VCH Weinheim, Germany
6. **Bodenhausen, G., and D. J. Ruben.** 1980. Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. Chem. Phys. Lett. **69:**185-188.
7. **Brünker, P., W. Minas, P. T. Kallio, and J. E. Bailey.** 1998. Genetic engineering of an industrial strain of *Saccharopolyspora erythraea* for stable expression of the *Vitreoscilla* haemoglobin gene (*vhb*). Microbiol. **144:**2441-2448.
8. **Chen, R., and J. E. Bailey.** 1994. Energetic effect of *Vitreoscilla* hemoglobin expression in *Escherichia coli*: an online ^{31}P NMR and saturation transfer study. Biotechnol. Prog. **10:**360-364.
9. **Cramm, R., R. A. Siddiqui, and B. Friedrich.** 1994. Primary sequence and evidence for a physiological function of the flavohemoprotein of *Alcaligenes eutrophus*. J. Biol. Chem. **269:**7349-7354.
10. **Emmerling, M., J. E. Bailey, and U. Sauer.** 1999. Glucose catabolism of *Escherichia coli* strains with increased activity and altered regulation of key glycolytic enzymes. Metabol. Engin. **1:**117-127.
11. **Fiaux, J., C. I. J. Andersson, N. Holmberg, L. Bülow, P. T. Kallio, T. Szyper-ski, J. E. Bailey, and K. Wüthrich.** 1999. ^{13}C NMR flux ratio analysis of *Escherichia coli* central carbon metabolism in microaerobic bioprocesses. J. Am. Chem. Soc. **121:**1407-1408.
12. **Frey, A. D., J. E. Bailey, and P. T. Kallio.** 2000. Expression of *Alcaligenes eutrophus* flavohemoprotein and engineered *Vitreoscilla* hemoglobin-reductase fusion protein for improved hypoxic growth of *Escherichia coli*. Appl. Environ. Microbiol. **66:**98-104.
13. **Gardner P. R., A. M. Gardner, L. A. Martin, Y. Dou, T. Li, J. S. Olson, H. Zhu, and A. F. Riggs.** 2000. Nitric-oxide dioxygenase activity and function of flavohemoglobins. J. Biol. Chem. **275:**31581-31587.
14. **Güntert, P., V. Dötsch, G. Wider, and K. Wüthrich.** 1992. Processing of multidimensional NMR data with the new software Prosa. J. Biomol. NMR **2:**619-629.
15. **Hardison, R.** 1998. Hemoglobins from bacteria to man: evolution of different patterns of gene expression. Exp. Biology **201:**1099-1117.
16. **Harrison, D. E. and B. Chance.** 1970. Fluorimetric technique for monitoring changes in the level of reduced nicotinamide nucleotides in continous cultures of microorganisms. Appl. Microbiol. **19:**446-450.
17. **Hart, R. A., and J. E. Bailey.** 1991. Purification and aqueous 2-phase partitioning properties of recombinant *Vitreoscilla* hemoglobin. Enzyme Microb. Technol. **13:**788-795.

18. **Holmberg, N., G. Lilius, J. E. Bailey, and L. Bülow.** 1997. Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. Nat. Biotechnol. **15:**244-247.
19. **Holms, W. H.** 1986. The central metabolic pathways of *Escherichia coli*: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. Curr. Top. Cell. Regul. **28:**69-105.
20. **Kallio, P. T., D. J. Kim, P. S. Tsai, and J. E. Bailey.** 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur. J. Biochem. **219:**201-208.
21. **Kallio, P. T., and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin (VHb) enhances total protein secretion and improves the production of α-amylase and neutral protease in *Bacillus subtilis*. Biotechnol. Prog. **12:**31-39.
22. **Kallio, P. T., P. S. Tsai, and J. E. Bailey.** 1996. Expression of *Vitreoscilla* hemoglobin is superior to horse heart myoglobin or yeast flavohemoglobin expression for enhancing *Escherichia coli* growth in a microaerobic bioreactor. Biotechnol. Prog. **12:**751-757.
23. **Karplus, P. A., M. J. Daniels, and J. R. Herriott.** 1991. Atomic structure of ferredoxin-$NADP^+$ reductase: prototype for a structurally novel flavoenzyme family. Science **251:**60-66.
24. **Kim, S. O., Y. Orii, D. Lloyd, M. N. Hughes, and R. K. Poole.** 1999. Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett. **445:**389-394.
25. **Kita, K., K. Konishi, and Y. Anraku.** 1986. Purification and properties of two terminal oxidase complexes of *Escherichia coli* aerobic respiratory chain. Methods Enzymol. **126:**94-113.
26. **Knappe, J., F. A. Neugebauer, H. P. Blaschkowski, and M. Gänsler.** 1984. Post-translational activation introduces a free radical into pyruvate formate-lyase. Proc. Natl. Acad. Sci. USA **81:**1332-1335.
27. **Kornberg, H. L.** 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. 99:1-11.
28. **Liu, S.-C., B. Ogretmen, Y. Y. Chuang, and B. C. Stark.** 1992. Selection and characterization of α-amylase-overproducing recombinant *Escherichia coli* containing the bacterial hemoglobin gene. Appl. Microbiol. Biotechnol. **38:**239-242.
29. **Liu, S.-C., D. A. Webster, M. L. Wei, and B. C. Stark.** 1996. Genetic engineering to contain the *Vitreoscilla* hemoglobin gene enhances degradation of benzoic acid by *Xanthomonas maltophilia*. Biotechnol. Bioeng. **49:**101-105.
30. **Magnolo, S. K., D. L. Leenutaphong, J. A. DeModena, J. E. Curtis, J. E. Bailey, J. L. Galazzo, and D. E. Hughes.** 1991. Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of a bacterial hemoglobin. Bio/Technol. **9:**473-476.
31. **Membrillo-Hernández, J., M. D. Coopamah, M. F. Anjum, T. M. Stevanin, A. Kelly, M. N. Hughes, and R. K. Poole.** 1999. The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. **274:**748-754.
32. **Minas, W., P. Brünker, P. T. Kallio, and J. E. Bailey.** 1998. Improved erythromycin production in a genetically engineered industrial strain of *Saccharopolyspora erythraea*. Biotechnol. Prog. **14:**561-566.
33. **Morikawa, M., K. Izui, M. Taguchi, and H. Katsuki.** 1980. Regulation of *Escherichia coli* phosphoenolpyruvate carboxylase by multiple effectors in vivo. Estimation of the activities in the cells grown on various compounds. J. Biochem. **87:**441-449.
34. **Orii, Y., and D. A. Webster.** 1986. Photodissociation of oxygenated cytochrome *o* (*Vitreoscilla*) and kinetic studies of reassociation. J. Biol. Chem. **261:**3544-3547.
35. **Pendse, G. J., and J. E. Bailey.** 1994. Effect of *Vitreoscilla* hemoglobin expression on growth and specific tissue plasminogen activator productivity in recombinant chinese hamster ovary cells. Biotechnol. Bioeng. **44:**1367-1370.
36. **Poole, R. K.** 1983. Bacterial cytochrome oxidases: a structurally and functionally diverse group of electron transfer proteins. Biochim. Biophys. Acta **726:**205-243.

37. **Probst, I., G. Wolf, and H. G. Schlegel.** 1979. An O_2-binding flavohemoprotein from *Alcaligenes eutrophus*. Biochim. Biophys. Acta **576**:471-478.
38. **Puustinen, A., M. Finel, T. Haltia, R. B. Gennis, and M. Wikström.** 1991. Properties of the two terminal oxidases of *Escherichia coli*. Biochemistry **30**:3936-3942.
39. **Sambrock, J., E. F. Frisch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd. ed. Cold Spring Harbor Laboraory Press, Cold Spring Harbor, N. Y.
40. **Sauer, U., V. Hatzimanikatis, J. E. Bailey, M. Hochuli, T. Szyperski, and K. Wüthrich.** 1997. Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. Nat. Biotechnol. **15**:448-452.
41. **Sauer, U., D. R. Lasko, J. Fiaux, M. Hochuli, R. Glaser, T. Szyperski, K. Wüthrich, and J. E. Bailey.** 1999. Metabolic flux ratio analysis of genetic and environmental modulations of *Escherichia coli* central carbon metabolism. J. Bacteriol. **181**:6679-6688.
42. **Shaka, A. J., J. Keeler, T. Frenkiel, and R. Freeman.** 1983. An improved sequence for broadband decoupling: Waltz-16. J. Magn. Reson. **52**:335-338.
43. **Szyperski, T.** 1995. Biosynthetically directed fractional ^{13}C-labeling of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism. Eur. J. Biochem. **232**:433-448.
44. **Szyperski, T., J. E. Bailey, and K. Wüthrich.** 1996. Detecting and dissecting metabolic fluxes using biosynthetic fractional ^{13}C labeling and two-dimensional NMR spectroscopy. Trends Biotechnol. **14**:453-459.
45. **Szyperski, T.** 1998. ^{13}C-NMR, MS and metabolic flux balancing in biotechnology research. Q. Rev. Biophy. **31**:41-106.
46. **Szyperski T., R. W. Glauser, M. Hochuli, J. Fiaux, U. Sauer, J. E. Bailey, and K. Wüthrich.** 1999. Bioreaction network topology and metabolic flux ratio analysis by biosynthetic fractional ^{13}C labeling and two-dimensional NMR spectroscopy. Metabol. Engin. **1**:189-197.
47. **Tsai, P. S., G. Rao, and J. E. Bailey.** 1995. Improvement of *Escherichia coli* microaerobic oxygen metabolism by *Vitreoscilla* hemoglobin: new insights from NAD(P)H fluorescence and culture redox potential. Biotechnol. Bioeng. **47**:347-354.
48. **Tsai, P. S., M. Nägeli, and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o*. Biotechnol. Bioeng. **49**:151-160.
49. **Tsai, P. S., V. Hatzimanikatis, and J. E. Bailey.** 1996. Effect of *Vitreoscilla* hemoglobin dosage on microaerobic *Escherichia coli* carbon and energy metabolism. Biotechnol. Bioeng. **49**:139-150.
50. **Tyree, B., and D. A. Webster.** 1987. The binding of cyanide and carbon monoxide to cytochrome *o* reductase associated with cytochrome *o* purified from *Vitreoscilla*. J. Biol. Chem. **249**:4257-4260.
51. **Webster, D. A.** 1987. Structure and function of bacterial hemoglobin and related proteins, p. 245-265. *In* Advances in Inorganic Biochemistry, Elsevier, N. Y.
52. **Wider, G., and K. Wüthrich.** 1993. A simple experimental scheme using pulsed field gradients for coherence pathway rejection and solvent suppression in phase-sensitive heteronuclear correlation spectra. J. Magn. Reson. **102**:239-241.

Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli*

Abstract

Escherichia coli MG1655 cells expressing novel bacterial hemoglobin and flavohemoglobin genes from a medium copy number plasmid were grown in shake flask cultures under nitrosative and oxidative stress. *E. coli* cells expressing these proteins display enhanced resistance against the NO• releaser sodium-nitroprusside (SNP) compared to the control strain bearing the parental plasmid. The expression of bacterial hemoglobins originating from *Campylobacter jejuni* (CHb) and *Vitreoscilla* sp. (VHb) conferred resistance to SNP challenged cells. In addition, it has been shown that NO• detoxification is also a common feature of flavohemoglobins originating from different taxonomic groups and can be transferred to a heterologous host. These observations have been confirmed in a specific *in vitro* NO• consumption assay. Protein extracts isolated from *E. coli* strains overexpressing flavohemoglobins more readily consumed authentic NO• than protein extracts from the wildtype strain. An oxidative challenge to the cells evoked a non-uniform response of the various cell cultures. Improved oxidative stress-sustaining properties had also been observed when the flavohemoglobins from *E. coli, Klebsiella pneumoniae, Deinococcus radiodurans* and *Pseudomonas aeruginosa* were expressed in *E. coli*.

Introduction

Oxidative and nitrosative stresses are encountered in all living organisms. Reactive oxygen species (ROS) and nitrogen species (RNS) are either formed as undesired and noxious metabolic side products or organisms might also be challenged by such stresses generated exogenously. Oxidative stress is an unavoidable side effect of aerobic lifestyle. ROS are formed *in vivo* during respiration when molecular oxygen is reduced to water (19,52). In particular reduced flavoproteins have been implicated in this redox process in *Escherichia coli* (25, 32). RNS, such as the nitric oxide radical (NO•) (nitrogen monoxide is the IUPAC recommended name) and the more potent peroxynitrite ($ONOO^-$) cause directly or indirectly nitrosative stress (27).

In mammals elevated levels of NO• in combination with ROS serve as potent toxins in cellular responses against infections, foreign bodies and neoplastic tissues. In plants, ROS appear to play key roles in early and late stages of plant response to pathogens. ROS seem to act both as cellular signals and killing molecules (12). The presence of NO• in plants is also well documented and recent results suggest clearly that NO• appears to play important roles during the plant-pathogen interaction (4). Therefore, infectious bacteria may be challenged by ROS and RNS as part of host defense mechanisms, when invading mammals and plants.

In denitrifying bacteria, NO• is produced during growth when nitrate or nitrite are used as terminal electron acceptors (14, 20). Additionally, in non-denitrifying bacteria such as *E. coli,* NO• may be produced by non-enzymatic reactions (56) or by reduction of nitrite to NO• by an oxidase (5).

Oxidative and nitrosative damage to proteins, nucleic acids and cellular membranes can occur when the concentration of reactive species exceeds the capacity of the cell's mechanisms for elimination (16). *E. coli* has developed (constitutive and inducible) enzymatic mechanisms to detoxify ROS and RNS under the control of the *sox*RS and *oxy*R regulatory networks (9, 13, 30,46).

Whereas the induction of *hmp* expression by NO• was known (47), the implication of *E. coli* flavohemoprotein HMP in the inducible response against nitrosative stress was derived later (21, 28). HMP plays a significant role in the detoxification of RNS in *E. coli* under aerobic conditions (43, for a review: 48). The reaction catalyzed by HMP converts NO• into nitrate with consumption of O_2 and NADH. Although the overall reaction balance is widely accepted, two different mechanisms for the HMP mediated reaction have been proposed. A route including a dioxygenase (21, 28) where the heme-bound oxygen reacts with NO• to yield nitrate, has been considered to be the main route for NO• degradation. Alternatively a 'denitrosylase' activity at physiologically more relevant $NO^{\bullet}/O_2$ concentrations has been proposed (29), where heme-bound NO• reacts with oxygen to form nitrate.

The impact of bacterial flavohemoglobins on oxidative stress is a subject of discussion. It has been shown in *E. coli* that overexpression of native HMP leads to the accumulation of superoxide and peroxide (1, 44). In contrast, an *E. coli hmp* null mutant has been found to be more sensitive against ROS (43).

Similar functions or putative roles in nitrosative or oxidative stress response have also been proposed or demonstrated for the flavohemoglobins from diverse microorganisms such as *Saccharomyces cerevisiae* (55), *Mycobacterium tuberculosis* (31), *Salmonella typhimurium* (10, 11), and *Ralstonia eutropha* (22).

While most bacterial species express flavohemoglobins, some bacteria such as *Vitreoscilla* sp. and *Campylobacter jejuni* express single domain hemoglobins (53, 3). The most thoroughly investigated bacterial hemoglobin is the one from *Vitreoscilla* sp. (VHb) (53, 39). VHb is a homodimeric hemoglobin. It has been postulated that expression of heterologous VHb yields increased intracellular oxygen concentration and in turn oxygen dependent cellular activities are stimulated in recombinant host cells (35). The VHb protein has been successfully expressed in various biotechnologically relevant organisms such as *E. coli, Bacillus subtilis, Streptomyces coelicolor, S. cerevisiae* and Chinese hamster ovary (CHO) cells. The expression of VHb results in an improved growth and enhanced productivity of recombinant

proteins or improved synthesis of primary or secondary metabolites (for review: 37). Recently, similar growth-stimulating effects and improved carbon utilization have also been observed when novel flavohemoglobins, which have been isolated from several bacterial species, have been expressed in *E. coli* (3, 17).

In this study, we have expressed the flavohemoglobin genes from *Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Deinococcus radiodurans, E. coli* and *R. eutropha* (previously *Alcaligenes eutrophus*) and the hemoglobin genes from *Vitreoscilla* sp. and *C. jejuni* in *E. coli* MG1655. Our goal was to study the feasibility of these hemoglobins to provide protection to a heterologous host from nitrosative and oxidative stress. In view of further biotechnological applications like bioremediation (45), the NO consumption activities of these hemoglobins have also been tested *in vitro*.

Materials and Methods

Bacterial strain, plasmids, and growth conditions

E. coli MG1655 K-12 (F^-, λ^-; Cold Spring Harbor Laboratory) has been used as a host throughout this study. Plasmids used in this study are listed in Table I. All cultivations were performed either in M9 minimal medium (50) supplemented with 4 g/l glucose, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, thiamine (5 mg/l), trace elements (36), vitamin mix (36) or in Luria-Bertani medium (LB, 50). Ampicillin (100 mg/l) was added to all cultures for plasmid maintenance. To obtain identical cultivation conditions for precultures and for oxidative and nitrosative stress experiments, shake flasks were filled with media to a fixed ratio of 1/10 of medium to total shake flask capacity. All cultivations were performed at 37°C on a rotatory shaker at 300 rpm. Cell growth was followed by measuring culture turbidity at 600 nm (A_{600}) with a Perkin Elmer λ1 spectrophotometer.

Table 1. Plasmids used in this study.

Plasmid	Flavohemoglobin/ Hemoglobin*	Origin of protein	Reference
pKQV4	None		(36)
pPPC1	VHb*	*Vitreoscilla* sp.	(36)
pAX1	FHPg*	*R. eutropha*	(17)
pAX4	VHb-Red[1)]	Chimeric protein	(17)
pAX5	FHP	*R. eutropha*	(17)
pECS14	HMP	*E. coli*	(3)
pBSS15	HmpBs	*B. subtilis*	(3)
pPAS6	HmpPa	*P. aeruginosa*	(3)
pDRS3	HmpDr	*D. radiodurans*	(3)
pCJS8	CHb*	*C. jejuni*	(3)
pSTS28	HmpSt	*S. typhimurium*	(3)
pKPS10	HmpKp	*K. pneumoniae*	(3)

[1)] Fusion protein of *Vitreoscilla* VHb and the C-terminal reductase domain of *R. eutropha* flavohemoglobin FHP.

Determination of resistance to SNP, and paraquat

Precultures were inoculated to A_{600} of 0.1 from overnight grown cultures of the various bacterial strains. At A_{600} of 0.5 the precultures were induced with IPTG to obtain a final concentration of 0.1 mM and the precultures were grown to an A_{600} of 1. To determine the resistance of hemoglobin- and flavohemoglobin-expressing strains and control strain against toxic ROS and RNS species, IPTG induced precultures were used to inoculate fresh minimal medium containing either 1 mM nitroprusside-sodium-dihydrate (SNP, Fluka), 1 mM $K_4Fe(CN)_6$ or 100 µM 1,1'-dimethyl-4,4'-bipyridinium dichloride (paraquat, PQ, Fluka), respectively, to an initial A_{600} of 0.2. As a reference, stressor free cultures were also prepared from each preculture. Both, stressor-containing and stressor-free cultures were grown further under identical conditions and A_{600} was routinely measured during a three hours period.

Specific growth rates (µ) were calculated by applying linear regression analysis on the natural logarithms of A_{600} values plotted against the time course of the

cultivations. The ratio of μ from a culture subjected to a stressor ($\mu_{stressor}$) to that from the respective reference culture ($\mu_{no\ stress}$) was calculated ($\mu_{stressor}$ / $\mu_{no\ stress}$) to quantify the effects of the stressors on growth of the *E. coli* cultures. The assay was performed in triplicate and repeated at least twice for each experiment. One-way ANOVA and Dunnett's multiple comparison test was applied for statistical analysis.

Growth of cells and preparation of soluble protein fraction for *in vitro* NO•-assay

E. coli cells were grown in LB medium in the presence of ampicillin (100 μg/ml). Precultures were diluted into fresh LB medium to A_{600} of 0.1 and grown to A_{600} of 1 where the expression of the hemoglobins and flavohemoglobins was induced with the addition of IPTG to a final concentration of 1 mM. The cultures were grown for 10 hours at a low shaking rate of 170 rpm to ensure production of the heme cofactor.

Cells were harvested by centrifugation and resuspended in assay buffer (50 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, pH 7). Soluble protein fractions were obtained as described (17).

Determination of protein and heme concentration

Total soluble protein concentration of the extracts was determined by the Bradford method (6) using reagents obtained from BioRad and bovine serum albumin as a standard.

Heme content in crude extracts was determined using the pyridine hemochrome method (2). Briefly, an equal volume of a stock solution containing 200 mM NaOH, 40% pyridine and 0.8 mM $K_3Fe(CN)_6$ was added to an aliquot of the soluble protein fraction and mixed. Absorbance of this oxidized sample was measured at 556 nm and 539 nm. A few grains of sodium dithionite were added to reduce the hemichromes to hemochromes and the maximum and minimum absorbance values

(556 nm and 539 nm, respectively) were measured after 3 min of incubation at room temperature. The absorbance values of the oxidized form were subtracted from the values recorded for the reduced sample, and the minimum absorbance was subtracted from the maximum absorbance. A pyridine hemochrome standard curve was generated using hemin (Sigma) as a standard. An extinction coefficient of 19 mM^{-1} cm^{-1} was obtained for hemin and was used to calculate heme contents of the samples.

Measurements of nitric oxide consumption

Gaseous nitric oxide was obtained from Linde (Höllriegelskreuth, Germany) and passed through a degassed NaOH solution and a column packed with NaOH pellets to remove higher nitrogen oxides before use. NO• solutions were prepared by degassing assay buffer for at least 30 min with N_2 and then saturating it with NO•.

NO• decay was measured with an ANTEK Instruments nitric oxide analyzer with a chemiluminiscence detector, which was adapted to liquid measurements using a 2 ml stirred cuvette connected to a bundle of capillaries that allow gas diffusion. 1µM of NO• corresponds to a signal of 42 mV.

An appropriate amount of crude extract as indicated in the results section was added to the cuvette and filled to 2 ml with assay buffer. NADH was added to a final concentration of 200 µM unless otherwise stated. The signal of this mixture was set as baseline and after 10 seconds NO• was added from an NO• saturated solution (2 mM) using a gastight SampleLock Hamilton syringe. The decay of NO• was recorded until the baseline was reached. All measurements were performed at room temperature and in duplicates. NO consumption rates in the presence of crude extracts were calculated by subtracting the rate of NO• consumption in buffer and expressed relative to heme content.

Quantification of nitrate and nitrite

Nitrate and nitrite concentration in samples was analyzed by using the Griess reagent. Nitrate was enzymatically converted to nitrite and quantified using colorimetric assay kit (Roche Molecular Biochemicals). Nitrite was determined directly with the Griess reagent (Molecular Probes, Eugene, Oregon). Nitrate and nitrite standards were used for calibration. Each sample was measured twice.

Results

Determination of resistance of hemoglobin and flavohemoglobin expressing *E. coli* cells against SNP and paraquat

Table 2. Ratios $\mu_{stressor}/\mu_{no\ stress}$ of cultures exposed to SNP or paraquat and of stressor free cultures

Strain[a)]	1 mM SNP[b)]	
Control	0.68 ± 0.06[c)]	
FHP	0.88 ± 0.02	P ≤ 0.001[d)]
VHb-Red	0.88 ± 0.05	P ≤ 0.001
HmpKp	0.84 ± 0.03	P ≤ 0.001
HmpPa	0.86 ± 0.04	P ≤ 0.001
HmpSt	0.89 ± 0.05	P ≤ 0.001
HmpBs	0.92 ± 0.06	P ≤ 0.001
HMP	0.90 ± 0.08	P ≤ 0.001
HmpDr	0.93 ± 0.02	P ≤ 0.001
FHPg	0.92 ± 0.02	P ≤ 0.001
VHb	0.91 ± 0.06	P ≤ 0.001
CHb	0.87 ± 0.07	P ≤ 0.001
HMP[e)]	0.99 ± 0.08	P ≤ 0.001
VHb[e)]	0.96 ± 0.06	P ≤ 0.001

[a)] Strains expressing various hemoglobins and flavohemoglobins in *E. coli* MG1655 cells. The control strain contained the parental plasmid pKQV4.
[b)] Ratio of growth rates from a culture exposed to SNP and a stressor free control culture.
[c)] Mean values and standard deviation are given. Values are calculated from the results of at least two independent experiments performed in triplicates.
[d)] Statistical significance data has been analyzed using ANOVA and compared using Dunnett's multiple comparison test assuming a confidence interval of 99%. All data has been compared to the control cultures.
[e)] Cultures of *E. coli* MG1655:pPPC1 and MG1655:pECS14 grown in the presence of 1 mM $K_4(Fe(CN)_6)$.

E. coli cells expressing either flavohemoglobins or hemoglobins achieved significantly higher μ_{SNP} / $\mu_{no\ stress}$ ratios relative to the ratio obtained with the control strain (0.68) (Table 2). All recombinant *E. coli* strains investigated show μ_{SNP} / $\mu_{no\ stress}$ ratios of approximately 0.9. Thus, growth rates of *E. coli* cultures expressing either flavohemoglobins or hemoglobins are only minimally affected by nitrosative stress, clearly indicating that the expression of these hemoproteins helps the cells to resist the toxicity exerted by SNP. However it is not possible to distinguish between the effects exerted by hemoglobins and flavohemoglobins. *E. coli* cells expressing either type of proteins are equally capable of protecting the cells from nitrosative stress. To exclude the eventuality that cyanide liberated from SNP is responsible for growth inhibition, experiments using 1 mM $K_4(Fe(CN)_6)$ have been performed. Growth rate of $K_4(Fe(CN)_6)$ containing cultures expressing either VHb or FHP were not affected by the presence of this complex relative to control cultures (Table 2).

Table 3. Ratios $\mu_{stressor}/\mu_{no\ stress}$ of cultures exposed to 100 µM paraquat and of stressor free cultures

Strain[a)]	100 µM paraquat[b)]	
Control	0.32 ± 0.06[c)]	
FHP	0.36 ± 0.04	P ≥ 0.05[d)]
HmpKp	0.33 ± 0.08	P ≥ 0.05
HmpPa	0.43 ± 0.05	P ≥ 0.05
HmpSt	0.48 ± 0.05	P ≤ 0.001
HmpBs	0.24 ± 0.01	P ≥ 0.05
HMP	0.52 ± 0.07	P ≤ 0.001
HmpDr	0.52 ± 0.05	P ≤ 0.001
VHb	0.32 ± 0.06	P ≥ 0.05
CHb	0.28 ± 0.05	P ≥ 0.05

a) Strains expressing various hemoglobins and flavohemoglobins in *E. coli* MG1655. The cells, control strain contained the parental plasmid pKQV4.
b) Ratio of growth rates from a culture exposed to paraquat and a stressor free control culture.
c) Mean values and standard deviation are given, standard deviation is calculated from the results of at least two independent experiments performed in triplicates.
d) Statistical significance, data has been analyzed using ANNOVA and compared using Dunnett's multiple comparison test assuming a confidences interval of 95%. All data has been compared to the respective control cultures.

The effect of the paraquat treatment on the growth of the cultures is heterogeneous. Increased resistance against $O_2^{\bullet-}$ generated from paraquat was only seen in the MG1655 strains expressing the flavohemoglobins HMP, HmpSt, HmpDr and HmpPa. In these cultures the specific growth rates in the presence of paraquat were decreased maximally by 50% relative to the stressor free culture resulting in $\mu_{paraquat}/\mu_{no\ stress}$ ratios of about 0.5. HmpBs, HmpKp, CHb or VHb expressing cultures were more severely affected by the paraquat treatment and the ratios $\mu_{paraquat}/\mu_{no\ stress}$ were approximately 0.3, a value which does not significantly differ from the ratio of the control strain (0.32) (Table 3).

***In vitro* NO• consumption assay**

NO• decay was followed with the nitric oxide analyzer. Soluble protein fractions were added to the reaction chamber and the reaction was started with the addition of an aliquot of a NO• saturated solution. In the absence of a reducing agent, the consumption of NO• by flavohemoglobins and hemoglobins was only slightly accelerated compared to protein-free buffer. Therefore, NADH was added to the reaction mixture.

The addition of NADH dramatically increased the consumption of NO• relative to the decay observed in protein-free solutions in the presence of NADH. The addition of NADH was necessary for both flavohemoglobins, such as FHP, and classical hemoglobins, such as CHb (Figure 1). The control cell extract, at the same heme concentration as CHb and FHP, did not show any acceleration in NO• decay in the presence of additional NADH (data not shown).

NO• consumption activity of various cell extracts is displayed in Figure 2. NO• turnover of the different flavohemoglobins and hemoglobins was calculated by subtracting rate of NO decay in buffer and standardizing to the heme content (Table 4). The NO• consumption rates for VHb, CHb, HmpDr, FHPg and VHb-Red were not significantly different from the rate of the control. The protein extracts

containing FHP and HmpPa had intermediary rates, leading to an acceleration of NO• turnover of approximately 10-fold relative to controls. The protein extracts containing the flavohemoglobins of *K. pneumoniae* , *S. typhimurium, B. subtilis* and *E. coli* showed highest activities being up to 100-fold higher than the turnover numbers for the native hemoglobins (Table 4).

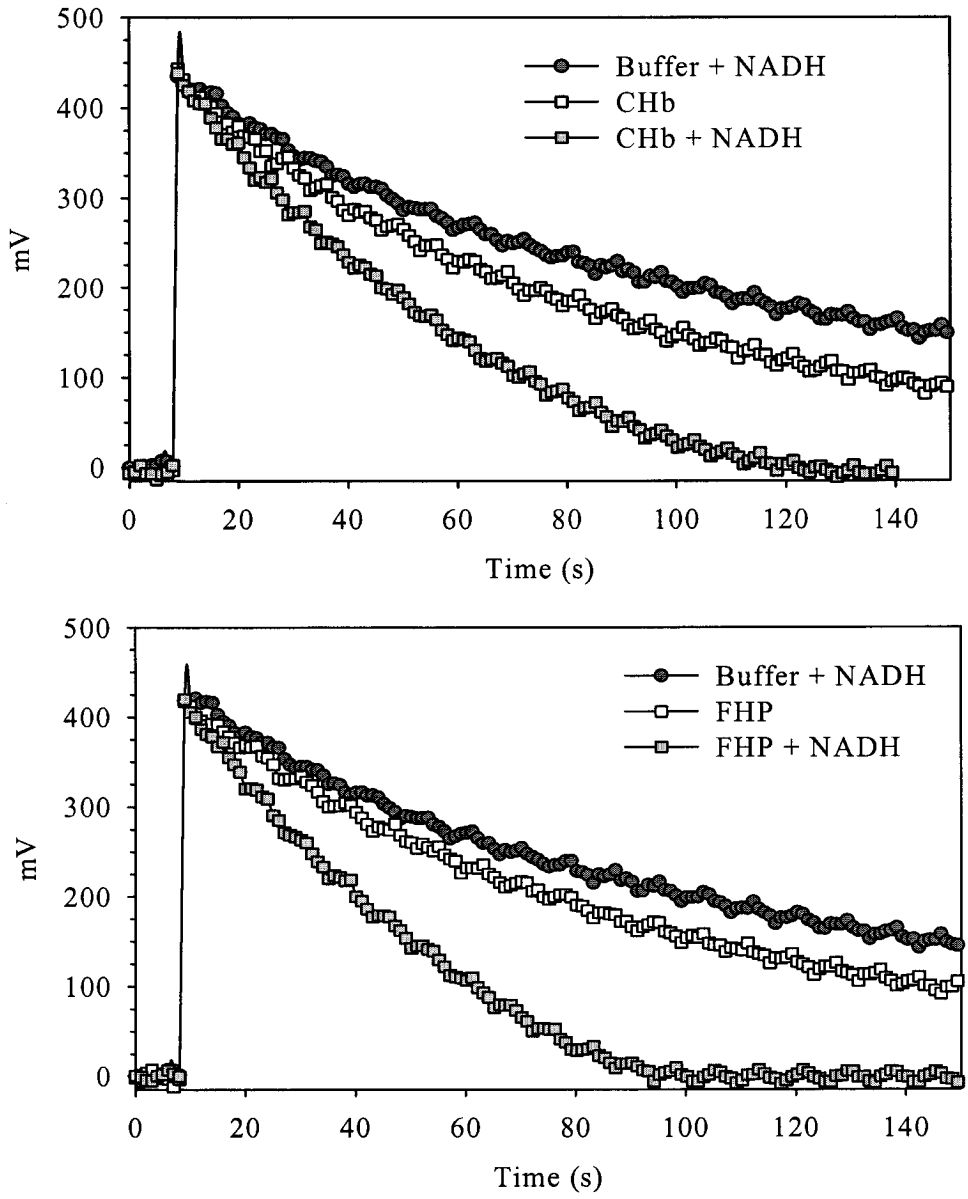

Figure 1. NO• consumption curves of soluble protein fraction of cells expressing *C. jejuni* hemoglobin CHb and *R. eutropha* FHP in the presence and absence of NADH. NO• consumption was measured with a nitric oxide analyzer using a chemiluminiscence detector. The assay was performed with or without the addition of 200 µM NADH. 42 mV corresponds to 1 µM NO•.

Due to the fast NO• removal, the NO• turnover rate of cell extracts of *S. typhimurium*, *K. pneumoniae*, *B. subtilis* and *E. coli* was also determined at a NO• concentration of 20 μM. The rates at a higher NO• concentration were not significantly different from the turnover determined at 10 μM besides for the extract of *B. subtilis*, which reached a substantially higher rate at 20 μM relative to the one at 10 μM (Table 4).

Table 4. NO• consumption of soluble protein fractions.

protein extract[a]	NO• turnover rates [s^{-1}] [b]	
	10 μM NO•	20 μM NO•
control	1.9 ± 0.1	n. d.[c]
control	2.0 ± 0.1	n. d.
CHb	0.4 ± 0.1	n. d.
FHPg	3.4 ± 1.3	n. d.
VHb	0.9 ± 0.1	n. d.
VHb-Red	4.3 ± 1.0	n. d.
HmpDr	3.1 ± 1.5	n. d.
FHP	15 ± 1	n. d.
HmpPa	26 ± 4	n. d.
HmpKp	95 ± 1	86 ± 9
HmpSt	76 ± 4	78 ± 6
HmpBs	68 ± 11	128 ± 6
HMP	91 ± 4	90 ± 4

[a] Soluble protein fractions were obtained from *E. coli* MG1655 cells overexpressing either hemoglobins or flavohemoglobins.
[b] NO• turnover rates were calculated with a correction for backgroud rates of NO• decomposition and expressed relative to heme content.
[c] n. d.: not determined. See text for details.

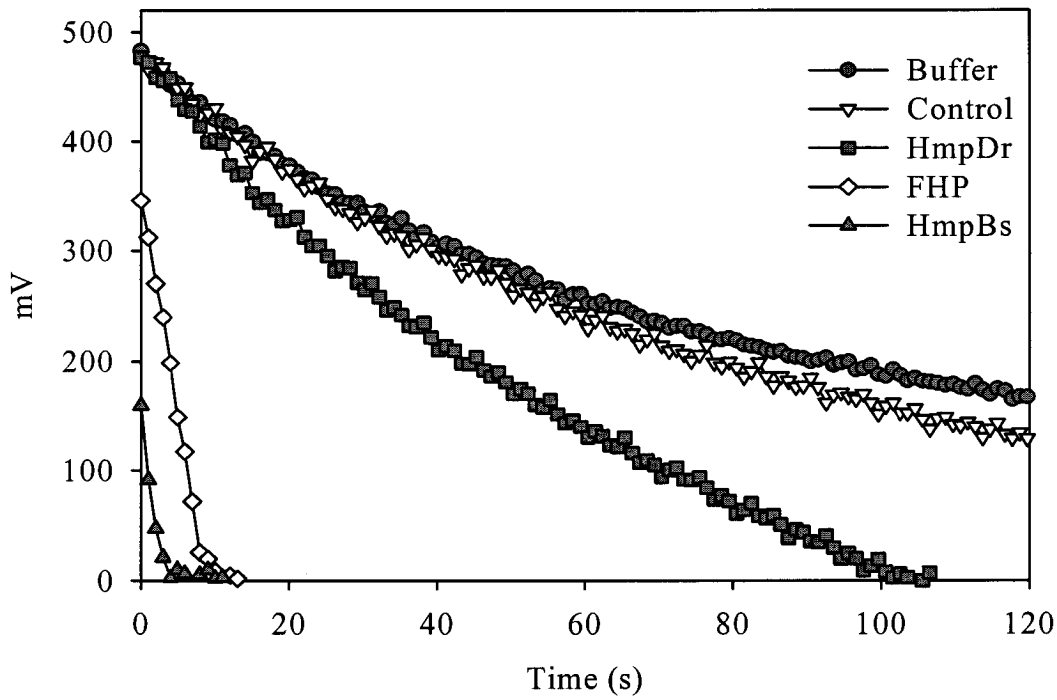

Figure 2. NO• consumption curves of soluble protein fractions containing various bacterial hemoglobin and flavohemoglobin proteins. Protein extracts were added to a final concentration of 0.05 mg/ml in an air equilibrated buffer containing 200 μM NADH. The reaction was initiated by addition of an aliquot of a NO• saturated solution to a final concentration of 10 μM. NO• consumption was followed with a nitric oxide analyzer using a chemiluminiscence detector. 42 mV corresponds to 1 μM NO•.

Previously, it has been shown that the aerobic detoxification of NO• by purified *E. coli* HMP stoichiometrically yields nitrate (21, 23, 28). In contrast, the nonenzymatic reaction of NO• and O_2 in the aqueous-phase should produce nitrite (54). To further prove the involvement of flavohemoglobins and hemoglobins in the observed NO• removal, nitrate and nitrite concentrations were determined. Identical mixtures, such as used for determination of NO• consumption, including air equilibrated buffer and 200 μM NADH have been prepared in sealed glass vials and a NO• saturated solution was injected to give a final concentration of 20 μM. After 30 minutes of incubation the samples were analyzed for nitrite and nitrate. Mainly all NO• was recovered as nitrate, nitrite was almost undetectable in all samples (0 - 1 μM)(Table 5). Thus, these data confirm that the acceleration of NO• decay by

hemoglobin- and flavohemoglobin- containing protein extracts is due to the presence of these proteins and not to any unspecific reaction.

Table V. Nitrate production of cell extracts during NO• turnover.

Protein extract[a)]	Nitrate [μM][b)]
wt	17 ± 4
pKQV4	18 ± 5
FHPg	19 ± 5
VHb-Red	15 ± 5
FHP	19 ± 6
VHb	17 ± 4
CHb	16 ± 3
HMP	22 ± 1
HmpSt	23 ± 3
HmpKp	18 ± 4
HmpBs	24 ± 4
HmpPa	24 ± 7
HmpDr	26 ± 3

a) Soluble protein fractions were obtained from *E. coli* MG1655 cells overexpressing either hemoglobins or flavohemoglobins.
b) Nitrate recovery after conversion of 20 μM NO• by cell extracts. Mean value of two independent assays and standard deviation are given. Each measurement has been done twice.

Discussion

Several lines of evidence indicate that hemoglobin and flavohemoglobin proteins analyzed in this study are either directly or indirectly involved with the cellular response against oxidative and nitrosative stress. In our *in vivo* experiments we have evaluated the effect of hemoprotein expression on the resistance against oxidative and nitrosative stress.

E. coli cells expressing the different flavohemoglobins and hemoglobins had a non-uniform response to oxidative stress. When growing cells in a paraquat containing environment, statistically significant growth ameliorating effects were only observed for *E. coli* cells expressing HMP, HmpSt and HmpDr. The other strains,

including VHb and CHb producing strains, showed the same growth pattern than that observed for the control strain (Table III).

Although the reports on the association of flavohemoglobins from various organisms and oxidative stress are numerous, no clear indication for the role of these proteins is obvious. In *S. typhimurium* no significant differences in the minimal inhibitory concentrations of paraquat and H_2O_2 were observed between an *hmp* null mutant and a wildtype strain (10). Overexpression of *E. coli* HMP from a multicopy plasmid caused oxidative stress *in vivo*. *In vitro* experiments with purified HMP also demonstrated the generation of superoxide (41). Anjum and colleagues (1) confirmed these results and stated that a strain overproducing HMP was significantly more sensitive to paraquat relative to the plasmid-bearing wild-type strain. In contrast, an *E. coli hmp* null mutant has been found to be more sensitive towards paraquat than the corresponding wildtype *E. coli* strain. Therefore, it has been concluded that HMP might exhibit a regulatory role rather than being part of the direct stress response in *E. coli* mechanism under oxidative stress (43). All these issues point to an important role for the dose of flavohemoglobin on the response against oxidative stress. This could be one of the reasons for the differences observed between the different flavohemoglobins and hemoglobins tested in the present study.

All flavohemoglobins and hemoglobins tested in this study were equally capable of sustaining growth of a heterologous host relative to the non-expressing control strain under the experimental conditions.

When tested *in vitro* the capability of these proteins to degrade NO•, the cell extracts containing the flavohemoglobins of *B. subtilis* and of the putative pathogenic bacteria *E. coli*, *K. pneumoniae*, *S. typhimurium*, and *P. aeruginosa* proved to be the fastest. This is not surprising since pathogenic bacteria are potentially exposed to high amounts of reactive nitrogen species when infecting their host organisms. It is reported that the level of NO• in macrophages is up to the millimolar range, although the exact concentration of NO• in the bacteria is not known. Furthermore,

in the denitrification process NO• is supposed to attain concentrations of 10^{-9} -10^{-7} M (26). NO• turnover of purified HMP or of HMP containing cell extracts as well as of FHP and yeast flavohemoglobin (22, 23, 28, 44) has been reported. Our numbers are in good agreement with the reported numbers of Gardner et al. (22).

NO• consumption was small for cell extracts harboring the hemoglobins from *Vitreoscilla* and *C. jejuni*. The absence of a significant NO• degrading activity is in contrast to the results of the *in vivo* experiments, where a clear nitrosative stress remediating effect was shown. This could be due to the fact that a membrane bound protein is involved in the enzymatic reduction of these hemoglobins, like in the reduction of myoglobin (40). As the *in vitro* assay mainly contained the soluble protein fraction, the re-reduction of the proteins would not be as effective as *in vivo*. Another feasible explanation is that the conditions of the *in vitro* assay, such as O_2 and NO• concentration, differ from the ones encountered in the *in vivo* assays.

The NO• detoxification by HMP converts equistochiometric amounts of NO•and O_2 to nitrate with the consumption of NADH (21, 23, 23, 28). The NO• removal mediated by our hemoproteins yields mainly nitrate. Thus, it is plausible to conclude that the observed acceleration of NO• removal by protein extracts is indeed due to the hemoprotein-mediated conversion of NO• to nitrate and not caused by any unspecific interaction of NO• with other proteins contained in the cell extracts.

The results of the *in vivo* experiments suggested that bacterial hemoglobins (VHb, CHb) and flavohemoglobins are equally capable of supporting the *E. coli* cells to overcome the nitrosative stress produced by SNP *in vivo*. To study the effect of the reductase domain more carefully, the following proteins described before have been included: a strain engineered to solely express the hemoglobin domain of *R. eutropha* FHP, termed FHPg and a strain producing a novel fusion protein consisting of N-terminal VHb fused to the C-terminal reductase domain of FHP, termed VHb-Red (17).

The results of the *in vivo* experiments suggest, that the removal of the C-terminal reductase domain from FHP or the addition of the C-terminal reductase domain (VHb-Red) do not significantly change the protective effect under nitrosative stress conditions in respect to the corresponding native proteins under our experimental conditions. Both pairs of proteins (FHP and the hemoglobin domain thereof or VHb and its fusion protein) perform equally well under our experimental conditions when challenged with SNP. However, the *in vitro* NO• consumption activity is clearly improved in the presence of the reductase domain. The inclusion of a reductase domain improves the performance of FHPg and VHb hemoglobins by a factor of 4-5. Again this different behaviour between the effects exerted *in vivo* and *in vitro* could be explained by the lack of an appropriate external reductase system necessary for the full activity of hemoglobins in the *in vitro* analysis. It would also explain the superior performance of flavohemoglobins in respect to hemoglobins under those conditions, since the necessary re-reduction of the heme iron for the NO• turnover is going to be probably more effective if the reductase domain is bound to the globin domain. In previous studies on VHb, an NADH-cytochrome *o* reductase was also copurified from *Vitreoscilla* (53, 24, 33), which was able to reduce VHb as well as a number of artificial electron acceptors in an NADH dependent manner. The observed acceleration of NO• degradation by the addition of NADH (Figure 4) allows us to suggest that in analogy to *Vitreoscilla* an endogenous reductase system of *E. coli* might be able to reduce the heme-iron of hemoglobins to the biochemically active ferrous form. A variety of possible reductase systems exists in *E. coli,* such as ferredoxin NAD(P)H oxidoreductase, NAD(P)H:flavin reductase and ferric reductase, which could potentially function as heme reductases.

In a recent study, Kaur *et al.* (38) have also analyzed the effect of addition of a reductase domain to VHb in SNP stressed cells, by using a very similar construct to that of VHb-Red, previously described by Frey et al. (17). They also showed a faster NO• consumption activity in the extracts containing the chimeric protein relative to the VHb protein. Unfortunately, they do not report any NO• turnover value, which would have allowed the direct comparison of the data. Similar as in this study,

growth experiments under nitrosative stress conditions do not seem to achieve significant differences between the growth performance of VHb and VHb-R expressing *E. coli* cells.

VHb and more recently FHP and related proteins have been routinely used to improve growth and recombinant protein production in various bacterial species and also higher eukaryotes (37). VHb has been assumed to increase the intracellular oxygen levels. Concomitantly, this leads to improved efficiency of the respiratory chain and resulted in higher ATP turnover rate, increased ATPase activities, increased levels of the cytochromes, and higher oxygen uptake rates relative to globin-free *E. coli* cultures (37). Recently, Stevanin et al. (51) reported the inducible protection of cytochromes and respiration by HMP from NO•. Also myoglobin in skeletal and cardiac muscles is proposed to act as a scavenger of intracellular NO•, to avoid or reduce inhibition of cytochrome-*c* oxidase, and thereby protect the energy-producing machinery (7). It is therefore tempting to speculate that the role of VHb and FHP is rather the protection of the respiratory chain from endogenously produced NO• radicals than increasing the intracellular oxygen uptake. In good agreement, Kaur et al (38) showed increased oxygen uptake rates of hemoglobin expressing cells in the presence of NO•. Furthermore, a FHP-or FHPg-expressing strain had higher oxygen uptake rates than *E. coli* strains producing VHb or VHb-Red under microaerobic conditions (18). These results are in good agreement with the observed higher NO• turnover rate of FHP/FHPg relative to VHb/VHb-Red. Prevention of the inactivation of cytochromes by NO• and related molecules would result in the same effects as attributed to VHb in an oxygen providing role. Both potential functions would require a close interaction of VHb with the cytochromes and indeed VHb has been shown to be preferentially localized in the close surrounding of the respiratory chain in both the native host *Vitreoscilla* and also in *E. coli* (49).

In this study novel hemoglobins and flavohemoglobins originating from various bacterial species have been successfully expressed in the heterologous wildtype *E.*

coli MG1655 host. This strain harbors a chromosomal copy of the endogenous *hmp* gene, which probably provides a background HMP level in our stress experiments due to the induction of the endogenous *hmp* copy after the addition of the stressors paraquat and SNP (47, 42). Nevertheless our results show clearly that the overexpression of different flavohemoglobins and hemoglobins increase the natural resistance of *E. coli* cells under nitrosative stress.

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References

1. **Anjum, M. F., N. Ioannidis, and R. K. Poole.** 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.
2. **Berry, E. A., and B. L. Trumpower.** 1987. Simultaneous determination of hemes a, hemes b, and hemes c from pyridine hemochrome spectra. Anal. Biochem. **161:**1-15.
3. **Bollinger, C. J. T., J. E. Bailey, and P. T. Kallio.** 2001. Novel hemoglobins to enhance microaerobic growth and substrate utilization in *Escherichia coli*. Biotechnol Prog. **17**:798-808.
4. **Bolwell, G. P.** 1999. Role of active oxygen species and NO in plant defence responses. Curr. Op. Plant Biol. **2:**287-294.
5. **Bonner, F. T., M. N. Hughes, R. K. Poole, and R. I. Scott.** 1991. Kinetics of the reactions of trioxodinitrate and nitrite ions with cytochrome *d* in *Escherichia coli*. Biochim. Biophys. Acta **1056:**133-138.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the princible of protein-dye binding. Anal. Biochem. **72:**248-254.
7. **Brunori, M.** 2001. Nitric oxide, cytochrome *c* oxidase and myoglobin. Trends Biochem. Sci. **26**:21-23.
8. **Buisson, N., and R. Labbe-Bois**. 1998. Flavohemoglobin expression and function in *Saccharomyces cerevisiae* - No relationship with respiration and complex response to oxidative stress. J. Biol. Chem. **273**:9527-9533.
9. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753-762.
10. **Crawford, M. J., and D. E. Goldberg.** 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.
11. **Crawford, M. J., and D. E. Goldberg.** 1998. Role for the *Salmonella* flavohemoglobin in protection from nitric oxide. J. Biol. Chem. **273:**12543-12547.
12. **Dangl, J.** 1998. Innate immunity - Plants just say NO to pathogens. Nature **394:**525.
13. **Demple, B.** 1996. Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon: a review. Gene **179:**53-57.

14. **Dermastia, M., T. Turk, and T. C. Hollocher.** 1991. Nitric oxide reductase: Purification from *Paracoccus denitrificans* with use of a single column and some characteristics. J. Biol. Chem. **266:**10899-10905.
15. **Dikshit, K. L., and D. A. Webster.** 1988. Cloning, characterization and expression of the bacterial globin gene from *Vitreoscilla* in *Escherichia coli*. Gene **70:**377-386.
16. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561-585.
17. **Frey, A. D., J. E. Bailey, and P. T. Kallio.** 2000. Expression of *Alcaligenes eutrophus* flavohemoprotein and engineered *Vitreoscilla* hemoglobin-reductase fusion protein for improved hypoxic growth of *Escherichia coli*. Appl. Environ. Microbiol. **66:**98-104.
18. **Frey, A. D., J. Fiaux, T. Szyperski, K. Wuthrich, J. E. Bailey, and P. T. Kallio.** 2001. Dissection of central carbon metabolism of hemoglobin-expressing *Escherichia coli* by ^{13}C nuclear magnetic resonance flux distribution analysis in microaerobic bioprocesses. Appl. Environ. Microbiol. **67:**680-7.
19. **Fridovich, I.** 1998. Oxygen toxicity: a radical explanation. J. Exp. Biol. 201:1203-1209.
20. **Fujiwara, T., and Y. Fukumori.** 1996. Cytochrome *cb*-type nitric oxide reductase with cytochrome *c* oxidase activity from *Paracoccus denitrificans* ATCC 35512. J. Bacteriol. **178:**1866-1871.
21. **Gardner, P. R., A. M. Gardner, L. A. Martin, and A. L. Salzman.** 1998. Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc. Natl. Acad. Sci. USA **95:**10378-10383.
22. **Gardner, P. R., A. M. Gardner, L. A. Martin, Y. Dou, T. Li, J. S. Olson, H. Zhu, and A. F. Riggs.** 2000. Nitric-oxide dioxygenase activity and function of flavohemoglobins. Sensitivity to nitric oxide and carbon monoxide inhibition. J. Biol. Chem. **275:**31581-7.
23. **Gardner, A. M., L. A. Martin, P. R. Gardner, Y. Dou, and J. S. Olson.** 2000. Steady-state and transient kinetics of *Escherichia coli* nitric oxide dioxygenase (flavohemoglobin) - The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. J. Biol. Chem. **275:**12581-12589.
24. **Gonzalez-Prevatt, V., and D. A. Webster,** 1980. Purification and properties of NADH-cytochrome *o* reductase from *Vitreoscilla*. J. Biol. Chem. **255:**1478-1482.
25. **Gonzalez-Flecha, B., and B. Demple.** 1995. Metabolic sources of hydrogen-peroxide in aerobically growing *Escherichia coli*. J. Biol. Chem. **270:**13681-13687.
26. **Goretski, J., O. C. Zafiriou, and T. C. Hollocher.** 1990. Steady-state nitric oxide concentrations during denitrification. J. Biol. Chem. **265:**11535-8.
27. **Hausladen, A., C. T. Privalle, T. Keng, J. DeAngelo, and J. S. Stamler.** 1996. Nitrosative stress: activation of the transcription factor OxyR. Cell **86:**719-729.
28. **Hausladen, A., A. J. Gow, and J. S. Stamler.** 1998. Nitrosative stress: metabolic pathway involving the flavohemoglobin. Proc. Natl. Acad. Sci. USA **95:**14100-14105.
29. **Hausladen, A., A. Gow, and J. S. Stamler.** 2001. Flavohemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. Proc. Natl. Acad. Sci. U S A **98:**10108-12.
30. **Hidalgo, E., H. G. Ding, and B. Demple.** 1997. Redox signal transduction via iron-sulfur clusters in the SoxR transcription activator. Trends Biochem. Sci. **22:**207-210.
31. **Hu, Y. M., P. D. Butcher, J. A. Mangan, M. A. Rajandream, and A. R. M. Coates.** 1999. Regulation of *hmp* gene transcription in *Mycobacterium tuberculosis*: effects of oxygen limitation and nitrosative and oxidative stress. J. Bacteriol. **181:**3486-3493.
32. **Imlay, J. A., and I. Fridovich.** 1991. Assay of metabolic superoxide production in *Escherichia coli*. J. Biol. Chem. **266:**6957-6965.
33. **Jakob, W., D. A. Webster, and P. M. H. Kroneck.** 1992. NADH-dependent methemoglobin reductase from the obligate aerobe *Vitreoscilla* - Improved method of purification and reexamination of prosthetic groups. Arch. Biochem. Biophys. **292:**29-33.
34. **Ji, X. B., and T. C. Hollocher.** 1988. Reduction of nitrite to nitric oxide by enteric bacteria. Biochem. Biophys. Res. Commun. **157:**106-8.

35. **Kallio, P. T., D. J. Kim, P. S. Tsai, and J. E. Bailey.** 1994. Intracellular expression of *Vitreoscilla* hemoglobin alters *Escherichia coli* energy metabolism under oxygen-limited conditions. Eur. J. Biochem. **219:**201-208.

36. **Kallio, P. T., P. S. Tsai, and J. E. Bailey.** 1996. Expression of *Vitreoscilla* hemoglobin is superior to horse heart myoglobin or yeast flavohemoglobin expression for enhancing *Escherichia coli* growth in a microaerobic bioreactor. Biotechnol. Prog. **12:**751-757.

37. **Kallio, P. T., A. D. Frey, and J. E. Bailey.** 2001. From *Vitreoscilla* hemoglobin (VHb) to a novel class of growth stimulating hemoglobin proteins. In: Merten, O.-W., D. Mattanovich, G. Larsson, P. Neubauer, C. Land, D. Porro, J. Teixeira de Mattos, P. Postma, and J. Cole (ed.). "Recombinant protein production with prokaryotic and eukaryotic cells. A comparative view on host physiology". Kluewer Academics Publishers, Dordrecht, The Netherlands. pp. 75-87.

38. **Kaur, R., R. Pathania, V. Sharma, S. C. Mande, and K. L. Dikshit.** 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.

39. **Khosla, C., and J. E. Bailey.** 1988. The *Vitreoscilla* hemoglobin gene - molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. Mol. Gen. Genet. **214:**158-161.

40. **Livingston, D. J., S. J. McLachlan, G. N. La Mar, and W. D. Brown.** 1985. Myoglobin: cytochrome b_5 interactions and the kinetic mechanism of metmyoglobin reductase. J. Biol. Chem. **260:**15699-707.

41. **Membrillo-Hernández, J., N. Ioannidis, and R. K. Poole.** 1996. The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide in vitro and causes oxidative stress *in vivo*. FEBS Lett. **382:**141-144.

42. **Membrillo-Hernández, J., S. O. Kim, G. M. Cook, and R. K. Poole.** 1997. Paraquat regulation of *hmp* (flavobemoglobin) gene expression in *Escherichia coli* K-12 is SoxRS independent but modulated by σ^s. J. Bacteriol. **179:**3164-3170.

43. **Membrillo-Hernández, J., M. D. Coopamah, M. F. Anjum, T. M. Stevanin, A. Kelly, M. N. Hughes, and R. K. Poole.** 1999. The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a nitric oxide releaser, and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. **274:**748-754.

44. **Mills, C. E., S. Sedelnikova, B. Soballe, M. N. Hughes, and R. K. Poole.** 2001. *Escherichia coli* flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of nitric oxide. Biochem. J. **353:**207-13.

45. **Patel, S. M., B. C. Stark, K. W. Hwang, K. L. Dikshit, and D. A. Webster.** 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.

46. **Pomposiello, P. J., and B. Demple.** 2001. Redox-operated genetic switches: the SoxR and OxyR transcription factors. Trends Biotechnol. **19:**109-114.

47. **Poole, R. K., M. F. Anjum, J. Membrillo-Hernández, S. O. Kim, M. N. Hughes, and V. Stewart.** 1996. Nitric oxide, nitrite, and Fnr regulation of *hmp* (Flavohemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. **178:**5487-5492.

48. **Poole, R. K., and M. N. Hughes.** 2000. New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. **36:**775-783.

49. **Ramandeep, K. W. Hwang, M. Raje, K. J. Kim, B. C. Stark, K. L. Dikshit, and D. A. Webster.** 2001. *Vitreoscilla* hemoglobin. Intracellular localization and binding to membranes. J. Biol. Chem. **276:**24781-9.

50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

51. **Stevanin, T. M., N. Ioannidis, C. E. Mills, S. O. Kim, M. N. Hughes, and R. K. Poole.** 2000. Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes *bo* or *bd*, from nitric oxide. J. Biol. Chem. **275:**35868-75.

52. **Storz, G., and J. A. Imlay.** 1999. Oxidative stress. Curr. Opin. Microbiol. **2:**188-194.

53. **Webster, D. A., and C. Y. Liu.** 1974. Reduced nicotinamide adenine dinucleotide cytochrome *o* reductase associated with cytochrome *o* purified from *Vitreoscilla*. Evidence for an intermediate oxygenated form of cytochrome *o*. J. Biol. Chem. **249**:4257-4260.

54. **Wink, D. A., J. F. Darbyshire, R. W. Nims, J. E. Saavedra, and P. C. Ford.** 1993. Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. Chem. Res. Toxicol. **6**:23-27.

55. **Zhao, X. J., D. Raitt, P. V. Burke, A. S. Clewell, K. E. Kwast, and R. O. Poyton.** 1996. Function and expression of flavohemoglobin in *Saccharomyces cerevisiae*. Evidence for a role in the oxidative stress response. J. Biol. Chem. **271**:25131-25138.

56. **Zweier, J. L., P. H. Wang, A. Samouilov, and P. Kuppusamy.** 1995. Enzyme-independent formation of nitric oxide in biological tissues. Nat. Med. **1**:804-809.

Expression of *Vitreoscilla* hemoglobin in hybrid aspen (*Populus tremula x tremuloides*): an attempt to improve growth of an economically relevant tree species

Abstract

We describe in this work for the first time *Vitreoscilla* hemoglobin (VHb) expression in an economically important boreal woody plant hybrid aspen (*Populus tremula x tremuloides*). VHb has been mainly expressed in unicellular organisms of both prokaryotic and eukaryotic origin. VHb is suggested to increase intracellular oxygen levels and enhance the activity of terminal oxidases leading to higher ATP levels and ATP turnover rates in bacteria. In the published plant papers tobacco plants with *vhb* gene (13) were characterized by fast germination rates, high yield, enhanced chlorophyll content, and a shift from anabasine towards nicotine production. Similar positive effects on cell growth were observed upon expression of VHb in a tobacco suspension culture (11). The mechanism of heterologous expression of VHb is unknown but VHb may contribute to a more stable oxygen level in the cells but other functions have also been proposed. In the present study, we were able to generate several transgenic VHb-lines but no clear dependence between copy numbers and VHb expression was found. VHb-expression, analyzed under different ambient conditions, such as different growth temperature and elevated UV-B illumination, did generally not result in statistically significant growth improvements relative to control lines. However, a trend indicating improved growth characteristics can be deduced. Generally, VHb-expression was dependent on plant genotype. Using light and electron microscopy, differences in the cell structure and cell numbers were detected between the transgenic and control lines. VHb-lines displayed a higher cell number per leaf area and furthermore a significantly increased amount of starch in the chloroplasts. The latter finding is of potential interest since starch would provide the plant a carbon source when growth conditions are not optimal for photosynthesis. Effects of elevated UV-B illumination on VHb lines were interesting because the lines responded individually. Several VHb-lines had elevated levels of total flavonoids, quercetin-, kaempferol- and myricetin-derivatives relative to controls and other transgenic lines.

Introduction

Hybrid aspen is an interspecific F_1 hybrid of European aspen (*Populus tremula* L.) and American aspen (*P. tremuloides* Michx.) produced by artificial hybridization. Originally, interspecific crosses have been made due to the superior growth characteristics of this hybrid. In recent years, special interest has been focused on the hybrid species by paper industry due to the wood quality properties, which are favourable in the manufacture of fine paper. Hybrid aspen as well as many other *Populus* species have also proved to be competent both for micropropagation and genetic transformation indicating the possibilities for molecular breeding.

The obligate aerobe *Vitreoscilla* produces a soluble hemoglobin (VHb) under oxygen limitation. This protein has been functionally expressed in prokaryotic and eukaryotic organisms, where oxygen availability has been considered to be a growth limiting factor. The beneficial effects of heterologous VHb expression on cell growth and product yields in bacteria and also in higher eukaryotic cells are known for more than a decade (15). In *Escherichia coli* VHb is suggested to increase intracellular oxygen levels and enhance the activity of terminal oxidases and thus, leading to higher ATP levels and ATP turnover rates (9, 15, 30). Furthermore, shifts in the ratio of NAD^+/NADH have been reported and a more oxidized cytoplasm has been reported for VHb-expressing *E. coli* cells (29). Very recently, it has been hypothesized that VHb and other bacterial hemoglobin proteins might provide a means for the detoxification of reactive nitrogen, e.g NO and related compounds, and presumably also reactive oxygen species (12, 16, 24).

Holmberg et al. (13) have also expressed VHb in tobacco plants and observed faster germination rates, higher yield on plant material, enhanced chlorophyll content, and a shift in the secondary metabolite production from anabasine towards nicotine, the synthesis of which is more O_2-demanding. Furthermore, a six-fold increase in the scopolamine production was reported upon transformation of *Datura innoxia* with VHb (7). Similar positive effects on growth were recently observed upon expression of VHb in suspension cultured tobacco cells, where a

shortened lag-phase and increased final cell dry weight for VHb-expressing cells relative to transformed control cells were reported (11). It is not known how VHb is promoting growth and affecting other cellular functions of the plant metabolism but it has been proposed that VHb may contribute to a more stable oxygen levels in the plant cells. However, other functions, such as scavenging oxygen radicals, functioning as terminal oxidase, inhibiting the oxidation of unsaturated fatty acids in seeds have also been proposed (7).

The positive results reported prompted us to test the VHb-technology in trees. The aim of our study was to generate transgenic VHb-positive lines and to analyze VHb-expression in a fast growing hybrid aspen (*P. tremula x tremuloides)* having industrial significance. Hybrid aspen as a long-living tree is also exposed to different environmental conditions both during the whole life-span as well as during the changing season. Thus, we focused our efforts to study the effect of different cultivation and stress conditions such as UV-B illumination on VHb-expression. Simultaneously we followed growth characteristics, anatomic features, and changes in secondary metabolites to find out possible mechanisms of VHb-action in hybrid aspen.

Materials and methods

Plant material

Apical and axillary buds derived from suckers of the selected hybrid aspen plus trees (*Populus tremula* x *tremuloides*), V613, V617, and V619 growing in southern Finland (61°48'N, 28°22'E and 61°48'N, 29°17'E) were used as explant material. Multiplication of bud material was performed according to Ryynänen (26) by using modified MS medium (22) under 16:8 h light/dark photoperiod with the light intensity (80-115 μmol m^{-2} s^{-1}), at 22°C.

Gene constructs

Disarmed *Agrobacterium tumefaciens* strain LBA 4404 (pBVHb) was used for genetic transformation of hybrid aspen. The *vhb* gene has originally been isolated by Khosla and Bailey (17). The plasmid pBVHb, a derivative of pBI121 (Clontech), includes the *vhb* gene driven by 35S CaMV promoter and the neomycin phosphotransferase (*npt2*) gene under control of nopaline synthetase (*nos*) promoter as selectable marker. The construction of pBVHb has been described in Farrés and Kallio (11) and the correctness of amplified *vhb* gene was verified as described (4). The plasmid construct is identical with the VHb-expression vector pBH4, which has been used in tobacco transformation by Holmberg and co-workers (13).

Production of transgenic plantlets

Wounded shoot and leaf pieces representing clones V613, V617, and V619 were precultured on modified MS medium designed for callus-production with 0.5 μM BA and 4 μM 2,4-D for three days before co-cultivation. Single bacterial colonies were grown overnight with shaking (200 rpm) in Luria Broth (LB) medium (21), pH 5.6, containing 100 μM acetosyringone. Overnight culture was diluted with modified MS medium 1:50 to an absorbance of 1 ± 0.2 at 550 nm. Precultured shoot and leaf pieces were soaked in diluted bacterial solution with gentle shaking for 30 min at room temperature, blotted dry on filter paper, and returned onto the callus production medium for two days. After cocultivation the explants, if overgrown with bacteria, were rinsed with 500 mg/l cefotaxime before being transferred to the callus production media including 50 mg/l kanamycin and 300 mg/l cefotaxime.

Elimination of *Agrobacterium* was verified by polymerase chain reaction (PCR) and regeneration of plantlets was started on modified MS medium supplemented with 0.1 μM TDZ and 50 mg/l kanamycin. From each regenerating callus one shoot was chosen to be multiplicated on MS medium supplemented with 2.22 μM BA and 2.85 μM IAA i.e. to start an individual line. Rooting of shoots was performed on growth

regulator-free MS medium. The rooted plantlets were transferred to soil and grown in a greenhouse.

Analysis of the transformed lines

PCR was used for preliminary testing the different hybrid aspen lines for the presence of *npt*2 and *vhb* genes. For PCR analyses 150 mg callus or *in vitro* shoot samples were taken and genomic DNA was isolated as described Doyle and Doyle (10), with minor modifications according to Aronen and Häggman (2). The primers used to amplify a 619 bp fragment of the *npt*2 gene were 5′-TGGGCACAACAGACAATCGG-3′ and 5′-CAGCAATATCACGGGTAGCC-3′, and the primers specific for a 437 bp fragment of the vhb were 5′-ATGTTAGACCAGCAAACCATTACC-3′ and 5′-TCAACCGCTTGAGCGTACAAATCT-3′. Genomic DNA isolated both from the plantlets of transformed and nontransformed lines (i.e. negative controls) were used as templates (0.5-1.0 μg) in the PCR as described by Aronen and Häggman (2). Furhermore, the absence of *Agrobacterium* in the regenerated material was confirmed using primer pair (5'-CACTCTTCGGAAGTAGTGGG-3', 5'-AAGCTATGCCGAACATCGGG-3'), specific for a 716 bp fragment of the *occ* region (bases 1315-2932) of the bacterial Ti-plasmid (32).

Genomic DNA for Southern hybridization was isolated from the leaves of the greenhouse-grown plants using the method of Lodhi et al. (19) further modified and described in detail by Valjakka et al. (30). Finally, the precipitated DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and treated with RNase. Genomic DNA samples from the nontransformed control lines and lines transformed with pBVHb were digested with *Hin*dIII. Twenty-μg DNA samples were separated on a 0.8% (w/v) agarose gel by standard procedures (27) and transferred to a nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany) by capillary transfer. Double stranded probes of *npt*2 (619 bp) and *vhb* (437 bp) were labeled with digoxigenin-11-dUTP. Prehybridisation and

hybridisation were performed in Easy Hyb solution (Boehringer Mannheim) at 42 °C with both *vhb* and *npt2* probes. After hybridisation, the membranes were washed twice for 5 min with 2x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.1% SDS at room temperature, and twice at 68°C for 15 min with 0.5x SSC containing 0.1% SDS. The digoxigenin-labeled hybridisation products were detected according to the manufacturer's instructions.

Expression of *vhb* gene was studied with Western blot analysis as described (13). Briefly, 200 mg leaf material was homogenized in 0.1 M NaCl, 0.2 mM EDTA (pH 8.0), 1 mM β-mercaptoethanol, 1 mM PMSF, 2% PVP and 50 mM Tris buffer at pH 8.0. The homogenates were centrifuged twice at 14000 rpm for 15 minutes. The protein concentration of the supernatants was measured by the method of Bradford (5) using Bio-Rad reagent and bovine serum albumine as standard. The proteins were separated on 15% SDS-PAGE gels and blotted onto Immun-Blot PVDF membrane supplied by Bio-Rad (Hercules, CA). The electrophoresis and blotting were performed as described by Laemmli (18) and Sambrook et al. (27), respectively. The VHb monomers were detected by VHb antiserum generated in rabbit (11), and visualised by HRP-conjugated goat antirabbit immunoglobulins (Bio-Rad, Hercules, CA). Either ECL-based (Amersham Pharmacia Biotech, Buckinghamshire, UK) or ChemiGlown-based (Finnzymes, Espoo, Finland) western blotting detection systems were used according to the manufacturer's instructions.

Chlorophyll and protein assays

For chlorophyll and protein measurements 100 mg frozen sample from the uppermost full-size leaves were homogenized in liquid nitrogen and diluted in 5 ml of extraction buffer (50 mM MES, 20 mM $MgCl_2$, 50 mM β-MeOH, 1% TWEEN 80, pH 6.8). 2 x 200 µl of the extract was used for chlorophyll assay according to Porra et al. (25). The rest of the extract was centrifuged (20'800 g, 4°C, 5 min) and 2 x 200 µl of the supernatant was used for soluble protein assay as described by Bradford (5). The amounts of chlorophylls and protein were measured using UV-2401 PC

Shimadzu spectrophotometer and the amounts of chlorophylls *a* and *b* were calculated according to Arnon (1).

Microscopy and morphometric analyses

For light and electron microscopic studies samples of 0.5 mm x 1 mm were cut from the middle of fully expanded leaves outside the mid vein, and fixed for 4 hours in 6% glutaraldehyde in 0.05 M sodium phosphate buffer containing 1 mM $MgCl_2$, pH 7.2, rinsed in the same buffer, postfixed in 1% OsO_4 in 0.1 M sodium phosphate buffer, dehydrated in ethanol and embedded in Epon (modified from (23)). 1.0 μm thick sections were stained with toluidine blue, and photographed with a Leitz Dialux 22 microscope. Ultrathin sections were cut at random block orientations, stained with uranyl acetate and lead citrate, and recorded at electron optical magnification of 10 000 with a Philips 208 transmission electron microscope.

Relative cell area and chloroplast number per relative cell area in palisade parenchyma layers of leaf mesophyll cells representing both control lines and transgenic hybrid aspen lines were measured from transverse sections using Olympus BK51 microscope and Image-Pro Plus 1.4 analysis program. Randomly chosen areas of mesophyll cells were used to analyze the proportions of mitochondria, chloroplasts, starch and plastoglobuli in cytoplasm of control lines and transgenic lines. For determination of total cytoplasm and chloroplast volumes a grid with lines spaced at 20 mm, corresponding to 0,667 μm at the specimen level, was placed over electron micrographs magnified to 30'000X (33). A square grid with lines spaced at 4 mm, corresponding to 0.133/μm at the specimen level, was used for morphometric analyses of plastoglobuli and mitochondria. Four to 12 micrographs from each of three or four samples of transgenic lines as well as control lines were analyzed. The total cytoplasm area analyzed was 2020/μm^2 for controls and 1400 / μm^2 for the transgenic lines.

Secondary metabolites

Phenolics were extracted from 5-10 mg of dried leaf material in Eppendorf tubes on ice by adding 0.6 ml of 100% cold methanol and homogenizing with Ultra-Turrax homogeniser with maximum speed for 30 s. The homogenate was allowed to stand on ice for 15 min, after which homogenization with Ultra-Turrax for 30 s was repeated and the homogenate was centrifuged for 3 min at 13'000 rpm. Supernatant was transferred immediately after centrifugation into 6 ml glass-vial and the previous extraction step with 0.6 ml methanol was repeated 3 more times. The extracts were combined, methanol was evaporated under nitrogen and finally the samples were stored at -20°C until analyzed.

Table 1. The conditions used in HPLC gradient elution for phenolics.

Time (min)	Solvent A (%)[a)]	Solvent B (%)
0	100	0
5	100	0
10	80	20
20	70	30
30	65	35
40	50	50
45	50	50
Rinsing	0	100
Equilibration	100	0

[a)] Aqueous 1.5% tetrahydrofuran+ 0.25% orthophosphoric acid in DAD and acetic acid in MS
[b)] 100% methanol

For HPLC analyses the samples were dissolved in methanol:H_2O (1:1) and then analyzed by DAD-HPLC. Retention time, UV-spectrum and HPLC/API-ES (positive ions) (14) was used to identify the components. The gradient used in HPLC is described in Table 1. The LC-quantification of the components was based on commercial standards or purified components. Salicin, salicortin, tremulacin, (+)-catechin, chlorogenic acid based on standards; Myricetins based on myricitrin, quercetins on hyperin, kaempferols on kaempferol-3-rhamnoside, cinnamic acids on chlorogenic acid, and neochlorogenic acid on chlorogenic acid.

Greenhouse experiments

Greenhouse experiments were established to study stable integration and expression of the *vhb* gene and its effect on growth characteristics, anatomy, morphology or production of secondary compounds during different cultivation conditions. Before the initiation of the experiments the *in vitro* plantlets were transferred into a mixture of an unfertililized and Ca-free peat (Kekkilä, Finland), perlite and forest soil (2:2:1) in 0.14 l pots and acclimatized at high humidity for two weeks.

The first greenhouse experiment

The first greenhouse experiment was designed to confirm stable integration of transgenes by Southern hybridizations and to study VHb-expression by Western blot analyses in transgenic aspen lines. Also morphology, fresh and dry weight and growth characteristics were examined. After acclimatization period in high humidity the plants were planted into 0.5 l pots and daily fertilization with commercial 0.2 % Superex fertilizer (Kekkilä, Finland) lasting for one month was started one month after transfer to greenhouse. Thereafter fertilization was decreased to take place every third day until the end of the experiment. In this experiment photoperiod was maintained for 16 h with SON-T AGRO 400 W Na-lamps (Philips AB). Average temperature was 20°C at day and 18°C at night. Relative humidity was more than 40%.

For the experiment only the transgenic lines with five or more plants were included. Thus, the experimental design consisted of 274 plantlets, which represented all three hybrid aspen clones and 15 VHb-expressing lines (7 lines from clone V613, 7 from clone V617 and 1 from clone V619) and 50 control plants (17 lines from clone V613, 19 from clone V617 and 14 from clone V619). The plants were divided into two randomly assigned blocks. The replicates were surrounded by extra hybrid aspens of the same age to equalize light conditions for all plants.

The experiment started on October 6 and it was finished on November 17. At the beginning of the experiment five plants from all VHb-expressing and control lines in both replica were randomly selected for regular (October 6 and 20, November 2 and 17) height and diameter measurements. Leaf samples, at least three samples per VHb or control line, for chlorophyll and protein assays were collected on November 2. Leaf samples (one full size leaf from 4 plants per line per replicate) for Western blot analyses were taken on November 2. Southern blot analyses, fresh weight and dry weight measurements of leaves, stems and roots, the number of side twigs and leaf area were analyzed from the samples collected on November 17. For leaf area analysis 6 leaves per plant and 5 plants per transgenic or control line were measured using a video camera connected to a graphic TV monitor.

The second greenhouse experiment

The second greenhouse experiment was designed to follow VHb-expression and secondary metabolism during the growing season and to study anatomy. Also fresh and dry weight and growth characteristics were examined. In this experiment the plants were fertilized with 0.2% 5-Superex commercial fertilizer (Kekkilä, Finland) twice at the end of the one-month-acclimatization period and then planted in commercial fertilized peat (Kasper, Kemira Agro Oy, Finland) 5 kg/m^3 including 9% N, 3,5% P, 5% K, 3.8% S, 4.8% Mg, 0.04% B, 0.08% Mn, 0.05% Cu, 0,05% Fe, 0.04% Zn, and 0.015% Mo. Photoperiod was maintained for 16 h with HPI-T 400 W lamps (Philips AB). Average temperature was 22°C at day and 18°C at night. Relative humidity was more than 40%.

For this experiment we selected four VHb-lines 3, 5, 18 and 28 representing clone V613 and four lines 45, 51, 60 and 62 representing clone V617. These lines showed the best growth characteristics during the first greenhouse experiment. Control lines representing both V613 and V617 clones were also included. Each line was represented by 48 individual plants and they were divided into four randomly assigned blocks.

The second greenhouse experiment was started 1st of August and it was finished 13th of October. At the beginning of the experiment five plantlets from all VHb-lines and controls in four replica were randomly selected for regular (August 14 and 28, September 11 and 25, October 10) height and diameter measurements. For VHb-expression studies, leaf samples (one full size leaf from 4 plants per line per replicate) were collected thrice on 11th of September, 25th of September and 10th of October. For secondary compound as well as for Western blot analysis samples were collected on days August 14, September 11 and October 10. For anatomical studies leaf samples were collected from four plants randomly selected from each of the four replicates representing VHb-lines V613/5 and V617/60 as well as from control lines V613 and V617. Two fully developed leaves in the upper part of the stem per plant were sampled.

UV-B experiment in the greenhouse

The aim of the experiment was to study the stress effect caused by UV-B illumination and its effect on VHb-expression, growth, dry matter content and secondary metabolism. At the beginning of the experiment, when the buds had not flushed yet and the elongation growth had not started, the plants were planted in commercial fertilized peat (Kasper, Kemira Agro Oy, Finland) as in the second greenhouse experiment. Half of the plants were at first acclimatized for one week on UV-B illumination 1.8 kJ m^{-2} per day and then for subsequent three weeks on ambient UV-B illumination level 3.6 kJ m^{-2} per day. The other half of the plants were at first acclimatized for one week on UV-B illumination 3.6 kJ m^{-2} per day and then for subsequent three weeks on elevated UV-B illumination level 7.2 kJ m^{-2} per day. The ambient UV-B level selected corresponds with the natural, local UV-B illumination level at the end of June. The radiation was provided by Phillips UV-B lamps, which were covered with cellulose-diacete film (0.115 mm, FilmSales Ldt, London, UK) in order to remove UV-C radiation emitted by the lamps. Because the UV-B lamps emitted some UV-A radiation a polyester film (0.125 mm, FilmSales

Ltd) was used to give to both the control and UV-B plants the same amount of UV-A over the experiments.

The one-year-old plants from the second greenhouse experiment were used for this experiment, i.e. VHb-lines 3, 5, 18 and 28 representing clone V613, lines 45, 51, 60 and 62 representing clone V617 and control lines from V613 and V617 clones. Each line was represented by 10 individual plants and they were divided into two randomly assigned blocks. This experiment was established 10th of April and it lasted for one month ending up on 7th of May. Leaf samples derived from the top and from the middle of the plant for Western blot analyses were collected and elongation and diameter of all plants were measured on April 23 and May 7. Leaf samples for secondary compound analyses and leaf, stem and root samples for fresh and dry weight analyses were collected at the end of the experiment May 7.

Statistical analyses

Statistical comparisons among data from growth measurements, fresh and dry weights, chlorophyll and protein amounts, amount of secondary compounds as well as relative cell area and chloroplast number per relative cell area were made by analysis of variance, and the means were compared by the Student-Neuman-Keuls multiple range test. Proportions of mitochondria, chloroplasts, starch and plastoglobuli were analyzed using Student's t-test.

Results

Production of transgenic lines

Hybrid aspen clones differed in their competency for genetic transformation. In the case of clones V613, V617 and V619 we produced altogether 14, 24 and 4 independent, PCR positive, and regeneration capable lines, respectively. From these lines two turned out to be partly escapes, including only the selectable marker gene *npt*2. The escape lines were omitted for Southern blot analyses and subsequent

greenhouse experiments and the lines with good regeneration ability were selected only. Stable integration of both *npt2* and *vhb* genes was confirmed in 12, 8, and 1 lines representing clones V617, V613 and V619, respectively (Figure 1). The copy number of transgenes varied from 1 to 4 (Figure 1).

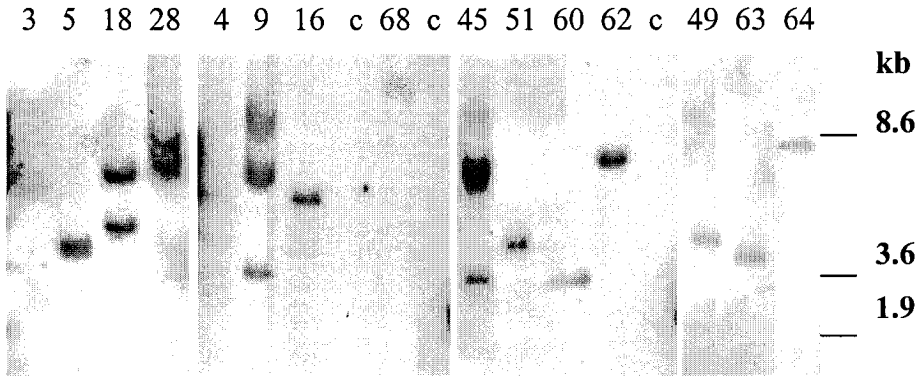
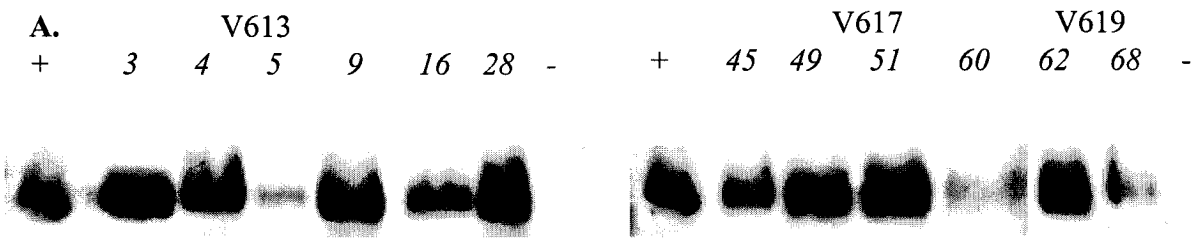


Figure 1. Southern analysis of pBVHb transformed hybrid aspen (*Populus tremula x tremuloides)* lines with a *vhb* probe. Lines numbered 3-16 represent hybrid aspen clone V613, line 68 represents clone V619 and lines 45-64 represent clone V617. C = non-transformed control plants from left to right representing clones V613, V617, and V619.

Characterization of VHb-lines in the first greenhouse experiment

Western blot analyses were performed four weeks after the initiation of the first greenhouse experiment at the time when all lines were still growing. According to the Western blot analyses all tested plants (three randomly chosen plants / line) were expressing VHb and the expression level was line dependent (Figure 2A).



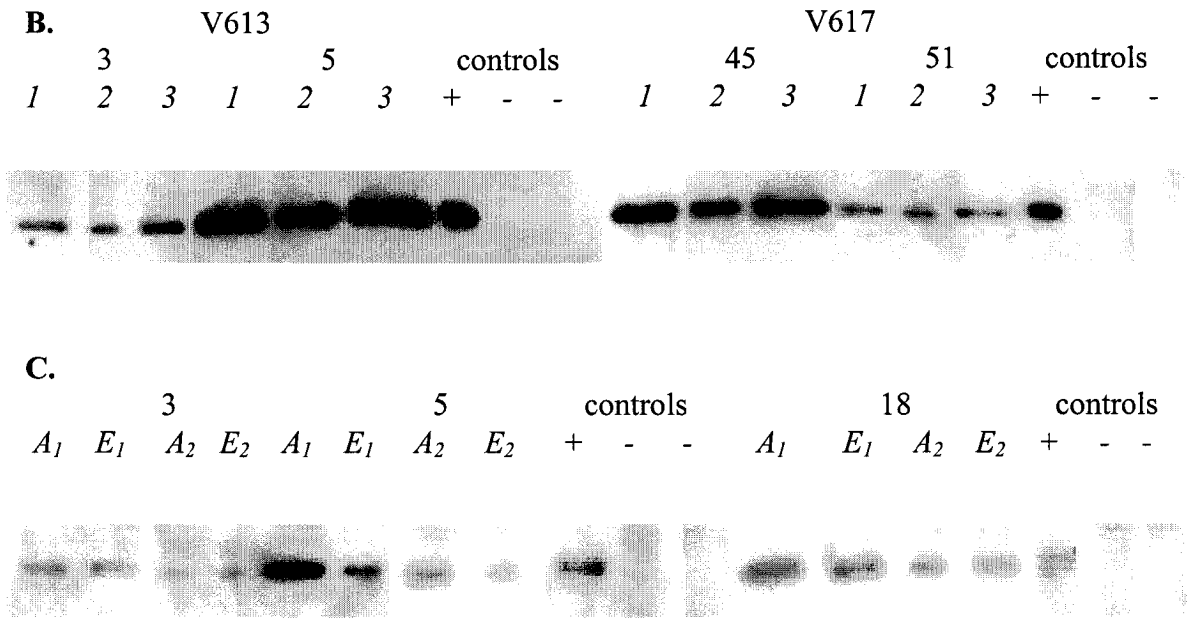
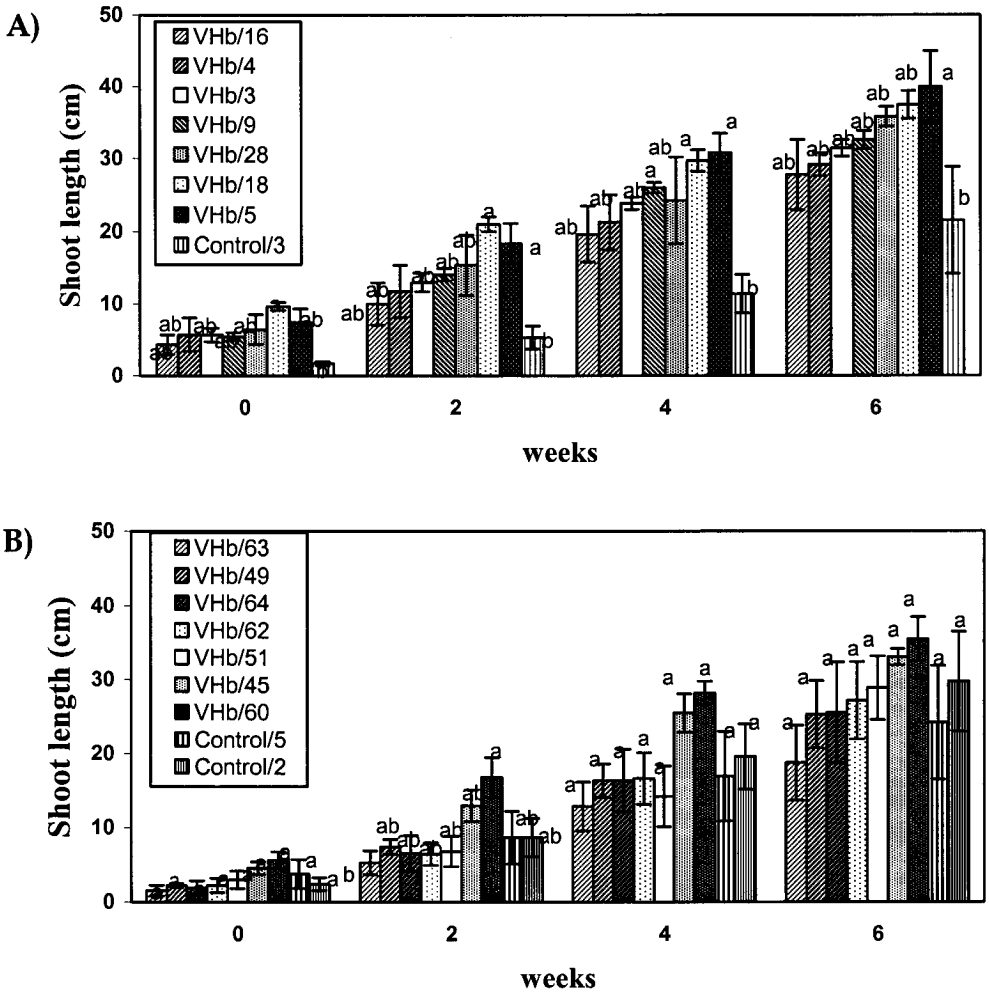


Figure 2. Western blot analyses indicate that all tested transgenic VHb-lines are able to express VHb protein. **A.** VHb expression was studied 4 weeks after the initiation of the first greenhouse experiment at the time when the plants were still growing. VHb-expression turned out to be line dependent. **B.** VHb-expression was followed in the greenhouse grown plants throughout the growing season during the second greenhouse experiment. Samples were taken thrice *1* = 12.9., *2* = 26.9. and *3* = 10.10. **C.** Also the effect of UV-B illumination on VHb expression was studied. Half of the plants were at first acclimatized for one week on UV-B illumination 1.8 kJ m^{-2} per day and then for subsequent three weeks on ambient (A) UV-B illumination level 3.6 kJ m^{-2} per day. The other half of the plants were at first acclimatized for one week on UV-B illumination 3.6 kJ m^{-2} per day and then for subsequent three weeks on elevated (E) UV-B illumination level 7.2 kJ m^{-2} per day. 1 = mature leaf close to the top of the plant, 2 = mature leaf located in the middle of the plant.

When the elongation growth of the lines were followed the ranking of the tested lines at the end of the experiment in clone V613 was the following: 5>18>28>9>3>4>16>control. The ranking in clone V617 was 60>45>control>2>51>62>64>49>control>5>63. The significant differences found are presented in Figure 3. At clonal level the plants of V617 were shorter ($p < 0.05$) and thinner ($p < 0.05$) than the other clones. There was no statistical difference in stem diameter within the clones.

At the end of the experiment, fresh and dry weight measurements indicated that leaf, shoot and root fresh weights and the corresponding dry weights were statistically lower ($p < 0.05$) in clone V617 than in the other clones. Generally, VHb-expressing lines had higher dry weight and fresh weight values relative to control lines but the difference was significant only in the case of clone V613 ($p < 0.05$). No significant differences were found in chlorophyll content or in the amount of soluble proteins, in the number of leaves or in the number of side branches. In leaf area measurements in clone V617 and V619 no significant differences were found among lines and within clone V613 only VHb-line 3 had significantly bigger ($p < 0.05$) leaf area than in the control plants. At clonal level, leaf size was, significantly smaller ($p < 0.05$) in clone V617 than in clones V613 and V619.



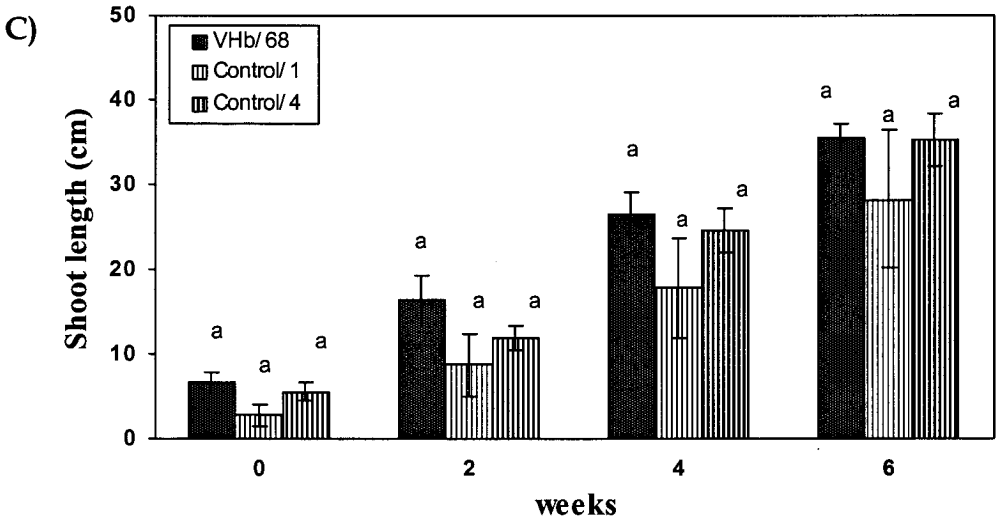
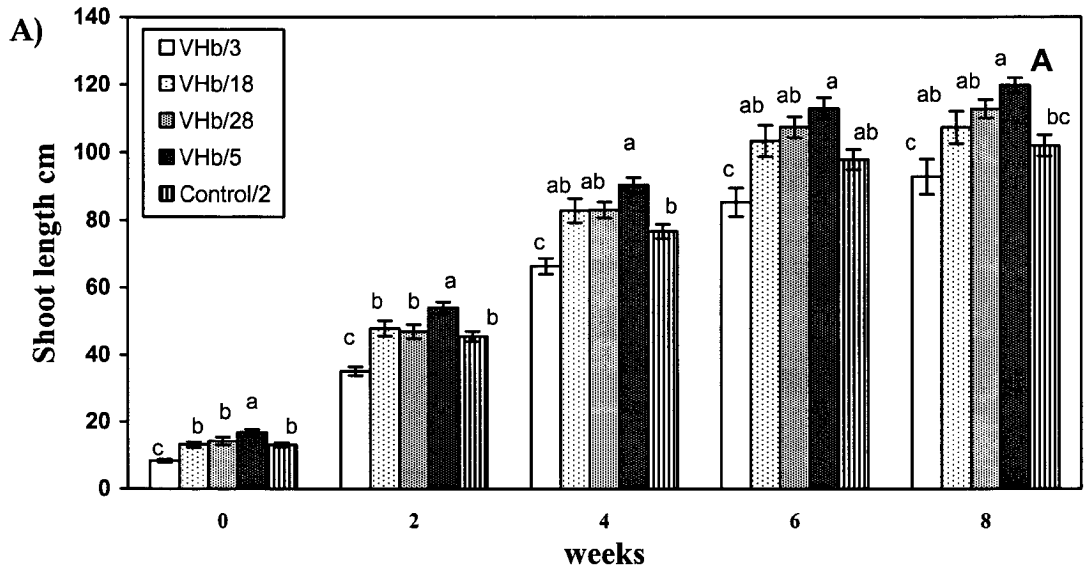


Figure 3. Increase in shoot length of transgenic VHb-lines of hybrid aspen (*P. tremula x tremuloides)* were followed and compared to controls during the first greenhouse experiment. The measurements were performed at two weeks intervals October 6 and 20, November 2 and 17. **A.** Lines of clone V613. **B.** Lines from clone V617 and **C.** lines from clone V619. Different letters indicate significant differences within the sampling time.

Second greenhouse experiment to follow VHb-expression and to study anatomy and secondary metabolism

VHb-expression was measured 4, 6 and 8 weeks after the initiation of the experiment. Expression levels did not vary between the sampling days (Figure 2B). Variation in the expression level was found within lines.



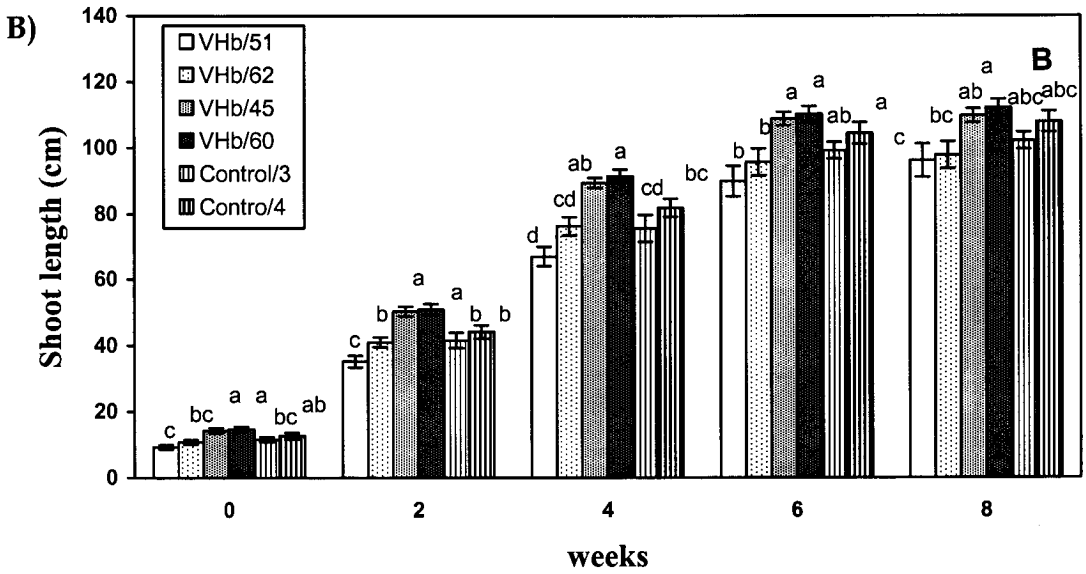


Figure 4. Increase in shoot length of transgenic VHb-lines of hybrid aspen (*P. tremula x tremuloides)* were followed and compared to controls during the second greenhouse experiment in 2000. In this experiment the cultivation conditions were more optimal than during the previous year. The measurements were performed at two weeks intervals August 14 and 28, September 11 and 25, October 10. **A.** Lines of clone V613. **B.** Lines from clone V617. Different letters indicate significant differences within the sampling time.

When the elongation growth of the lines was followed the ranking of the tested lines in clone V613 was the following 5>28>18>control>3. The corresponding ranking in clone V617 was 60>45>control 4>control 3>62>51. The significant differences found are presented in Figure 4. In this experiment when the cultivation conditions were optimal the plants were taller than in the first greenhouse experiment and no significant clonal differences in elongation growth were observed.

The concentrations of kaempferol-dervivatives as well as of phenolic acids was significantly decreasing in both clones (613, V617) during the experimental period (Figure 5). Furthermore the content of apigenins was significantly decreasing between the first two sampling dates (Figure 5). The two clones showed only minor variations in the contents of salicylates, quercetin-derivatives and total flavonoids during the experimental period and thus no general trend could be observed for these types of secondary metabolites.

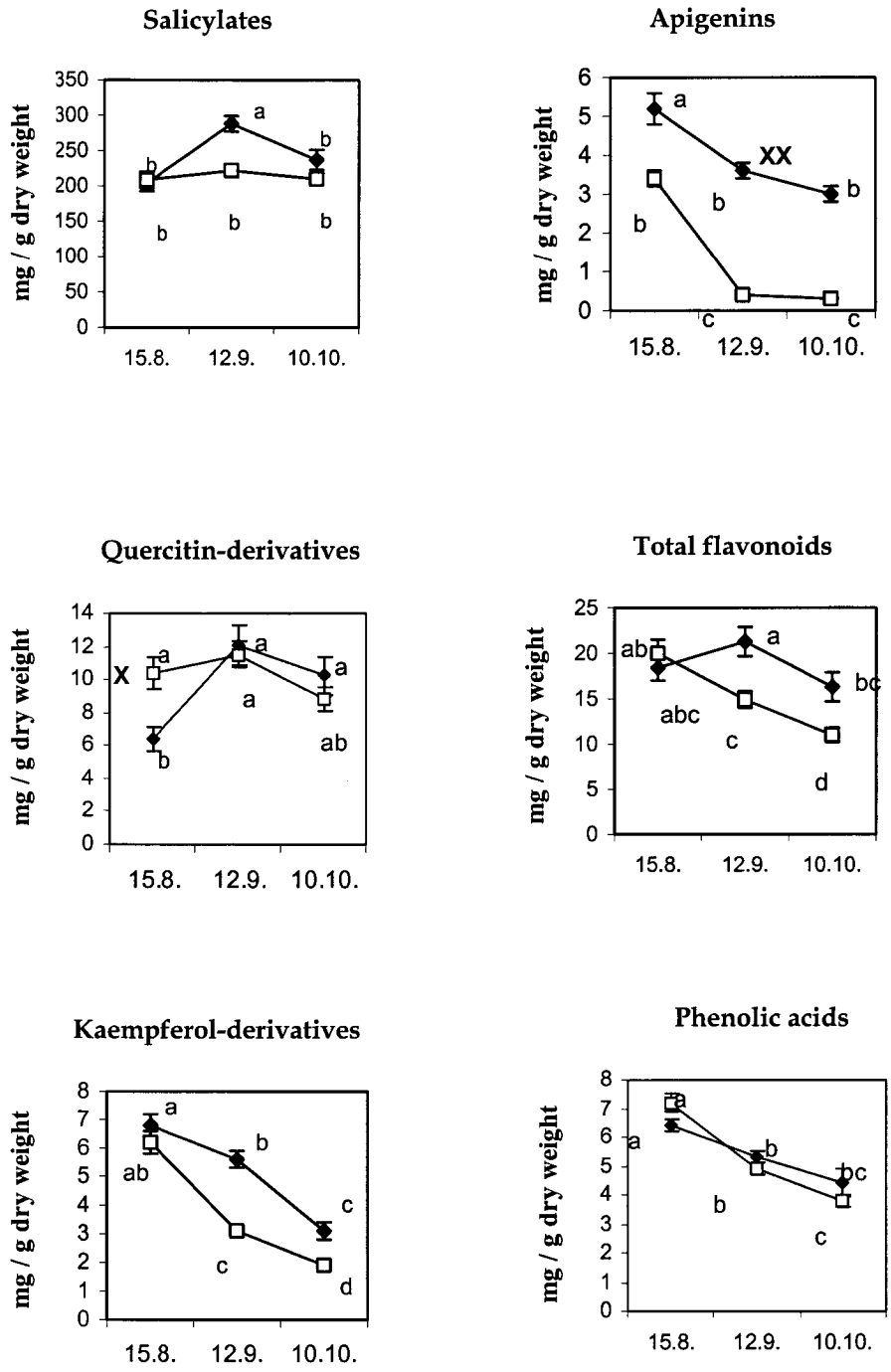


Figure 5. Intraclonal fluctuation in secondary metabolite concentrations during the experimental period of hybrid aspen (*P. tremula x tremuloides)* clones V◆13 ( ) and V617 (□). Different letters indicate significant intra- and interclonal differences ($p < 0.05$) at different sampling times. The significant differences ($p < 0.05$) found in quercetin-derivatives and in apigenins between VHb-lines and control lines are also indicated in the figure by X (V617 control line 13.5 ±1.9 mg/g dw > V617 VHB-lines 8.8 ±1.0 mg/g dw) and XX (V613 control line 4.1 ±0.4 mg/g dw> V613 VHb-lines 3.4 ±0.2 mg/g dw), respectively.

Controls and VHb-lines differed significantly ($p < 0.05$) only in the following two cases: The concentration of quercetin-derivatives was higher in V617 control lines (13.5 ± 1.9 mg/g dw) than in V617 VHb-lines (8.8 ± 1.0 mg/g dw) during the first sampling time and the concentration of apigenins was higher in V613 control line (4.1 ±0.4 mg/g dw) than in V613 VHb-lines (3.4 ± 0.2 mg/g dw) during the second sampling time.

Analysis of one-µm-sections, stained with toluidine blue, revealed generally no differences in leaf anatomy between the cross-sections of the transgenic VHb-line leaves and control leaves. Beneath the upper epidermal cells, where the cells were larger than in the lower epidermis, there was two-cell-layer-palisade parenchyma. The cells below the epidermis were larger and formed a more compact layer than the lower cells. However, in VHb-lines cell size in palisade parenchyma was smaller than in controls ($p < 0.05$). Because the leaf size was similar in both transgenic lines and controls this indicates that the total number of cells was higher in VHb lines. Chloroplast number was higher in the inner palisade parenchyma cell layer than in the outer one ($p < 0.05$) but significant differences between VHb-lines and control lines could not be found. About half of the leaf cross section was occupied by a loose spongy parenchyma of small cells and conspicuous intercellular spaces.

Table 2. Morphometric determinations of volumes of mitochondria, peroxisomes, chloroplasts, starch and plastoglobuli for control and VHb-lines of hybrid aspen.

Material	n[a)]	Percentage of cytoplasm			Percentage of chloroplasts	
		Mitochondria[b)]	Peroxisomes	Chloroplasts[b)]	Starch[c)]	Plastoglobuli[c)]
Control-lines	68	2.61 ± 0.39	0.64 ± 0.21	68.5 ± 2.47	11.6 ± 3.18	0.94 ± 0.14
VHb-lines	51	3.18 ± 0.59	0.42 ± 0.19	68.6 ± 1.97	20.4 ± 4.28*	1.10 ± 0.12

[a)] n = number of measured electron micrographs taken from two different control lines and VHb lines, each line containing 3 or 4 different plants. * = $P < 0.05$.
[b)] Mitochondria and chloroplasts are expressed as means ± SE of total cytoplasmic volume.
[c)] Starch and plastoglobuli are presented as means ± SE of the corresponding chloroplast volume.

In depth studies on ultrastructural morphology of mesophyll cells revealed some interesting differences between VHb and control lines. Mesophyll cells had a single, large central vacuole that was surrounded by a thin cytoplasm. Chloroplasts occupied the largest portion of the cytoplasm and morphometric determinations showed that it was almost exactly as large in transgenic and in the control leaf cells (Table 2). However, the volume of starch in the VHb-line chloroplasts was significantly ($P<0.05$) bigger than in control chloroplasts. Furthermore, individual starch grains also appeared to be relatively large in VHb-lines when compared to those ones in control lines (Figure 6). Osmiophilic plastoglobuli were also bigger and covered a larger volume in VHb-line chloroplasts than in control chloroplasts, but these differences were not significant. The percentage volume of mitochondria was larger in VHb-lines than in controls and the corresponding value of peroxisomes was higher in controls than in VHb-lines, but these differences were not significant. Only occasionally Golgi bodies as well as lipid droplets were visible in the cytoplasm. RER was present only as single cisternae. No differences were observed in the occurrence of these organelles between different lines.

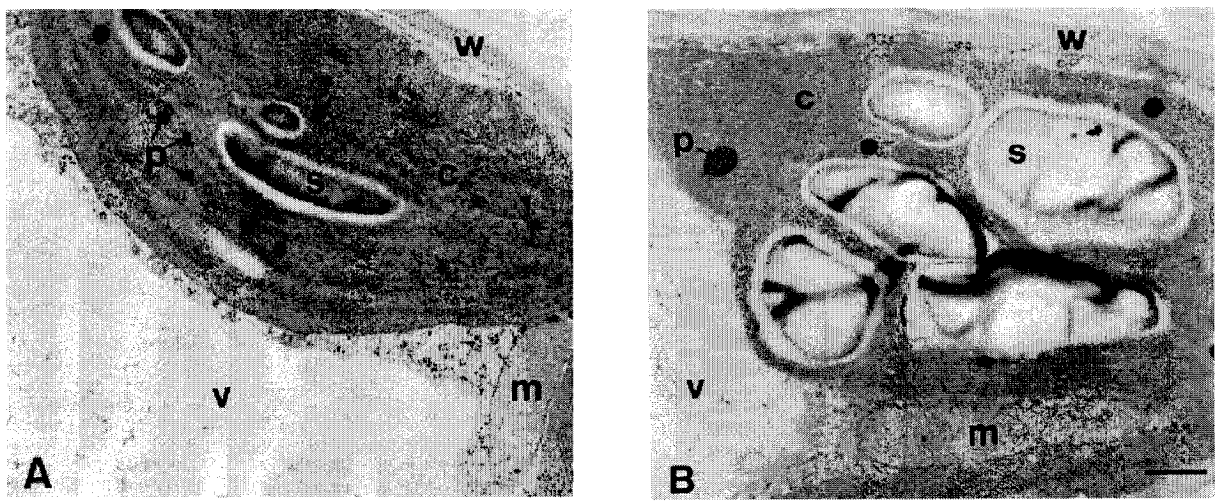


Figure 6. Transmission electron micrographs of mesophyll cells of hybrid aspen (*P. tremula x tremuloides*). Non-transgenic control line has several small starch grains in a chloroplast (A) while in transgenic VHb-line chloroplast starch grains are relatively large (B). Mitochondria are rich in cristae. c, chloroplast; m, mitochondria; p, plastoglobulus; s, starch; v, vacuole; w, cell wall. Bar = 0.5 μm.

Effect of UV-B illumination

Transgenic lines both in ambient and elevated UV-B illumination showed consistent VHb-expression (Fig. 2C). The same consistent expression level was found regardless of the position of the explant leaf, i. e. near the top of the plant or in the middle of the plant. Variation in the expression levels was dependent on the transgenic line (Fig. 2C). For instance, VHb-expression in line V613/3 was in most of the analyses weak whereas the expression in V617/45 was mostly good.

Table 3. Significant differences found in the contents of secondary compounds in leaves of transgenic VHb and of non-transgenic hybrid aspen cultivated under ambient and elevated UV-B illumination[a)]

Secondary compound groups	Growth conditions	
	Ambient UV-B	Elevated UV-B
Apigenins	none	none
Catechins	none	none
Condensed tannins	none	none
Kaempferol-derivates	none	V617/45: 1,7 ± 0,1[b)] V617/60: 1,4 ± 0,1[c)] V617/control: 0,5 ± 0,1 V617/control: 0,5 ± 0,1 V617/62: 0,7 ± 0,1 V617/62: 0,7 ± 0,1 V617/control: 0,7 ± 0,1 V617/control: 0,7 ± 0,1
Myricetin-derivates	none	V617/45: 0,8 ± 0,2 V617/60: 0,6 ± 0,1 V617/control: 0,1 ± 0.0 V617/control: 0,1 ± 0.0 V617/control: 0,2 ± 0,1 V617/control: 0,2 ± 0,1 V617/62: 0,2 ± 0,0 V617/62: 0,2 ± 0,0 V617/51: 0,3 ± 0,1 V617/51: 0,3 ± 0,1
Phenolic acids	V617/62: 8,5 ± 1,3 V617/45: 4,1 ± 0,8 V617/51: 3,5 ± 0,5	none
Quercetin-derivates	V613/5: 9,5 ± 1,6 V613/3: 3,0 ± 0,4	V617/45: 10,9 ± 1,5 V617/60: 8,8 ± 0,7 V617/control: 3,2 ± 0,3 V617/control: 3,2 ± 0,3 V617/control: 5,7 ± 0,9 V617/62: 4,1 ± 0,3 V617/51: 5,4 ± 1,2 V617/62: 4,1 ± 0,3
Salicylates	V613/18: 111,7 ± 17,4 V613/5: 231,7 ± 15,2 V613/28: 248,0± 24,0	none
Total flavonoids	V613/3: 3,9 ± 0,5 V613/5: 11, 7 ± 1,9	V617/45: 13,4 ± 1,8 V617/60: 10,9 ± 0,9 V617/control: 3,8 ± 0,3 V617/control: 3,8 ± 0,3 V617/62: 5,1 ± 0,4 V617/62: 5,1 ± 0,4 V617/51: 6,7 ± 1,4 V617/control: 6,7 ± 1.1

[a)] Significant differences in experimental groups display $P < 0.05$.
[b)] Groups of lines, within which statistically significant differences have been determined.
[c)] Concentration of secondary metabolites is given in mg/g dry weight, means and standard error are given.

When considering the growth data within the treatment only in elevated UV-B illumination the line V617/45 (22.8 ± 3.4 cm) grew significantly ($p < 0.05$) better than one control line (9.9 ± 2.9 cm) and this was also the only significant case when all lines in both treatments were analysed. In fresh and dry weight percentages of leaf and shoot samples there was no statistical differences either within the treatment or between the treatments.

In UV-B illumination, however, VHb-lines responded individually. In ambient UV-B especially V613/5 VHb-line had more salicylates, quercetin-derivatives and total flavonoids than VHb-lines V613/18, V613/3 and V613/3, respectively (Table 3) but the values did not differ from the controls. In elevated UV-B illumination significant differences were found only in lines of the clone V617. VHb lines V617/45 and V617/60 had generally more quercetin-derivatives, kaempferol-derivatives, myricetin-derivatives and total flavonoids than the controls or other V617 VHb lines (Table 3).

Discussion

In this paper we are for the first time describing *Vitreoscilla* hemoglobin (VHb) expression in an economically important boreal woody plant hybrid aspen (*Populus tremula x tremuloides*). This far VHb has been mainly expressed in unicellular organisms of both prokaryotic and eukaryotic origin (15). Holmberg et al. (13) generated *vhb* carrying tobacco plants showing reduced germination time, increased growth rates and content of dry matter. In addition, higher production of nicotine with a concomitant decrease in anabasine content is detected in VHb-positive tobacco plant relative to non-transformed control plants. Furthermore, improved growth of suspension cultures derived from VHb-expressing tobacco plants relative to transformed control cultures has been reported very recently (11).

We were able to generate several transgenic lines with slightly varying copy numbers but no clear dependence between copy numbers and VHb-expression

levels could be found. VHb-expression was stronger in lines with 3 or 4 gene copies. Due to the limited amount of this kind of lines it is not possible to predict if the copy number has direct effect on the VHb-expression level. Generally, VHb-expression was found to be dependent on the genetic background of the lines. Expression of VHb did not vary significantly according to the sampling day or leaf position in our experiments. In addition, we could not find any clear correlation between elongation growth and the amount of VHb expressed.

Some significant differences in elongation growth were recorded between transgenic lines (Figure 3). However, no general growth improvements were observed in VHb-expressing lines relative to control lines. This finding is in clear contradiction to the results of Holmberg et al. (13) showing growth promoting effects in VHb-expressing tobacco. Our results revealed that some transgenic lines show highly improved growth characteristics relative to control lines. However, we cannot exclude the possibility that the effect of transgene expression is dependent either on integrational position of the *vhb* gene or on the genotype of the various hybrid aspen lines.

The effect of different cultivation conditions, i. e. 1st and 2nd greenhouse experiment, on growth characteristics of transgenic lines was, however, obvious. Differences in size, i. e. within a single line as well as between the different lines from the identical clonal backgroud, are markedly more prominent in the first greenhouse experiment relative to the 2nd experiment, which was conducted under more elaborate growth conditions. For the second greenhouse experiment we optimized the cultivation conditions using pre-fertilized peat and HPI-T multimetal lamps instead of regular fertilization and Na-lamps used in the first greenhouse experiment. Furthermore, average temperature was increased 2°C during the light period. The shoot length at the end of the second growing season was almost doubled relative to the first experiment. One might speculate that under the optimized culture conditions the effects of VHb-expression are not observable, whereas under less permissive growth conditions differences would become more obvious. Our unpublished

results clearly show that the effect of VHb-expression on the growth properties of homozygous VHb-positive tobacco plants are only obvious under various stress conditions such as reduced temperature, oxidative and nitrosative stress, whereas under optimized conditions, in contradiction to the work of Holmberg et al. (13), no statistically significant differences could be observed (Frey, A.D. and Kallio P.T., unpublished). In addition, no differences in other characteristics, such as chlorophyll and protein content were obvious in our experiments (data not shown). Clearly, these findings are in contradiction to the previous VHb work in tobacco where an increase in chlorophyll content has been reported (13).

UV-B illumination is known to induce oxidative stress trough the generation of reactive oxygen species. Plants produce upon the exposure to UV-B radiation as set of protective pigments, such as flavonoids and apigenins. Since it has been hypothesized that the presence of VHb might buffer these highly reactive radicals (7), VHb-lines and control lines were exposed to UV-B radiation. In the UV-B illumination experiment with two-years old plants no significant differences in shoot length between control lines and VHb-lines could found. Analyses of other parameters such as, growth (fresh weight, dry weight) or morphological (number of leaves and side branches, leaf area) characteristics revealed only significant differences in a few cases.

When changes at the secondary metabolite levels were analyzed VHb-expressing lines did not differ from the control lines. When exposed to UV-B illumination the lines generally responded individually. In elevated UV-B illumination especially VHb lines V617/45 and V617/60 had more quercetin-derivatives, kaempferol-derivatives, myricetin-derivatives and total flavonoids than the controls or other V617 VHb-lines. However, this was not a general increase in all lines and therefore we would like to propose that VHb-lines may use different mechanisms to protect themselves against oxidative stress. It seems possible that VHb has a protective effect on the plant cell, i. .e. VHb might bind / detoxify the reactive oxygen species and thus no changes in secondary metabolite level would be induced by the UV-B illumination. Thus the level of flavonoids production is not increased significantly.

This suggestion is also supported by the recent findings that bacterial hemoglobins and flavohemoglobins are part of the stress response against oxidative stress in various microorganisms (12, 20, 34). On the other hand an increased level of flavonoids (such as kaempherols) suggests an increased protection of these transgenic lines against UV-B illumination, indicating better ability of those VHb-lines to allocate energy to protection.

We may speculate, based on ultrastructural measurements, that the higher number of peroxisomes (calculated from the total experimental cytoplasmic area) in the cytoplasm of the control materials indicate metabolic exchange between peroxisomes, mitochondria, and chloroplasts. This exchange could be triggered by higher phosphoglycolate production in chloroplasts and therefore results in higher photorespiration in control lines relative to VHb lines.

Interestingly, we were able to measure enhanced accumulation of starch in chloroplasts of VHb-lines. Higher starch content of VHb-lines could be explained either by an enhanced carbon fixation or an improved energy and carbon utilization. According to the prevailing opinion the Pi/3-phosphoglycerate ratio is the principal regulator of starch synthesis in plants and trees. This mechanism links ADP-glucose pyrophosphorylase activity with the supply of photosynthate (i.e. products of photosynthesis), because the enzyme is activated when the Calvin cycle intermediate 3-phosphoglycerate is abundant. Thus, it might be possible that the carboxylase function of Rubisco (ribulose-1,5-bisphosphate carboxylase / oxygenase) enzyme is more prominent in the VHb-lines than in the control lines (6).

Interestingly, the experiments of Barata et al. (3) and Chaparro-Giraldo et al. (8) aimed at to improve the efficiency of photosynthesizing activity by targeting the soybean leghemoglobin (Lba) to the chloroplasts. It has been hypothesized that the oxygenase reaction of Rubisco could be controlled by changing the CO_2/O_2 ratio in the chloroplasts, which thus, could prevent the wasteful photorespiration activity. By targeting the oxygen avid Lba to the chloroplast the authors assumed to impair oxyxgenase reaction and therefore improve carbon fixation rate. The results in

tobacco revealed no differences in photosynthesis, starch, sucrose and enzyme levels involved in aerobic metabolism (3). Surprisingly, the Lba-expression generates even deleterious effects on plant growth and tuber production in potato (8). Both groups suggested that the observed effects could be due to either insufficient Lba concentrations in the chloroplasts or inappropriate O_2 binding kinetics of Lba.

An improved metabolic efficiency and energy household in VHb-lines could also result in the accumulation of starch. VHb-expression has been shown to result in increased ATP levels (29). Interestingly, constitutive expression of the non-symbiotic barley hemoglobin in maize cells resulted in enhanced energy charge, determined as the total ATP level, relative to wildtype maize cells under oxygen limitation (28). The authors suggested that non-symbiotic hemoglobins accomplish this effect by promoting glycolytic flux through NADH oxidation, resulting in increased substrate-level phosphorylation.

An increased starch content as observed in the VHb-expressing lines would potentially favor the growth of hybrid aspen at non-optimal growth conditions, e. g either at the beginning or at end of the growing season or under non-optimal light conditions, where the *de novo* synthesis of carbon sources is restricted and the accumulated starch would help transgenic VHb-expressing plants to sustain cellular activities and growth.

References

1. **Arnon, D. I.** 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24:**1-15.
2. **Aronen, T., and H. *Häggman.*** 1995. Differences in *Agrobacterium* infections in silver birch and scots pine. Eur. J. Forest Pathol. **25:**197-213.
3. **Barata, R. M., A. Chaparro, and S. M. Chabegas.** 2000. Targeting of soybean leghemoglobin in tobacco chloroplasts: effects on aerobic metabolism in transgenic plants. Plant Science **155:**193-202.
4. **Bollinger, C. J., J. E. Bailey, and P. T. Kallio.** 2001. Novel hemoglobins to enhance microaerobic growth and substrate utilization in *Escherichia coli*. Biotechnol. Prog. **17:**798-808.

5. **Bradford, M. M.** 1976. Rapid and sensitive method for quantitation of microgram quantities of protein Utilizing principle of protein-dye binding. Anal. Biochem. **72:**248-254.
6. **Buchanan, B. B., W. Gruissem, and R. L. Jones.** 2001. Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville, Maryland.
7. **Bülow, L., N. Holmberg, G. Lilius, and J. E. Bailey** 1999. The metabolic effects of native and transgenic hemoglobins on plants. Trends Biotechnol. **17:**21-24.
8. **Chaparro-Giraldo, A., R. M. Barata, S. M. Chabregas, R. A. Azevedo, and M. C. Silva.** 2000. Soybean leghemoglobin targeted to potato chloroplasts influences growth and development of transgenic plants. Plant Cell Reports **19:**961-965.
9. **Chen, R. Z., and J. E. Bailey.** 1994. Energetic effect of *Vitreoscilla* hemoglobin expression in *Escherichia coli* - an online P^{31} NMR and saturation-transfer study. Biotechnol. Prog. **10:**360-364.
10. **Doyle, J. J., and J. L. Doyle.** 1990. Isolation of plant DNA from fresh tissue. BRL Focus. **12:**13-15.
11. **Farres, J., and P. T. Kallio.** 2002. Improved growth in tobacco suspension cultures expressing *Vitreoscilla* hemoglobin. Biotechnol. Prog. 18:229-233.
12. **Frey, A. D., J. Farres, C. J. T. Bollinger, and P. T. Kallio.** Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli.* submitted for publication.
13. **Holmberg, N., G. Lilius, J. E. Bailey, and L. Bülow** 1997. Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. Nature Biotechnol. **15:**244-247.
14. **Julkunen-Tiitto, R., and S. Sorsa.** 2001. Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. J. Chem. Ecol. **27:**779-789.
15. **Kallio, P. T., A. D. Frey, and J. E. Bailey.** 2001. From *Vitreoscilla* hemoglobin (VHb) to a novel class of growth stimulating hemoglobin proteins. In: Merten, O.-W., D. Mattanovich, G. Larsson, P. Neubauer, C. Land, D. Porro, J. Teixeira de Mattos, P. Postma, and J. Cole (ed.). "Recombinant protein production with prokaryotic and eukaryotic cells. A comparative view on host physiology". Kluewer Academics Publishers, Dordrecht, The Netherlands. pp. 75-87.
16. **Kaur, R., R. Pathania, V. Sharma, S. C. Mande, and K. L. Dikshit.** 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.
17. **Khosla, C., and J. E. Bailey.** 1988. The *Vitreoscilla* hemoglobin gene: molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli.* Mol Gen Genet. **214:**158-61.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature **227:**680.
19. **Lodhi, M. A., G. N. Ye, N. F. Weeden, and B. I. Reisch.** 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol. Biol. Rep. **12:**6-13.
20. **Membrillo-Hernandez, J., M. D. Coopamah, M. F. Anjum, T. M. Stevanin, A. Kelly, M. N. Hughes, and R. K. Poole** 1999. The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "nitric oxide releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. **274:**748-754.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press., Cold Spring Harbor, NY.
22. **Murashige, T., and F. Skoog.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum **15:**473.
23. **Pihakaski-Maunsbach, K., K. B. Nygaard, K. H. Jensen, and O. Rasmussen.** 1993. Cellular-changes in early development of regenerating thin cell layer-explants of rapeseed analyzed by light and electron-microscopy. Physiol. Plantarum **87:**167-176.
24. **Poole, R. K., and M. N. Hughes.** 2000. New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. **36:**775-783.
25. **Porra, R. J., W. A. Thompson, and P. E. Kriedemann.** 1989. Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll *a* and

chlorophyll *b* extracted with 4 different solvents - Verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy. Biochim. Biophys. Acta **975**:384-394.

26. **Ryynänen, L.** 1991. Micropropagation of hybrid aspen (*Populus* tremula x *tremuloides*). In: Breeding of broad-leaved trees and micropropagation of forest trees. Proceedings of the meeting of the nordic group of tree breeding in Finland 1989. pp. 61-68.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
28. **Sowa, A. W., S. M. G. Duff, P. A. Guy, and R. D. Hill.** 1998. Altering hemoglobin levels changes energy status in maize cells under hypoxia. Proc. Natl. Acad. USA **95**:10317-10321.
29. **Tsai, P. S., G. Rao, and J. E. Bailey.** 1995. Improvement of *Escherichia coli* microaerobic oxygen-metabolism by *Vitreoscilla* hemoglobin - New insights from NAD(P)H fluorescence and culture redox potential. Biotechnol. Bioeng. **47**:347-354.
30. **Tsai, P. S., M. Nageli, and J. E. Bailey.** 1996. Intracellular expression of *Vitreoscilla* hemoglobin modifies microaerobic *Escherichia coli* metabolism through elevated concentration and specific activity of cytochrome *o*. Biotechnol. Bioeng. **49**:151-160.
31. **Valjakka, M., T. Aronen, J. Kangasjarvi, E. Vapaavuori, and H. Häggman.** 2000. Genetic transformation of silver birch (*Betula pendula*) by particle bombardment. Tree Physiol. **20**:607-613.
32. **Von Lintig, J., D. Kreusch, and J. Schroder** 1994. Opine-regulated promoters and lysr-type regulators in the nopaline (Noc) and octopine (Occ) catabolic regions of Ti plasmids of *Agrobacterium tumefaciens*. J. Bacteriol. **176**:495-503.
33. **Weibel, E. R.** 1979. Stereological methods. 1. Practical methods for biological morphometry. Academic Press, NY, New York.
34. **Zhao, X. J., D. Raitt, P. V. Burke, A. S. Clewell, K. E. Kwast, and R. O. Poyton** 1996. Function and expression of flavohemoglobin in *Saccharomyces cerevisiae* - Evidence for a role in the oxidative stress response. J. Biol. Chem. **271**:25131-25138.

Conclusions and final remarks

In the last few year research on bacterial hemoglobin and flavohemoglobin has gained momentum. This protein family has been shown to be widespread in prokaryotic organisms. Surprisingly, bacterial hemoglobins and flavohemoglobins have been shown to serve other functions than pure oxygen storage and delivering tasks. Indeed they are able to perform catalytic activities and their main task might be the detoxification of NO and related reactive nitrogen species. This proposed function is dependent on a reductase system, which in the case of flavohemoglobins is integrated into the same protein.

One member out of this group of proteins, *Vitreoscilla* hemoglobin VHb, has been expressed for several years to promote growth of various heterologous hosts, which were known to lack oxygen during growth. VHb has been proposed to increase effective intracellular oxygen concentration and therefore the more energy yielding aerobic metabolic pathways would be favored over the anaerobic pathways.

However, taking into account the most recent findings, which reported NO detoxification activity of bacterial flavohemoglobin from various organisms and also of VHb, it might be necessary to adapt the model of VHb action to explain the observed effects on growth and productivity. Considering the various effects of VHb on the metabolism of *E. coli* and connecting them with the latest results reported about VHb and flavohemoglobins, it might be plausible that VHb might protect the cytochromes, as shown for *E. coli* flavohemoglobin. Instead of delivering oxygen to the respiratory chain, VHb might provide a means to detoxify NO and reactive nitrogen species. Therefore, the activity of the cytochromes would be protected from the inhibitory action of NO. By engineering a reductase domain to the C-terminus of VHb, the effect of this artificial flavohemoglobin (VHb-Red) regarding growth promotion was significantly enhanced relative to VHb. This finding might be an indication that the growth promoting effect of VHb / VHb-Red is dependent on a catalytic reaction, such as the NO detoxification reaction.

VHb and VHb-Red have been demonstrated to be able to protect cells from the negative effects of nitrosative stress. Furthermore, NO degrading activity has been clearly shown for VHb-Red and to some extent also for VHb. Moreover, recent data indicate the preferred localization of VHb in the close neighborhood of the cytochromes in both *Vitreoscilla* and *E. coli* cells. However, a direct interaction of VHb and cytochromes has not yet been shown.

Altogether it seems likely that VHb has an important role in the detoxification of reactive nitrogen species and in the protection of the cellular metabolism from these noxious compounds. Therefore, it can be hypothesized that the growth supporting effect of VHb is based on its protective role. However it cannot be completely ruled out that VHb is able to serve other functions, such as oxygen delivery.

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List of publications

Frey, A. D., Bailey, J. E., and P. T. Kallio. 2000. Expression of *Alcaligenes eutrophus* flavohemoprotein and engineered *Vitreoscilla* hemoglobin-reductase fusion protein for improved hypoxic growth of *Escherichia coli*. Appl. Envrion. Microbiol. 66:98-104.

Frey, A. D., Fiaux, J., Szyperski, T., Wüthrich, K., Bailey, J. E., and P. T. Kallio. 2001. Dissection of central carbon metabolism of hemoglobin-expressing *Escherichia coli* by ^{13}C nuclear magnetic resonance flux distribution analysis in microaerobic bioprocesses. Appl. Envrion. Microbiol. 67:680-687.

Frey, A. D., Rimann, M., Bailey, J. E., Kallio, P. T., Thompson, C. J., and M. Fussenegger. 2001. Novel pristinamycin-responsive expression systems for plant cells. Biotech. Bioeng. 74:154-163.

Kallio, P. T., Frey, A. D., and J. E. Bailey. 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. Kleuwer Academics Publishers. pp. 75-87.

Frey, A. D., Farrés, J. Bollinger C. J. T., and P. T. Kallio. Bacterial hemoglobins and flavohemoglobins for alleviation of nitrosative stress in *Escherichia coli*. In press.

Häggman, H., Aronen, T., Frey, A. D., Ryynänen, L., Julkunen-Tiitto, R., Timonen, H., Pihakaski-Maunsbach, K., Chen, X., and P. T. Kallio. Expression of *Vitreoscilla* hemoglobin in hybrid aspen (*Populus tremula* x *tremuloides*): an attempt to improve growth of an economically relevant tree species. Submitted for publication.