J. Microbiol. Biotechnol. (2007), **17**(12), 2066–2070

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Overexpression of *Shinorhizobium meliloti* Hemoprotein in *Streptomyces lividans* to Enhance Secondary Metabolite Production

KIM, YOON JUNG[1], SOON OK SA[1], YONG KEUN CHANG[1]*, SOON-KWANG HONG[2], AND YOUNG-SOO HONG[3]

[1]*Department of Chemical and Biomolecular Engineering, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea*
[2]*Division of Bioscience and Bioinformatics, Myongji University, Yongin 449-728, Korea*
[3]*Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea*

Received: February 13, 2007 Accepted: April 15, 2007

Abstract It was found that *Shinorhizobium meliloti* hemoprotein (SM) was more effective than *Vitreoscilla* hemoglobin (Vhb) in promoting secondary metabolites production when overexpressed in *Streptomyces lividans* TK24. The transformant with *sm* (*sm*-transformant) produced 2.7-times and 3-times larger amounts of actinorhodin than the *vhb*-transformant in solid culture and flask culture, respectively. In both solid and flask cultures, a larger amount of undecylprodigiocin was produced by the *sm*-transformant. It is considered that the overexpression of SM especially has activated the pentose phosphate pathway through oxidative stress, as evidenced by an increased NADPH production observed, and that it has promoted secondary metabolites biosynthesis.

Keywords: Bacterial hemoproteins, *S. lividans*, secondary metabolites production

Limited oxygen availability is one of the serious problems facing large-scale cultures for secondary metabolite production by *Streptomyces* and other mycelia-forming microorganisms, and results in poor cell growth and a reduced product yield. In 1988, Khosla and Bailey [22, 23] reported a new genetic strategy based on cloning the *Vitreoscilla* hemoglobin gene (*vhb*), which then prompted further research related to bacterial hemoglobins and hemoproteins [1, 11, 13, 18–21, 31). Essentially, Magnolo introduced *vhb* into *S. coelicolor* and *S. lividans* and observed enhanced actinorhodin production and cell growth, respectively [28]. Bacterial hemoglobins belong to the large superfamily of hemoproteins. Many hemoproteins have been identified in such organisms as protozoa, plants, fungi, and bacteria: *Escherichia coli* [33], *Ralstonia eutropha* (*alcaligenes eutrophus*) [8], *Erxinia chrysanthemi* [12], *Bacillus subtilis* [26], *Salmonella typhimurium* [9], *Mycobacterium tuberculosis* [16], and *Pseudomonas aeruginosa*, *Krebsiella pneumoniae*, *Deinococcus radiodurans*, and *Campylobacter jejuni* [7]. Moreover, it has been demonstrated that overexpression of these hemoproteins also enhances cell growth and byproduct formation, similar to the *Vitreoscilla* hemoglobin (Vhb). Accordingly, this paper describes the first application of a newly isolated hemoprotein from *Shinorhizobium meliloti* (SM) as a vehicle for oxygen-transfer *in vivo* to enhance the production of secondary metabolites in a *Streptomyces* strain.

From a protein BLAST, it was found that a flavor hemoprotein (GenBank Accession No. AAP93662) from *Shinorhizobium meliloti* (SM) had a high homology to Vhb. Thus, the *sm* gene from *S. meliloti* (ATCC 9930) was isolated and cloned, together with *vhb* from *Vitreoscilla* (ATCC 15218) [17]. When compared with Vhb, SM has two additional domains (oxidoreductase FAD-binding and NAD-binding domains) and is about three times larger. Furthermore, the *S. coelicolor* genome harbors an SM homolog with a 39% identity and 57% similarity. Thus, *S. lividans*, which has a 97% genome homology with *S. coelicolor*, was expected to include an ortholog. The primers used for the PCR were supplied by DyneBio Science (Korea): *vhb* (forward), 5'-CTTAAGGAAGACC***CATATG***-TTAGACCAGC-3' (NdeI); *vhb* (reverse), 5'-CAATATTT-GTCCC***AAGCTT***TGGCAACAG-3' (HindIII); *sm* (forward), 5'-AGGAGAAAC***CATATG***CTCACTCAGAAG-3' (NdeI); and *sm* (reverse), 5'-TGCCCCTCG***GAATTC***GCTCGAA-ACAGC-3' (EcoRI). The PCR amplification was carried out in a thermocycler (Applied Biosystem, GeneAmp PCR System 2700, CA, U.S.A.) with Taq polymerase (TaKaRa, Japan). After an initial denaturation step (5 min at 96°C), 30 cycles of amplification with 2 steps (20 sec at 98°C, 1 min at 68°C) were followed by a final extension period

*Corresponding author
Phone: 82-42-869-3927; Fax: 82-42-869-3910;
E-mail: ychang@kaist.ac.kr

of 10 min at 72°C. The amplified DNA of *vhb* (564 bp) and *sm* (1,269 bp) was cloned into pGEM-T (Promega), and then transformed into *E. coli* DH5α. Thereafter, the cloned hemoglobin genes were subcloned into pUWL201PW, a streptomycetes expression vector that has an *ermE* strong promoter and ribosomal binding site. The resulting recombinant plasmids, pUWL201PW-*vhb* and pUWL201PW-*sm*, were then transformed into *S. lividans* TK24. An R2YE medium [24] was used for the liquid and solid cultivation of *S. lividans* TK24, where the solid cultures were performed on an agar plate for 7 days at 28°C, whereas the flask cultures were performed in a 500-ml baffled flask containing 100 ml of an R2YE liquid medium supplemented with thiostreptone (50 μg/ml) at 28°C and 200 rpm in a rotary shaker. A 1-cm^2 fragment (cells+agar) of the solid culture after 7 days of cultivation was used as the inoculant for the seed culture, and 5 ml of the 2-day-old seed culture was used as the inoculant for the flask culture [5, 25, 33].

To check the expression of the *vhb* and *sm* genes, the *S. lividans* TK24 transformed with pUWL201PW-*vhb* or pUWL201PW-*sm* was cultured in an R2YE broth, and then an RT-PCR [5] was carried out using the primer pairs listed in Table 1. A SuperScript One-Step RT-PCR (Invitrogen, U.S.A.) with *Ex Taq* (TaKaRa, Japan) was used to generate a product from each mRNA with 0.5 μg of the total RNA as the template. The experimental conditions were as follows: first-strand cDNA synthesis, 38°C for 50 min, followed by 95°C for 3 min; amplification, about 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. As a result of the RT-PCR, transcript signals for the *vhb* and *sm* genes were detected in the transformants with pUWL201PW-*vhb* and pUWL201PW-*sm*, respectively, yet no signal was found in the control (Fig. 1A). Therefore, these data clearly show that the *vhb* and *sm* genes were successfully transcribed in the *S. lividans* transformants.

To confirm the transcriptional analysis result, 1% SDS-15% PAGE of the total cellular proteins prepared from the transformants was performed. From their amino acid sequences, the molecular masses of Vhb and SM were expected to be 15.724 kDa and 44.673 kDa, respectively. Consistently, two proteins with approximate molecular masses of 15 kDa and 45 kDa were detected in the transformants with pUWL201PW-*vhb* and pUWL201PW-*sm*, respectively (Fig. 1B), thereby supporting that the *vhb* and *sm* genes had been successfully expressed in the host strain on the transcriptional and proteomic levels.

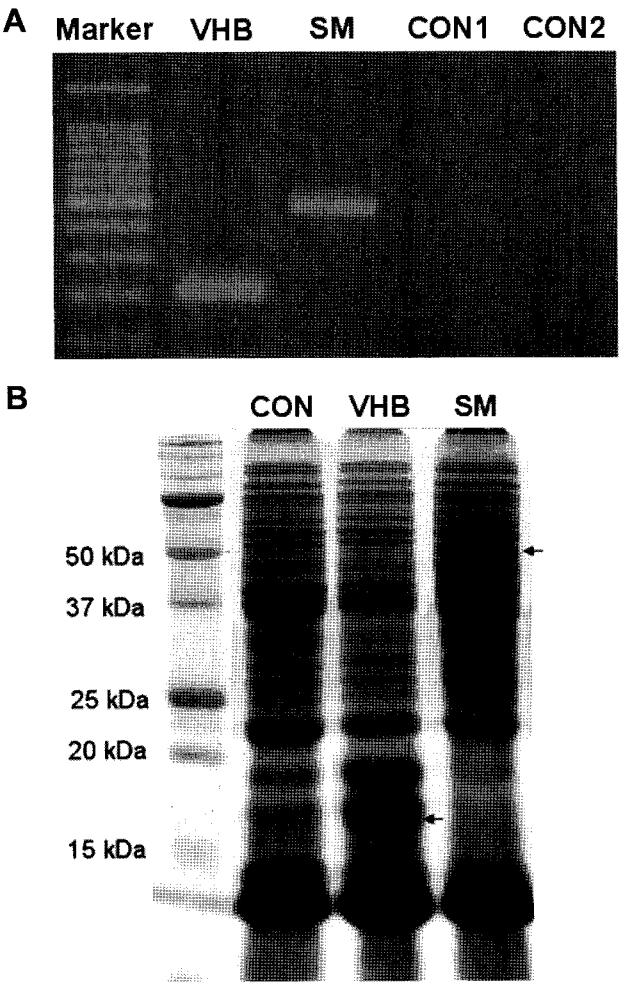

Fig. 1. Transcriptional (**A**) and proteomic (**B**) analyses of *S. lividans* transformed with expression vectors.
A. Expression analysis of *vhb* and *sm* based on RT-PCR analysis of transformants. Total RNA was isolated from the *S. lividans* transformants, and then an RT-PCR was performed as explained in the text. Lane 1, protein ladder (100 bp); lane 2 (VHB), RT-PCR of *vhb*-transformant with *vhb* primers; lane 3 (SM), RT-PCR of *sm*-transformant with *sm* primers; lane 4 (CON1) and lane 5 (CON2), RT-PCR of vector-transformant with *vhb* and *sm* primers, respectively. **B.** SDS-PAGE analysis of total cellular proteins: Lane 1, protein size marker; lane 2 (CON), vector only; lane 3 (VHB), *vhb*-transformant; lane 4 (SM), *sm*-transformant.

Table 1. Primers used for RT-PCR.

Function	Gene	Oligonucleotide
Bacterial hemoproteins	*vhb*	Forward: 5'-gga gca gcc taa ggc ttt ggc g-3'
		Reverse: 5'-gtc atc ggt tgc ggc atc gcc g-3'
	sm	Forward: 5'-cgc cgt gca ggt gcc taa gct cg-3'
		Reverse: 5'-cgt cga cga ggc ccg caa agt cg-3'
Antioxidant enzymes	*catA*	Forward: 5'-gcc tcc tac cgg cac cat gca cgg-3'
		Reverse: 5'-ctg gtt gcc gtc gac gcg cat gg-3'
	sodF	Forward: 5'-gcc gga ggg gat ccg cca tgt cc-3'
		Reverse: 5'-ggt gga gcc ctg gcc gac gtt gc-3'

Based on the confirmed expression of the *vhb* and sm genes in the transformants, the effects of Vhb and SM on the host were examined. Although the transformants showed the same growth and spore formation as the control, a large amount of the blue-pigmented antibiotic actinorhodin was produced by the transformant with *sm*, followed by the transformant with *vhb* on R2YE agar plates (data not

shown). Thus, the transformants were cultured in R2YE agar and liquid media, and their cell growth and the production of actinorhodin and undecylprodigiosin measured. To measure the cell mass or concentration, the cells were washed with a phosphate buffer. The washed cells were then dried at 80°C for 24 h and weighed at room temperature. The amounts of actinorhodin and undecylprodigiocin were measured according to previously reported procedures [2, 6, 27]. To analyze the intracellular actinorhodin, 20 mg of dried cells was extracted with 5 ml of chloroform in a test tube for 30 min at room temperature. Then, 5 ml of 1 N NaOH was added, and the mixture vortexed and spun in a microcentrifuge for 15 sec. The resulting aqueous phase contained actinorhodin, exhibiting a blue color at an alkaline pH of 12. The optical density of the aqueous phase was determined at 615 nm. To analyze the intracellular undecylprodigiosin, which turns red in an acidic pH, the chloroform phase was acidified with HCl, and the optical density determined at 540 nm. To analyze the extracellular actinorhodin and undecylprodigiocin in the flask culture, the optical density of the cell-free culture broth was measured at 615 nm (pH 12) and 468 nm (pH 2), respectively. To analyze the actinorhodin secreted into the agar in the solid culture, the agar was heat-melted before measuring the optical density.

The production of actinorhodin and undecylprodigiosin by the transformants on the R2YE agar plates revealed that the expression of the *sm* gene was more effective in enhancing secondary metabolite production than the *vhb* gene. As seen in Fig. 2, the *sm*-transformant produced the largest amount of total (intra- and extracellular) actinorhodin per unit of cell mass, which was about 4 times more than the control, whereas the *vhb*-transformant only produced 1.5 times more actinorhodin than the control (Fig. 2A). The

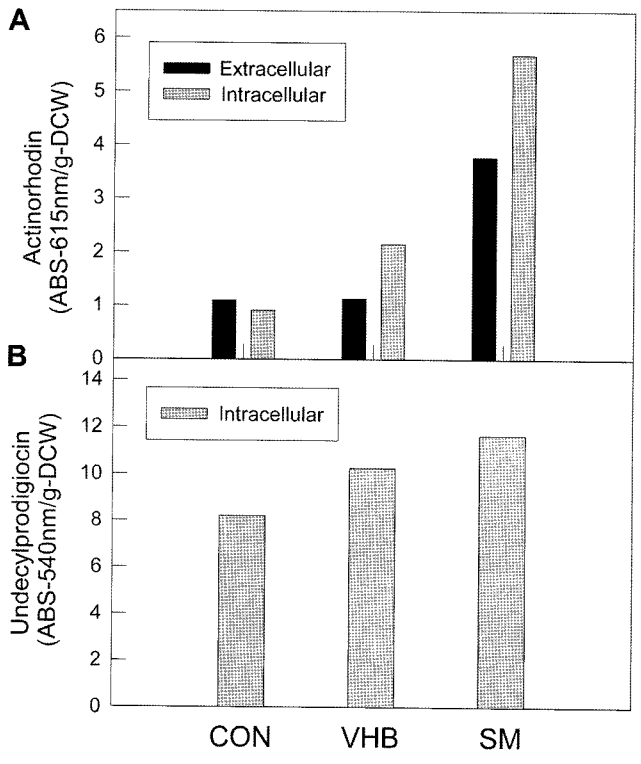

Fig. 2. Effects of Vhb and SM overexpression on secondary metabolism of *S. lividans* TK24.

S. lividans TK24 transformed with the expression vectors pUWL201PW (CON), pUWL201PW-*vhb* (VHB), and pUWL201PW-*sm* (SM) was cultivated on R2YE agar plates, and then the amount of actinorhodin (**A**) and undecylprodigiosin (**B**) produced was measured after 7 days of cultivation.

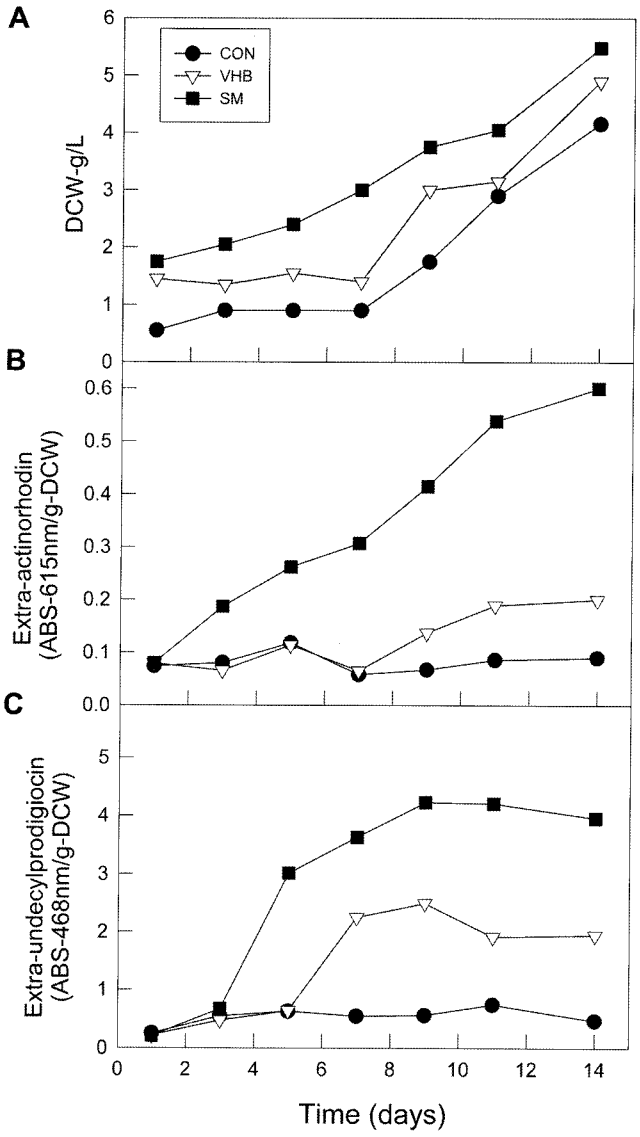

Fig. 3. Effects of Vhb and SM overexpression on growth and secondary metabolism of *S. lividans* TK24 in liquid culture.

S. lividans TK24 transformed with the expression vectors pUWL201PW (CON), pUWL201PW-*vhb* (VHB), and pUWL201PW-*sm* (SM) was cultivated in an R2YE broth for 14 days, and then the cell growth (**A**) and amount of actinorhodin (**B**) and undecylprodigiosin (**C**) produced were measured as explained in the text.

amount of extracellular undecylprodigiocin was negligible (Fig. 2B). The *sm*- and *vhb*-transformants produced 1.5 and 1.2 times more intracellular undecylprodigiocin, respectively, than the control.

The cell growth was slightly enhanced in the *vhb*- and *sm*-transformants, where the *sm*-transformant had the highest cell concentration at the end of the seed cultures. The differences in the initial cell concentration in the main cultures were simply due to different growth rates in the seed cultures (Fig. 3A). The amount of extracellular actinorhodin produced by the *sm*-transformant was about 6 times more than that produced by the control, whereas the *vhb*-transformant only produced 2 times more extracellular actinorhodin than the control (Fig. 3B). The *sm*- and *vhb*-transformants produced about 8 and 4 times more extracellular undecylprodigiocin, respectively, than the control (Fig. 3C). Therefore, overall, the *sm*- and *vhb*-transformants produced a lot more secondary metabolites than the control. In particular, the newly isolated hemoprotein SM significantly enhanced both the cell growth and the secondary metabolite production when compared with the well-characterized hemoprotein Vhb.

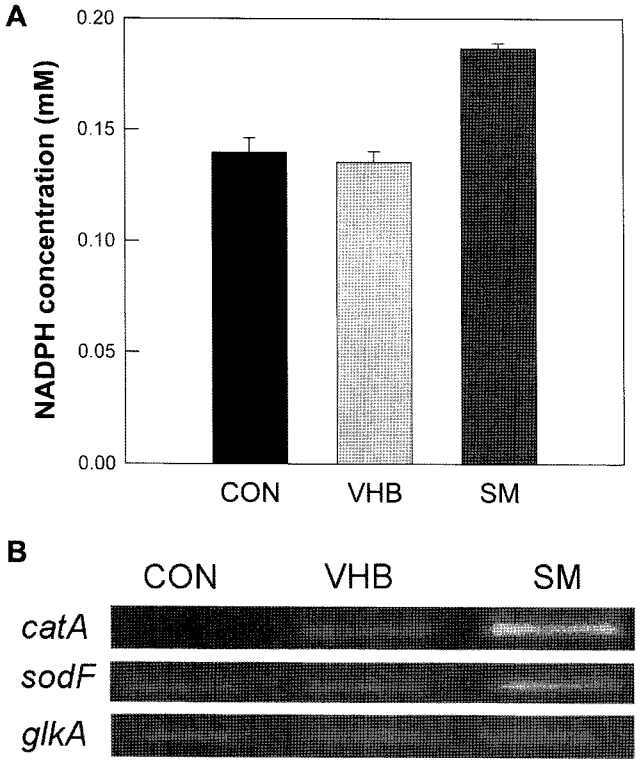

Fig. 4. Effects of Vhb and SM overexpression on antioxidant metabolism of *S. lividans* TK24.
S. lividans TK24 transformed with the expression vectors pUWL201PW (CON), pUWL201PW-*vhb* (VHB), and pUWL201PW-*sm* (SM) was cultivated in an R2YE agar broth, and then the intracellular concentration of NADPH (**A**) and transcription of the *catA* and *sodF* genes encoding antioxidant enzymes (**B**) and *glkA* internal standard were analyzed as explained in the text.

In previous reports by other groups [32], it has been suggested that the overexpression of Vhb activates the pentose phosphate pathway through oxidative stress, thereby increasing NADPH production and promoting secondary metabolism. Therefore, the intracellular concentration of NADPH was measured using an HPLC equipped with a supelcosil LC-18-T [25×4.6] column. The mobile phase used a 10% methanol-phosphate buffer solution (0.1 M KH_2PO_4 (pH 6.0): methanol=90:10) with a flow rate of 1.3 ml/min.

As a result, only the *sm*-transformant revealed a significantly enhanced NADPH production (Fig. 4A). In addition, a RT-PCR analysis of the *catA* and *sodF* genes encoding two typical antioxidant enzymes [4] revealed that their transcription was stimulated by the introduction of the *vhb* or *sm* genes, respectively (Fig. 4B). It has also been reported that oxidative stress activates glucose-6-phosphate dehydrogenases (*zwf1* and *zwf2*), which are the first enzymes in the oxidative pentose phosphate pathway (PPP) [3, 29]. Therefore, it would appear that the expression of SM imposed oxidative stress on the cells, thereby enhancing the PPP and increasing the production of NADPH for biosynthesis, which in turn may have positively affected the secondary metabolite production.

Acknowledgement

This work was supported by grant from 21C frontier R&D Programs (Microbial Genomics & Applications Center).

References

1. Alexander, D. F. and P. T. Kallio. 2003. Bacterial hemoglobins and flavohemoglobins: Versatile proteins and their impact on microbiology and biotechnology. *FEMS Microbiol.* **27:** 525–545.
2. Aydin, S. 2003. Menadione knocks out *Vitreoscilla* haemoglobin (Vhb): The current evidence for the role of Vhb in recombinant *Escherichia coli. Biotechnol. Appl. Biochem.* **38:** 71–76.
3. Bollinger, C. J., J. E. Bailey, and P. T. Kallio. 2001. Novel hemoglobins to enhance microaerobic growth and substrate utilization in *Escherichia coli. Biotechol. Prog.* **17:** 798–808.
4. Bruheim, P., M. Butler, and T. E. Ellingsen. 2002. A theoretical analysis of the biosynthesis of actinorhodin in a hyper-producing *Streptomyces lividans* strain cultivated on various carbon sources. *Appl. Microbiol. Biotechnol.* **58:** 735–742.
5. Butler, M. J., P. Bruheim, S. Jovetic, F. Marinelli, P. W. Postma, and M. J. Bibb. 2002. Engineering of primary carbon metabolism for improved antibiotic production in *Streptomyces lividans. Appl. Environ. Microbiol.* **68:** 4731–4739.
6. Cho, Y. H., E. J. Lee, and J. H. Roe. 2000. A developmentally regulated catalase required for proper differentiation and

osmoprotection of *Streptomyces coelicolor*. *Mol. Microbiol.* **35:** 150–160.
7. Choi, E.-Y., E. A. Oh, J.-H. Kim, D.-K. Kang, S. S. Kang, J. Chun, and S.-K. Hong. 2007. Distinct regulation of the *sprC* gene encoding *Streptomyces griseus* protease C from other chymotrypsin genes in *Streptomyces griseus* IFO13350. *J. Microbiol. Biotechnol.* **17:** 81–88.
8. Choi, S.-S., J. H. Kim, J.-H. Kim, D.-K. Kang, S.-S. Kang, and S.-K. Hong. 2006. Functional anaylsis of *sprD* gene encoding *Streptomyces griseus* protease D (SGPD) in *Streptomyces griseus*. *J. Microbiol. Biotechnol.* **16:** 312–317.
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:** 7345–7354.
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. Doumith, M., P. Weingarten, U. F. Wehmeier, K. Salah-bey, B. Benhamou, C. Capdevila, J.-M. Michel, W. Piepersberg, and M.-C. Raynal. 2000. Analysis of genes involved in 6-deoxyhexose biosynthesis and transfer in *Saccharopolyspora erythraea*. *Mol. Gen. Genet.* **264:** 477–485.
12. Erenler, S. O., S. Gencer, H. Geckil, B. C. Stark, and D. A. Webster. 2004. Cloning and expression of the *Vitreoscilla* hemoglobin gene in *Enterobacter aerogenes*: Effect on cell growth and oxygen uptake. *Appl. Biochem. Microbiol.* **40:** 241–248.
13. 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.
14. Geckil, H., S. Gencer, H. Kahraman, and S. O. Erenler. 2003. Genetic engineering of *Enterobacter aerogenes* with the *Vitreoscilla* hemoglobin gene: Cell growth, survival, and antioxidant enzyme status under oxidative stress. *Res. Microbiol.* **154:** 425–31.
15. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557–580.
16. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, D. P. Smith, and J. M. Ward. 1985. *Genetic Manipulation of Streptomyces: A Laboratory Manual*. The John Innes Foundation, Norwich, England.
17. Hu, Y., P. D. Butcher, J. A. Mangan, M. A. Rajandream, and A. R. Coates. 1999. Regulation of *hmp* gene transcription in *Mycobacterium tuberculosis*: Effects of oxygen limitation and nitrosative and oxidative stress. *J. Bacteriol.* **181:** 3486–3493.
18. 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:** 2220–2280.
19. Kallio, P. T., D. J. Kim, P. S. Tsai, and J. E. Bailey. 1994. Intracellular expression of *Vitreoscilla* haemoglobin activates *E. coli* energy metabolism under oxygen-limited conditions. *Eur. J. Biochem.* **219:** 201–208.
20. Khang, Y.-H., I.-W. Kim, Y.-R. Hah, J.-H. Hwangbo, and K.-K. Kang. 2003. Fusion protein of *Vitreoscilla* hemoglobin with D-amino acid oxidase enhances activity and stability of biocatalyst in the bioconversion process of cephalosporin C. *Biotechnol. Bioeng.* **82:** 480–488.
21. Khleifat, K. and M. M. Abboud. 2003. Correlation between bacterial haemoglobin gene (*vgb*) and aeration: Their effect on the growth and a-amylase activity in transformed *Enterobacter aerogenes*. *J. Appl. Microbiol.* **94:** 1052–1058.
22. 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*. *Biotechnology* **8:** 49–53.
23. Khosla, C. and J. E. Bailey. 1988. Heterologous expression of a bacterial hemoglobin improves the growth properties of recombinant *Escherichia coli*. *Nature* **331:** 633–635.
24. 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.
25. Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. *Practical Streptomyces Genetics*. John Innes Foundation, Norwich.
26. Kim, C.-Y., H.-J. Park, and E.-S. Kim. 2006. Functional dissection of sigma-like domain in antibiotic regulatory gene, afsR2, in *Streptomyces lividans*. *J. Microbiol. Biotechnol.* **16:** 1477–1480.
27. 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.
28. Lee, Y., J. Young, H.-J. Kwon, J.-W. Suh, J. Kim, Y. Chong, and Y.. Lim. 2006. AdoMet derivatives induce the production of actinorhodin in *Streptomyces coelicolor*. *J. Microbiol. Biotechnol.* **16:** 965–968.
29. 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. *Biotechnology* **9:** 473–476.
30. Pandolfi, P. P., F. Sonati, R. Rivi, P. Mason, F. Grosveld, and L. Luzzatto. 1995. Targeted disruption of the housekeeping gene encoding glucose-6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J.* **14:** 5209–5215.
31. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning*; 3rd Ed., Cold Spring Harbor, New York, U.S.A.
32. 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.
33. Vasudevan, S. G., W. L. 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.
34. Yang, H.-Y., S.-S. Choi, W.-J. Chi, J.-H. Kim, D.-K. Kang, J. Chun, S.-S. Kang, and S.-K. Hong. 2005. Identification of the sprU gene encoding an additional sprT homologous trypsin-type protease in *Streptomyces griseus*. *J. Microbiol. Biotechnol.* **15:** 1125–1129.