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MINI-REVIEW

Recent applications of *Vitreoscilla* hemoglobin technology in bioproduct synthesis and bioremediation

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Abstract Since its first use in 1990 to enhance production of α-amylase in *E. coli*, engineering of heterologous hosts to express the hemoglobin from the bacterium *Vitreoscilla* (VHb) has become a widely used strategy to enhance production of a variety of bioproducts, stimulate bioremediation, and increase growth and survival of engineered organisms. The hosts have included a variety of bacteria, yeast, fungi, higher plants, and even animals. The beneficial effects of VHb expression are presumably the result of one or more of its activities. The available evidence indicates that these include oxygen binding and delivery to the respiratory chain and oxygenases, protection against reactive oxygen species, and control of gene expression. In the past 4 to 5 years, the use of this "VHb technology" has continued in a variety of biotechnological applications in a wide range of organisms. These include enhancement of production of an ever wider array of bioproducts, new applications in bioremediation, a possible role in enhancing aerobic waste water treatment, and the potential to enhance growth and survival of both plants and animals of economic importance.

Keywords Bioproducts · Bioremediation · Genetic engineering · *Vitreoscilla* hemoglobin · Waste water

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Introduction

Vitreoscilla hemoglobin (VHb) was first studied in the 1960s (Webster and Hackett 1966) and recognized as a hemoglobin in 1986 (Wakabayashi et al. 1986). It was the first bacterial hemoglobin discovered, but in the following 28 years, it has been recognized, particularly due to the genomics revolution, that approximately two thirds of all bacterial species encode one or more hemoglobins (Vinogradov et al. 2006).

Bacterial hemoglobins fall into three main categories (Vinogradov and Moens 2008). VHb is a single domain hemoglobin (SDHb), having a single globin domain, the structure of which is closely similar to that of vertebrate globins (Bolognesi et al. 1999; Ratakonda et al. 2013). More common are the flavohemoglobins (FHbs), which have a *Vitreoscilla* type globin domain fused to a flavin-binding domain (Vinogradov and Moens 2008), and the truncated hemoglobins (trHbs), which have a single domain that is about 20 % smaller than SDHbs (Wittenberg et al. 2002).

As a broad group, the functions of bacterial hemoglobins are varied. These include, particularly for the FHbs and some of the trHbs, detoxification of NO through its conversion to nitrate (Pathania et al. 2002; Vinogradov and Moens 2008); oxygen sensing; and response to oxidative stress (Anand et al. 2010). Specifically, the roles of VHb have been extensively investigated. Its main role, both in native *Vitreoscilla* and heterologous hosts, appears to be to bind oxygen, particularly under low O_2 conditions, and deliver it to the respiratory chain by direct interaction with the terminal respiratory cytochrome (Webster 1987; Aydin et al. 2000; Ramandeep et al. 2001; Park et al. 2002; Chi et al. 2009). In this way, it likely enhances aerobic oxidative phosphorylation when O_2 is scarce. Other apparent roles include delivery of O_2 to oxygenases (Lin et al. 2003) and the response to oxidative stress (Anand et al. 2010). The latter function involves interactions with transcription factors, which is presumably connected to

transcriptional changes reported to be mediated by VHb (Roos et al. 2004). These will be discussed in more detail below.

The cloning of the VHb gene (*vgb*) in 1988 (Khosla and Bailey 1988; Dikshit and Webster 1988) ushered in the engineering of heterologous organisms with *vgb*/VHb to improve growth (Khosla and Bailey 1988) and production of useful products (Khosravi et al. 1990). The following quarter century has seen *vgb*/VHb engineering applied to a wide variety of bacteria, fungi, and even higher plants and animals to improve growth and survival, production of many proteins and metabolites, and bioremediation, particularly of aromatic compounds. These applications have been reviewed by us and others (Frey and Kallio 2003; Zhang et al. 2007; Stark et al. 2008; Stark et al. 2011). We have also recently reviewed what is known of the biochemical and molecular biological mechanisms which must underlie these improvements (Stark et al. 2012).

In this review, we are concerned with the biotechnological applications of engineering using *vgb*/VHb. We will summarize what was examined in detail in the earlier reviews but concentrate where possible on work that has been done in the last few years. Most of the work done using genetic engineering with *vgb*/VHb to improve host productivity has been of the "black box" variety, that is, engineering a host to express VHb and measuring an increase in production of a particular product or useful activity such as bioremediation. Since optimization of these strategies will be aided by the most comprehensive understanding possible of VHb's structure, biochemical activities, expression control, and interactions with other proteins, these topics are also considered.

Biochemistry and molecular biology of VHb

Structure and oxygen-binding properties

In *Vitreoscilla*, VHb exists as a single domain dimeric protein, having two identical subunits (15.7 kD each) and two heme *b*'s per dimer (Webster 1987). It forms a stable oxygenated species (kD of 7.2 μM), with an average oxygen association rate constant (k_{on}=78 μM^{-1} s^{-1}), but a high oxygen dissociation rate (k_{off}=5600 s^{-1}) (Webster 1987). This unusually high K_{off} correlates well with its proposed function of oxygen transfer to its host under oxygen limitation, especially to enhance respiration. Immunogold labelling studies, demonstrating localization of VHb near the cell membrane (Ramandeep et al. 2001), yeast two-hybrid studies showing direct interactions of VHb with the terminal respiratory oxidases cytochrome *bo* (Park et al. 2002) and cytochrome *d* (Duk 2007), and VHb enhancement of oxygen uptake and ubiquinol oxidase activities of respiratory membranes (Ramandeep et al. 2001) all support this role.

Recombinant VHb isolated from *E. coli* exists in both monomeric and dimeric forms (Giangiacomo et al. 2001; Kaur et al. 2002). The structure of the VHb monomer, although conforming to a three-over-three classical globin fold, has distal and proximal heme sites distinct from those of eukaryotic Hbs, lacking a proper E helix and E7 gate for the entry and exit of ligands (Tarricone et al. 1997; Bolognesi et al. 1999). GlnE7 does not participate in ligand stabilization and ProE8, which disrupts the structure of the E helix, does not significantly affect oxygen binding (Dikshit et al. 1998; Verma et al. 2005). In the initial crystal structure, the D region appeared disordered and flexible and could not be clearly resolved (Tarricone et al. 1997).

More recent crystal structures of VHb were able to determine the positions of most of the residues in the D region; these form a loop protruding towards the heme, which favors heme-globin interactions (Ratakonda et al. 2013). Site-directed mutants of VHb had previously identified specific residues of the D-loop (Asp44, Arg47, Glu49) that are involved in heme-globin interactions as well as binding of VHb with a flavoreductase partner (Lee et al. 2004). The proximal heme pocket of VHb also plays a key role in its function (Kaur et al. 2008).

Lipid-binding properties of VHb

Our immunogold labelling studies have shown that VHb remains associated with the cell membrane in recombinant *E. coli* and also in its native host, *Vitreoscilla* (Ramandeep et al. 2001). The membrane-associating properties of VHb have been studied by investigating the interaction of VHb with an artificial monolayer formed by phospholipids; this demonstrated that VHb is able to bind and penetrate the monolayer. Additionally, VHb displays reversible binding with phospholipids and free fatty acids in solution. UV-visible spectroscopy indicated the possibility of lipid binding at the distal site, which changes the five-coordinated state of VHb into a six-coordinated state (Rinaldi et al. 2006).

Such a possibility is supported by the crystal structure of VHb, which revealed that it has a unique distal site and a flexible DE loop region that can allow binding of other molecules in the distal pocket in addition to dioxygen. Thus, lipid binding at the distal site may interfere with VHb ligand binding ability, in turn, perhaps regulating the oxygen binding and physiological function of VHb in vivo. This is supported by a more than 20-fold decrease in oxygen affinity of VHb after lipid binding in vitro (Rinaldi et al. 2006).

VHb isolated from its native host, *Vitreoscilla*, displays several-fold lower oxygen affinity than what has been reported for the recombinant VHb, purified from *E. coli* (Giangiacomo et al. 2001). This difference might be due to differences in the lipid-ligated states of the protein isolated from the two different hosts. Recombinant VHb has been purified to a very high level and might be lacking lipid-

bound species, whereas VHb isolated from *Vitreoscilla* might retain the bound lipids as a result of its strong affinity to the cell membrane of its native host. These findings suggested that lipid/ligand-induced changes in the oxygen affinity of VHb may occur during its expression in native/heterologous hosts and may contribute to its functional diversity.

Regulation of VHb biosynthesis

Early work in *Vitreoscilla* showed that VHb expression is induced under low oxygen conditions (Webster 1987). Subsequent studies showed that oxygen-responsive regulation was transcriptional and operates similarly in *Vitreoscilla* and *E. coli* (Khosla and Bailey 1989; Dikshit et al. 1990). The bulk of this work has been done in *E. coli*, showing control by the oxygen sensitive regulators Fnr and ArcA, as well as by Crp (Joshi and Dikshit 1994; Yang et al. 2005). An OxyR binding site has also been identified within the *vgb* promoter, suggesting that multiple circuits may provide fine-level control of *vgb* expression in response to different oxygen levels and environmental stimuli (Anand et al. 2010).

VHb: one protein with many functions

As mentioned above, in addition to its proposed role in delivery of oxygen to the respiratory apparatus under hypoxia, VHb appears to have a number of other functions. These are likely related to its ability to exist in different oligomeric states (Giangiacomo et al. 2001; Kaur et al. 2002) and alter its oxygen-binding properties via interacting with cellular components, e.g., lipids, fatty acids, and especially "partner proteins". These functions, as they are likely to occur in bacteria, are summarized in Table 1 and Fig. 1 and discussed in more detail below.

Table 1 Activities of VHb with partner proteins

Activity	Partner protein(s)	Reference(s)
Electron transfer	Cytochromes *o*, *d*	Dikshit et al. 1992, Park et al. 2002, Duk 2007
Nitric oxide dioxygenase	Flavin reductase	Kaur et al. 2002, Lee et al. 2004
Antioxidant	OxyR	Geckil et al. 2003, Wang et al. 2009, Anand et al. 2010
Oxygenase enhancement	Oxygenases	Fish et al. 2000, Lin et al. 2003, Urgun-Demirtas et al. 2004
Transcriptional control, autoregulation	OxyR, Fnr, perhaps others	Roos et al. 2004, Isarankura-Na-Ayudhya et al. 2008, Anand et al. 2010

Electron transfer VHb's ability to interact directly with terminal respiratory oxidases may generate an alternate and/or efficient electron transfer pathway in the cell for facilitating energy generation. This may be related to its ability to act as an alternate terminal oxidase itself (Dikshit et al. 1992).

Nitric oxide dioxygenase (NOD) VHb closely resembles the heme domain of bacterial and yeast flavohemoglobins and carries conserved residues that are known to interact with the FAD-binding domain that may allow VHb to interact transiently with a suitable reductase partner(s) (Lee et al. 2004). In fact, a flavin-binding reductase was found associated with VHb when it was purified from its native host (Gonzales-Prevatt and Webster 1980), and a VHb chimera with the reductase domain of a flavohemoglobin acquired nitric oxide dioxygenase activity and was able to detoxify NO (Kaur et al. 2002). Thus, VHb may function as an NOD under certain conditions, if associated with a suitable partner protein.

Antioxidant A peroxidase like activity has been detected in VHb (Kvist et al. 2007), and it functions as an antioxidant in many heterologous hosts by conferring protection from oxidative stress (Geckil et al. 2003; Wang et al. 2009). This occurs through VHb interaction with OxyR and Fnr, modulating their activities in a redox dependent manner (Anand et al. 2010).

Oxygenase enhancement Several studies have shown that VHb appears to be able to stimulate oxygenase activities (Fish et al. 2000; Urgun-Demirtas et al. 2004), perhaps via direct oxygen delivery by VHb (Lin et al. 2003).

Transcriptional control Transcriptome and metabolic flux analyses showed that VHb expression may have significant effects on host gene expression and thus metabolism (Roos et al. 2004; Isarankura-Na-Ayudhya et al. 2008), for example, shifting metabolism to an energetically more efficient aerobic state (Ramachandran et al. 2012). VHb interaction with OxyR and Fnr (see above) transmits signals to OxyR to activate the oxidative stress regulon of the cell, simultaneously autoregulating its own biosynthesis (Anand et al. 2010); this sustains aerobic metabolism via a steady supply of oxygen (especially under hypoxia) while simultaneously balancing the toxic effects of reactive oxygen species. This study provided an insight into the mode of the protective effect conferred by VHb during expression in different heterologous hosts (Geckil et al. 2003; Wang et al. 2009).

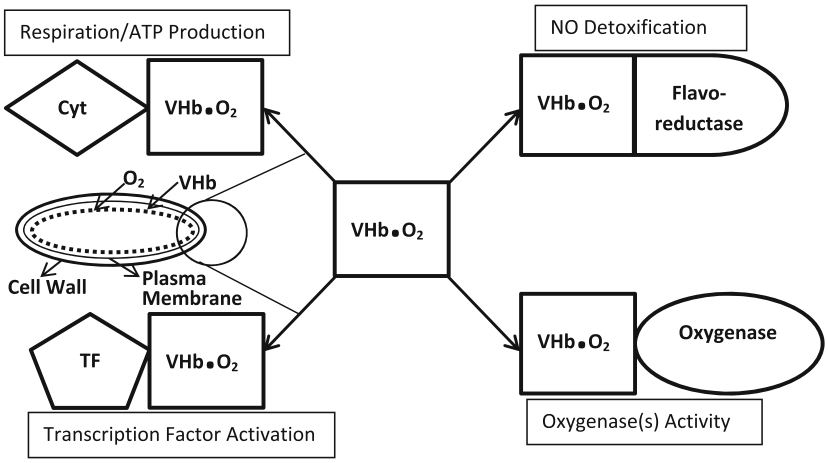

Fig. 1 Probable roles of VHb in enhancing performance of heterologous bacterial hosts. Oxygen enters the cell and binds to VHb (dots concentrated just underneath the plasma membrane). VHb bound to oxygen interacts with various partner proteins, either delivering oxygen to them (cytochromes, flavoreductases, oxygenases) to enhance their activity or activating transcription factors, thus enhancing expression of downstream functions

Protein engineering of VHb and engineering using the *vgb* promoter

VHb fusion proteins and use of the *vgb* promoter

A report by Park et al. (2003) described construction of a plasmid vector for use in *E. coli* which provides for fusion of a cloned gene downstream of a His-tag-*vgb*-protease cleavage site sequence. An improved version of the vector was described in Kwon et al. (2005). The resulting fusion proteins can be purified in one step using His-tag affinity chromatography and easily visualized during purification due to the red color of VHb. In addition, the VHb fusion can increase the solubility of proteins which, on their own, are sparingly soluble, and thus aid in increasing the yields of recombinant proteins. The protease cleavage site then can be used to remove the His-tag-VHb sequence. A related strategy used VHb fusions with a flavoreductase domain to enhance conversion of nitric oxide to nitrate (Kaur et al. 2002) and with D-amino acid oxidase, enhancing metabolism of cephalosporin C (Khang et al. 2003).

A recent report (Wu et al. 2014) investigated the use of the *vgb* promoter, which is inducible under low oxygen conditions, to enhance production of poly(hydroxybutyrate) (PHB) by recombinant *E. coli*. The rationale is that high density bacterial cultures will be starved for oxygen, and placing the genes of the PHB pathway under control of the *vgb* promoter will thus induce their expression under these conditions. In this case, a substantial increase in PHB production occurred using a promoter containing eight repeats of the *vgb* promoter organized in tandem.

VHb site-directed mutants

Several studies have investigated the effects of site-directed mutations in *vgb* on VHb structure and properties, with the secondary goal of producing a mutant VHb with improved ability to enhance the productivity of VHb-engineered organisms. Targeting two sites in the distal heme pocket resulted in mutant VHbs that were not better than wild-type VHb regarding oxygen binding (Dikshit et al. 1998; Verma et al. 2005). Lee et al. (2004) introduced a number of mutations into the (relatively disordered) D region of VHb, but again, none had improved ligand binding properties compared to those of wild-type VHb. The proximal heme site was investigated similarly and, while not true for all mutants, two VHbs with mutations in proximal site Tyr126 provided both improved growth properties and degradation of 2,4-dinitrotoluene (Kim et al. 2005) or production of recombinant protein (Kaur et al. 2008) for bacterial cells expressing them compared to those expressing wild-type VHb.

Enhancement of bioproduct synthesis using *vgb*/VHb

Earlier work

As mentioned above, the first case in which engineering of a heterologous host using *vgb*/VHb was used to increase production of a useful biomolecule (in this case, recombinant α-amylase in *E. coli*) was reported in 1990. In the following years, the same strategy was used in a wide variety of both prokaryotes and eukaryotes to enhance the production of many different biological molecules of practical importance. The recombinant organisms used included a variety of bacteria, a number of fungi, and several plants. The biomolecules produced included enzymes, amino acids, biofuels, and polymers that can replace petrochemical feed stocks (mostly in bacteria); and antimicrobials (in fungi). The applications in plants focused on improvements in growth and resistance to stress (improving, for example, tolerance to submergence in water). Detailed lists of these applications through about 2010 can be found in Stark et al. (2011). More recent work (discussed below) is summarized in Table 2. Most of the

Table 2 Recent applications of VHb technology regarding enhancement of bioproduct production

Species	Product (s)	Reference (s)
Bacteria		
P. aeruginosa	Rhamnolipid	Kahraman and Erenler 2012
S. spinosa	Spinosad	Luo et al. 2012
B. amyloliquefaciens	Poly-γ-glutamic acid	Zhang et al. 2013
A. hydrophila	Hydroxyalkanoates	Liu et al. 2011
C. freundii	Methionine γ-lyase	Kahraman et al. 2011
C. crenatum	L-arginine	Xu et al. 2011
S. elodea	Gellan gum	Wu et al. 2011
S. gilvosporeus	Natamycin	Wang et al. 2014
S. diastatochromogenes	Toyocamycin	Ma et al. 2014
C. freundii	Methionine γ-lyase	Kahraman et al. 2011
Streptomyces sp. FR-008	Candicidin D	Wang et al. 2012
E. coli	Biomass	Pablos et al. 2011
E. coli	Ethanol	Sanny et al. 2010; Arnaldos et al. 2012; Abanoz et al. 2012; Akbas et al. 2014
Yeast and fungi		
S. cerevisiae	Amorpha-4,11-diene	Shen et al. 2012
S. cerevisiae	Betulinic acid	Li and Zhang 2014
P. pastoris	β-galactosidase	Wu and Fu 2012; Wu et al. 2012
P. pastoris	*Yarrowia lipolytica* lipase	Wang et al. 2012
Higher eukaryotes		
Aspen	Stress response to herbivory	Sutela et al. 2013
A. membranaceus	Astragaloside IV	Wang et al. 2011
Aurantiochytrium	Astaxanthin, biodiesel	Suen 2013
Zebra fish	Survival under low dissolved oxygen levels	Guan et al. 2011

recent studies have been performed in various laboratories in China.

Recent work

Bacteria

As mentioned above, through 2010 *vgb*/VHb engineering had been used successfully to enhance production of a wide variety of biomolecules in a wide variety of bacterial species. Such applications have continued since then as well. Although they have been produced using conventional genetic engineering/expression strategies, the bioproducts in question have varied and important uses. Production by VHb-expressing *Pseudomonas aeruginosa* of rhamnolipid (a surfactant produced endogenously by *P. aeruginosa*) was studied by Kahraman and Erenler (2012), although no comparison with wild-type *P. aeruginosa* was reported. Luo et al. (2012) reported enhancement of production of the natural insecticide spinosad by *Saccharopolyspora spinosa* grown under both normal and limited aeration conditions. The production by *Bacillus amyloliquefaciens* of endogenous poly-γ-glutamic acid (a biomolecule used in a wide variety of commercial applications (Shih and Van 2001)) was increased by 30 % coincident with expression of VHb from *vgb* stably integrated into the host chromosome (Zhang et al. 2013), and VHb was correlated with 11 % higher biomass in *E. coli* cultures grown under conditions of limiting oxygen (Pablos et al. 2011).

Other recent applications in which *vgb*/VHb engineering has been correlated with increases in production of biochemicals include: hydroxyalkanoates by *Aeromonas hydrophila* (Liu et al. 2011); L-arginine by *Corynebacterium crenatum* (17 %; Xu et al. 2011); gellan gum by *Sphingomonas elodea* (up to 27 %; Wu et al. 2011); the antifungal medicinal natamycin by *Streptomyces gilvosporeus* (up to 175 %; Wang et al. 2014); and the antifungal and anticancer drug toyocamycin by *Streptomyces diastatochromogenes* (up to 210 %; Ma et al. 2014).

Expression of VHb in several bacterial species under a variety of growth conditions resulted in most cases in little or no increase in production of the anti-leukemia enzyme, L-asparaginase, although substantial increases (up to 2-fold) occurred in a few cases (Erenler and Geckil 2014); 2–3.1-fold increases in the production by *Citrobacter freundii* of the antileukemic methionine γ-lyase has also been reported (Kahraman et al. 2011). Engineering of *Streptomyces sp.* FR-008 contributed to increases in the production of the antifungal candicidin D (Wang et al. 2012).

A revisiting of whether VHb expression and consequent delivery of small amounts of oxygen to bacteria could increase the yield of biofuels via fermentation, although counter intuitive, showed promise (Sanny et al. 2010). Several studies using controlled supply of small amounts of oxygen to fermenting cultures have yielded similar results (Jansen et al. 1984; Okuda et al. 2007; Nieves et al. 2011). Cost-effective production of bioethanol and other biofuels will most likely require the use of lignocellulosic material and other waste products as carbon sources. Several recent studies in which rich media have been supplemented with such material for growth of ethanologenic *E. coli* have shown that substantial increases in ethanol production are correlated with *vgb*/VHb expression, although the amount of the increases varied with growth conditions. The supplements include hydrolyzed corn fiber (Arnaldos et al. 2012), hydrolyzed potato

processing waste (Abanoz et al. 2012), and cheese whey and molasses (Akbas et al. 2014).

Yeast and fungi

In the last several years, engineering of yeast and fungi to express *vgb*/VHb with the aim of enhancing production of useful biomolecules of various types and functions has continued. Shen et al. (2012), for example, increased the production of amorpha-4,11-diene (a precursor to the antimalarial agent artemisinin) in *S. cerevisiae* by 2–3-fold by the concomitant expression of VHb, while Li and Zhang (2014) reported a VHb-correlated increase of 3.2-fold in *S. cerevisiae* production of the cancer and HIV drug betulinic acid. Of special interest in this area, however, is the use of the fungus *Pichia pastoris*.

P. pastoris has become a particularly useful host for production of recombinant enzymes because of its abilities to produce recombinant proteins at high levels and modify proteins in a manner similar to that of higher eukaryotes, its vigorous growth in simple media, and its robust genetic system (Macauley-Patrick et al. 2005). Because of the high cell densities achieved by *P. pastoris*, oxygen transfer to the cultures can be a problem, and several recent studies have addressed this issue using the *vgb*/VHb strategy.

Wu and Fu (2012) increased production of recombinant β-galactosidase in *P. pastoris* by 9.9 % under low aeration conditions by simultaneous expression of VHb. Growth was also increased substantially as was oxygen uptake (by about 28 %). Wu et al. (2012) also looked at the effects of expression of *vgb*/VHb on recombinant β-galactosidase production in *P. pastoris* and found that the positive effects of VHb were enhanced when cultures were grown at 23 °C compared with 30 °C. Expression of VHb in *P. pastoris* was correlated with an increase in production of *Yarrowia lipolytica* lipase by 22–84 % (depending on the culture aeration rate; greatest relative increase at low aeration); again, growth and oxygen uptake were also increased in the VHb-expressing strain (Wang et al. 2012).

Eukaryotes other than fungi

A particularly interesting application of *vgb*/VHb technology has been the engineering of higher plants with *vgb* under the transcriptional control of higher plant promoters. Between 2004 and 2009, several such studies were reported (reviewed in Stark et al. 2011). The early studies focused mostly on plants of agricultural or biofuel use (rice, cabbage, and white poplar) and on improving characteristics of direct practical importance (growth; productivity; resistance to submergence, nitrosative, and oxidative stress).

Recent work on aspen has determined that VHb expression had little effect on the stress response of the tree to herbivory (in this case, by Lepidopteran larvae) or survival of these herbivores (Sutela et al. 2013). *Vgb*/VHb was used to engineer the legume *Astragalus membranaceus*, an important herb in traditional Chinese medicine, which produces astragaloside IV, a biochemical with various medicinal activities. *Vgb* was transferred to *A. membranaceus* by *Agrobacterium tumefaciens*, and the production of astragaloside IV in the resulting hairy roots increased by 5–6-fold compared to hairy roots not expressing VHb (Wang et al. 2011).

The eukaryotic microalga *Aurantiochytrium*, a source of fatty acids with potential for biodiesel and the antioxidant food supplement astaxanthin, was engineered to express VHb, resulting in an up to 44 % increase in the former product and a 9-fold increase in the latter product (Suen 2013). In another interesting pilot study, in this case related to aquaculture farming, zebrafish were engineered to express VHb and the resulting transgenics had greater survival rates than non-VHb-expressing fish when grown under the stress of low dissolved oxygen levels (Guan et al. 2011).

Applications of VHb for environmental biotechnology

Earlier work

Environmental biotechnology applications of *vgb*/VHb include systems to remediate pollution of air, water, and soil by biological treatment. The earliest reported use of *vgb*/VHb-engineered bacteria for bioremediation involved simulated studies with benzoic acid as a model pollutant using *Xanthomonas maltophilia*; in this case, there was limited enhancement compared to the wild type (Liu et al. 1996). Subsequent bioremediation work with *vgb*-engineered bacterial strains involved other organic contaminants, namely, 2,4-dinitrotoluene using *Burkholderia* strain DNT (Patel et al. 2000; Fish et al. 2000; Nasr et al. 2001; Lin et al. 2003; So et al. 2004); organophosphorus using *E. coli* (Kang et al. 2002); 2-chlorobenzoic acid using *Burkholderia cepacia* (Urgun-Demirtas et al. 2003; 2004; 2005; 2006); benzene, toluene, xylene (BTX) using *Pseudomonas aeruginosa* (Kahraman and Geckil, 2005); and benzoic acid using *Pseudomonas aeruginosa* (Chung et al. 2001; Kim et al. 2005).

The lab scale experimental systems used in these studies included batch shake flasks, continuous flow chemostat bioreactors, continuous flow membrane bioreactors, and continuous flow sand column bioreactors using *vgb*-bearing and wild-type strains of the respective species. The key findings included improvement in the extent and rate of contaminant degradation under hypoxic conditions, pathway modification to enable complete mineralization of the contaminant, improvement in the growth rate of the bacteria used, and

improved oxygen utilization rates under hypoxic conditions. The mechanistic basis for such improvements were found/reasoned to be due to VHb-related increases in the supply of oxygen to the oxygenases in the reaction pathways and/or increased expression of degradative enzymes as a result of increases in oxygen supply to the respiratory chain.

Recent work

The recent work on investigations and applications of hemoglobin technology involve enhancements under microaerobic or hypoxic conditions in the transformation of inorganics such as ammonia nitrogen, sequestering of metals, and solubilization of phosphate by increased production of organic acids (Table 3).

Nitrification of ammonia to nitrite/nitrate is one of the most important biochemical reactions in mineralization of naturally occurring organic nitrogen and in control of N pollution. Arnaldos et al. (2013; 2014) gave a new direction to bacterial hemoglobin technology by promoting/enriching the ability of native bacteria in mixed cultures to express hemoglobin type proteins under hypoxic conditions. The first step in oxidation of ammonia to nitrite/nitrate involves ammonia monooxygenase (AMO), the oxygen substrate requirements of which could theoretically be met, under low bulk dissolved oxygen (DO) concentrations, through delivery of oxygen by hemoglobin. This, in turn, could greatly reduce the oxygen supply requirements of nitrification and, hence, the energy needed to provide oxygen by conventional aeration.

Arnaldos et al. (2013) investigated mixed culture nitrifying activated sludge from a biological wastewater treatment plant and enriched it to nitrify at low DO concentration (~0.1 mg O_2/L) in a lab scale sequencing batch reactor (SBR). The low DO SBR completely nitrified the influent ammonia after a prolonged acclimation period (greater than 140 days) and performed comparably to a high DO SBR (near saturation DO). Achievement of complete nitrification in the low DO reactor coincided with the increased specific oxygen uptake rate of the biomass compared to the high DO reactor biomass and expression of a CO-binding soluble heme protein. The heme protein expressed was linked to ammonia oxidizing bacteria (AOB), which were dominant in the biomass, and was excluded as any one of the enzymes or proteins in the conventional account of ammonia oxidation by AOB.

Arnaldos et al. (2014) further investigated this heme protein by conducting bioassays targeting its function and activity, location in the cell, and the organisms that express it. It was shown that the heme protein is preferentially expressed in the cytoplasm by AOB, with heme *c* as its prosthetic group. Activity assays indicated that it is neither a peroxidase nor oxidase and none of the known heme proteins involved in ammonia oxidation. Whether this heme protein is a hemoglobin or not has not been established, but if it is, it would open a new area of research into methods to enhance wastewater treatment under low oxygen conditions.

Earlier, it was shown that VHb expression by *Gordonia amarae* enhanced production of extracellular biosurfactants (Dogan et al. 2006). The ability of such extracellular polymers to sequester heavy metals was studied by Kahraman et al. (2014). They showed that engineering *Pseudomonas aeruginosa* with *vgb* enhanced Pb, Co, and Cu uptake by the cells. Liao et al. (2014) showed that a *Bacillus sp*. expressing *vgb* had enhanced Mn oxidation in Mn (II)-contaminated water under oxygen-restricted conditions. It was hypothesized that VHb increases the concentration of intracellular oxygen needed to oxidize Mn (II).

Yadav et al. (2014) studied VHb enhanced solubilization of phosphate, as in soils, under hypoxic conditions through the increased expression of citrate, a chelating agent. An artificial citrate operon comprised of genes encoding citrate synthase and engineered to contain *vgb* was constructed and transformed into *Enterobacter hormaechei*. The transformant secreted citric acid and released soluble phosphate from rock phosphate, whereas the native strain could not. This may have applications in enhancing mineral phosphate solubilization under buffered, microaerobic conditions, such as in the rhizospheric environment.

Table 3 Recent applications of VHb technology related to bioremediation and waste water treatment

Species	Process	Reference (s)
Mixed culture of nitrifiers	Nitrification	Arnaldos et al. 2013, 2014
P. aeruginosa	Pb, Co, and Cu uptake	Kahraman et al. 2014
Bacillus sp.	Mn oxidation	Liao et al. 2014
E. hormaechei	Solubilization of phosphate	Yadav et al. 2014

Table 4 Possible future applications of VHb technology

Organisms	Applications
Crop plants (e.g., rice)	Improved growth (e.g., improved submergence tolerance for rice)
Animals used as food	Improved growth of food fish
Recombinant bacteria	Improved production of various biomolecules (enzymes, polymers, pharmaceuticals, insecticides)
Recombinant microbes (bacteria, yeast)	Improved production of biofuels
Aerobic wastewater bacteria	Efficient wastewater treatment at low aeration

Conclusions, insights, future directions

To date, the number and variety of bioproducts enhanced by VHb expression in heterologous hosts seems limited only by the imagination of the investigators, and we can expect these applications to grow in number in the future (Table 4). Specifically, regarding biofuel (e.g., ethanol, butanol) production, enhancement by VHb expression will need to be shown to occur both at large scale and in media in which low cost carbon sources such as food processing wastes and lignocellulosic material do not need to be supplemented with the expensive components of traditional rich microbiological media. The uses of VHb technology regarding bioremediation (including its potential in normal waste water treatment) are fewer in number, but future applications might also be expected in novel areas. Finally, as with any practical application in biology, continuing investigation into the biochemical activities of VHb, which seem to be many and varied and involve interaction with a variety of partner proteins with varied functions, will certainly aid in developing more systematic and successful employment of VHb technology.

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