Hemoglobin Biosynthesis in *Vitreoscilla stercoraria* DW: Cloning, Expression, and Characterization of a New Homolog of a Bacterial Globin Gene

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In the strictly aerobic, gram-negative bacterium *Vitreoscilla* strain C1, oxygen-limited growth conditions create a more than 50-fold increase in the expression of a homodimeric heme protein which was recognized as the first bacterial hemoglobin (Hb). The recently determined crystal structure of *Vitreoscilla* Hb has indicated that the heme pocket of microbial globins differs from that of eukaryotic Hbs. In an attempt to understand the diverse functions of Hb-like proteins in prokaryotes, we have cloned and characterized the gene (*vgb*) encoding an Hb-like protein from another strain of *Vitreoscilla*, *V. stercoraria* DW. Several silent changes were observed within the coding region of the *V. stercoraria* vgb gene. Apart from that, *V. stercoraria* Hb exhibited interesting differences between the A and E helices. Compared to its Hb counterpart from *Vitreoscilla* strain C1, the purified preparation of *V. stercoraria* Hb displays a slower autooxidation rate. The differences between *Vitreoscilla* Hb and *V. stercoraria* Hb were mapped onto the three-dimensional structure of *Vitreoscilla* Hb, which indicated that the four changes, namely, Ile7Val, Ile9Thr, Ile10Ser, and Leu62Val, present within the *V. stercoraria* Hb fall in the region where the A and E helices contact each other. Therefore, alteration in the relative orientation of the A and E helices and the corresponding conformational change in the heme binding pocket of *V. stercoraria* Hb can be correlated to its slower autooxidation rate. In sharp contrast to the oxygen-regulated biosynthesis of Hb in *Vitreoscilla* strain C1, production of Hb in *V. stercoraria* has been found to be low and independent of oxygen control, which is supported by the absence of a fumarate and nitrate reductase regulator box within the *V. stercoraria* vgb promoter region. Thus, the regulation mechanisms of the Hb-encoding gene appear to be quite different in the two closely related species of *Vitreoscilla*. The relatively slower autooxidation rate of *V. stercoraria* Hb, lack of oxygen sensitivity, and constitutive production of Hb suggest that it may have some other function(s) in the cellular physiology of *V. stercoraria* DW, together with facilitated oxygen transport, predicted for earlier reported *Vitreoscilla* Hb.

Hemoglobins (Hbs) or Hb-like proteins have been detected from organisms representing all kingdoms of life, such as vertebrates, invertebrates, higher plants, fungi, and bacteria (10), indicating a widespread requirement for this protein in cellular metabolism. Although the role of Hbs in higher eukaryotes as facilitators of oxygen diffusion is well established, the cellular function of the Hb and Hb-like flavohemoproteins in prokaryotes is still unknown and addresses an intriguing question yet to be solved. The Hb produced by the gram-negative bacterium *Vitreoscilla* strain C1, a member of the Beggiatoa family, has been extensively studied with respect to its structural and biochemical characteristics (5, 23, 29, 32, 33). However, the exact role that this protein plays in the cellular metabolism of its host has not been fully understood. It has been speculated, based on its oxygen binding kinetics (22, 23), that the function of *Vitreoscilla* Hb is to facilitate oxygen flux to the vigorously respiring membranes of *Vitreoscilla*, which is an obligate aerobe but is found in hypoxic habitats. This assumption is based on the fact that the cellular level of heme in *Vitreoscilla* increases many fold when the organism faces oxygen limitation (2). Recently, two-domain oxygen binding proteins carrying a heme binding N-terminal segment and flavin binding C-terminal segments have been reported for several bacteria and yeasts (3, 4, 24, 31, 35) and have been designated as flavohemoglobins. The functions of these bacterial globins have not been conclusively demonstrated, although available experimental evidence suggests that they may have multiple functions in the cellular metabolism of their host. Suggested roles for these proteins are facilitation of oxygen transport and storage (33), enhancement of energy status of cells (14), oxygen and nitrogen compound sensing (24), modulation of the redox status of the cell (4), and biological nitrogen fixation (25).

*Vitreoscilla* Hb is a homodimeric molecule, consisting of two identical subunits of 15.7 kDa along with two protohemes IX per molecule. The three-dimensional structure of *Vitreoscilla* Hb, obtained through X-ray crystallography (29), and recent studies of its site-directed mutants (8) have indicated that the structural conformation of the distal heme pocket of *Vitreoscilla* Hb is drastically altered by the perturbations in the E and F helices. The putative function of *Vitreoscilla* Hb is to facilitate respiration at low oxygen concentration by transporting oxygen to the terminal oxidases by the mechanism of facilitated diffusion (33). Additionally, it has been shown that *Vitreoscilla* Hb can support aerobic growth in *Escherichia coli* with impaired terminal oxidases (9). Its unique oxygen binding properties, especially its rate constant for oxygen dissociation ($k_{off}$), which is unusually large, are presumed to contribute to its postulated role.

The gene encoding the globin part of *Vitreoscilla* Hb has been cloned and expressed in *E. coli* (5, 15). Initial studies of the *vgb* gene expression and promoter activity in *E. coli* indicate that the expression of the *vgb* gene is regulated by oxygen (6). Conclusive evidence for this observation has been ob-
tained through studies of transcriptional fusion with the reporter genes cat and xylE (7, 17). Fumarate and nitrate reductase regulator (FNR [27]) and cyclic AMP receptor protein (CRP [19]) have been shown to participate coordinately in the regulation of oxygen specificity of the vgb promoter (13). The presence of Vitreoscilla Hb in a heterologous microbial system has been shown to facilitate the growth of its host at low oxygen concentrations (16). This effect is mediated through upgrading the oxygen utilization properties of Vitreoscilla Hb-carrying organisms, resulting in overall improvement of their energy status (14). Evidence for partial export of Vitreoscilla Hb into the periplasmic space of Vitreoscilla and E. coli has been presented (18) which suggests that its localization may provide an advantage by generating additional oxygen flux to the respiratory apparatus that may be physiologically relevant under oxygen limitation for this obligate aerobe.

Until recently, only one strain of Vitreoscilla, i.e., C1, had been studied with respect to Hb biosynthesis, and virtually nothing is known about other Hb-producing Vitreoscilla strains. While working with Vitreoscilla, we found that Vitreoscilla ster-
coraria synthesizes a very small amount of Hb. Exposure to low-oxygen conditions did not affect the relative level of Hb production, unlike in Vitreoscilla strain C1. This prompted us to explore the mechanism of Hb biosynthesis in this organism. In this communication, we report some interesting differences in the expression patterns of the bacterial globin gene and Hb biosynthesis in the two closely related species of Vitreoscilla. The gene (vgb) encoding the globin part of V. stercoraria has been cloned and expressed in E. coli, which revealed that the Hb biosynthesis and the regulation pattern of the globin gene are quite distinct in V. stercoraria compared to those in Vitreoscilla strain C1.

MATERIALS AND METHODS

Bacterial strains and plasmids. V. stercoraria was obtained from D. A. Web-
ter (Illinois Institute of Technology, Chicago, Ill.). One of the clonal isolates which consistently produced small round colonies on PYA (1% peptone, 1% yeast extract, 0.02% sodium acetate [pH 7.8]) was selected and designated as V. stercoraria DW. This strain was used for further experiments. All other strains and plasmids used in this study are listed in Table 1. Conditions for the culture of E. coli strains under different oxygen levels were essentially the same as described previously (6, 7). For shake flask experiments, 50 ml of culture broth in a 250-ml baffled flask was kept at 250 rpm to achieve well-aerated conditions, whereas for the low-aeration conditions a 150-ml cell culture was inoculated into a 250-ml flask and kept at 100 rpm. Wherever required, a precise level of dissolved oxygen (DO) was maintained by bubbling a mixture of air and nitrogen through the medium with a sterile filter.

Chemicals and enzymes. All restriction endonucleases, DNA-modifying en-
zymes, and the Packagene kit were obtained from Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.). DNA sequencing was done with a Se-
quenase version 2.0 kit (U.S. Biochemicals). All in vitro DNA manipulations were done according to a standard protocol (26). For detection of the Hb-
coding region, a 0.45-kb AflIII-Msal fragment of plasmid pUC8:16 (5), carrying a part of the vgb gene, was used as a probe. Localization and cloning of the V. stercoraria vgb gene. In an attempt to localize an Hb-encoding (vgb) gene on the V. stercoraria DW genome, we first performed Southern blotting analysis by using the vgb gene (5) as a probe. The pattern of vgb cross-reacting bands on the V. stercoraria genome is shown in Fig. 1. A genomic library of V. stercoraria DW was constructed on the cosmid vector pHC79 by following the standard cosmid cloning procedure (11). Utilizing the vgb gene as a probe, an Hb-positive clone was retrieved from the pool of the cosmid clone bank. This cosmid clone was named pMUC4. It carried a 40-kb genomic fragment on the cosmid vector pH79. A detailed restriction map of pMUC4 and its derivatives is given in Fig. 3A. After subcloning, a 1.1-kb EcoRI-SalI fragment carrying the entire V. stercoraria vgb gene was separated from the rest of the DNA segment and cloned on pH79, resulting in the construct pM11.

Preparation of Vitreoscilla Hb antiserum and Western blot analysis of bacte-
rial Hb. Hb expressed in E. coli was partially purified from the cell extracts as described previously (5, 34). A partially purified preparation of Vitreoscilla Hb was then run on a preparatory sodium dodecyl sulfate-polyacrylamide (15%) gel electrophoresis. A 15.7-kDa band, corresponding to Vitreoscilla Hb, was eluted from gel. This preparation was used to generate rabbit polyclonal anti-globin antibodies. Western blotting was done according to the standard protocol (30).

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>E. coli</td>
<td>hsdR24(lac-proAB) proAB lacZM15</td>
<td>Pharmacia</td>
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<td>XL-Blue</td>
<td>recA1 hsdR17 lacI2 M15 Tn10(ter)</td>
<td>Stratagene</td>
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<tr>
<td>LE392</td>
<td>hsdRS14 lacY1</td>
<td>Promega</td>
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<td>MC1000</td>
<td>lac crp mutant</td>
<td>13</td>
</tr>
<tr>
<td>JRG1728</td>
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<td>13</td>
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<tr>
<td><strong>Vitreoscilla</strong></td>
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<td></td>
</tr>
<tr>
<td>strain C1</td>
<td>vgb</td>
<td>5</td>
</tr>
<tr>
<td>DW</td>
<td>vgb</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>PHC79</td>
<td>Ap+ Te+</td>
<td>11</td>
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<tr>
<td>pUC8:15</td>
<td>Ap+ pUC8 carrying vgb on a 2.2-kb HindIII fragment</td>
<td>5</td>
</tr>
<tr>
<td>pUC8:16</td>
<td>Ap+, pUC8 carrying vgb on a 1.4-kb HindIII-SalI fragment</td>
<td>5</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap+ Te+</td>
<td>New England Biolabs</td>
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</table>

*Vitreoscilla strain C1 and V. stercoraria DW were obtained from the laboratory of D. A. Webster, Illinois Institute of Technology, Chicago.*

### Determination of heme and Hb concentration in vivo

Total cellular heme concentration was measured by preparing the pyridine hemochromogen of the heme extract as described by Boerman and Webster (2). Carbon monoxide difference spectra (courtesy of D. A. Webster) of whole cells were recorded at room temperature with a Cary model 210 spectrometer. After reduction with sodium dithionite, CO was bubbled through the sample cuvette at one bubble per second for 2 min, and difference spectra were recorded from 400 to 500 nm. Cuvettes of 1-cm path length were employed for all spectral measurements. The
cellular concentration of Vitreoscilla Hb was calculated as described previously (5).

Cell fractionation. To check the localization of Hb in E. coli and Vitreoscilla, total cellular proteins were separated into periplasmic and cytoplasmic fractions. Periplasmic proteins were released through chloroform treatment (1). The cytoplasmic protein fraction was obtained by subjecting the osmotically shocked cells to sonication in 0.5 mM MgCl2 at 0°C with four intermittent 20-s pulses at maximum output.

Purification of Hb from E. coli and determination of autooxidation rate. Purified preparations of Vitreoscilla Hb and V. stercoraria Hb were obtained from their respective natural hosts or from E. coli cells carrying the vgb and V. stercoraria vgb genes, respectively, according to the established protocol (22). During preparation of Hb from E. coli, it was observed that E. coli cells carrying the V. stercoraria vgb gene displayed a pinkish-brown color compared to the pink tinge generally observed in the case of the vgb gene. To check that this difference is related to their autooxidation pattern, we determined the rate of autooxidation of V. stercoraria Hb and Vitreoscilla Hb. The autooxidation rate was determined as the rate of conversion of the oxygenated form of Hb into the reduced form. Vitreoscilla Hb can be chemically reduced by excess dithionite and oxidized by ammonium persulfate. The spectrum of the oxygenated form of the reduced Hb has absorption maxima at 414 nm (OD 600) of 1.0. In contrast, the level of aeration (Fig. 2 and Table 2). In contrast to V. stercoraria DW, which was found to have a brownish-pink color, unlike Vitreoscilla strain C1, which normally gives a bright pink color. To check that this is due to the difference in the relative amounts of ferric and ferrous forms of the protein, we compared the rates of autooxidation of V. stercoraria Hb and Vitreoscilla Hb at 37°C in air-equilibrated buffer (Table 3) by simultaneously measuring of oxygen uptake and monitoring the pattern of spectral changes at 414 nm (see Materials and Methods). Results indicated that the rate of Vitreoscilla Hb autooxidation was about one and a half times faster than that of V. stercoraria Hb, indicating that these two proteins may have some differences in their oxygen binding density reached only to 0.4. This indicated that both Vitreoscilla strains are able to cope with oxygen-stressed conditions, and Hb present, in a relatively lower level, in V. stercoraria may be sufficient for survival under low-oxygen conditions. Respiratory activities of these two strains were compared by monitoring the specific oxygen consumption rate in samples withdrawn intermittently from the shake flask cultures. In the well-aerated cultures, there were no significant differences in the specific oxygen consumption rates of these two strains. With less aeration, however, the oxygen utilization rate of V. stercoraria was relatively slow, particularly at the later stages of growth (Table 2).

RESULTS

Pattern of Hb biosynthesis in V. stercoraria DW. Vitreoscilla strain C1 is the most-studied strain with respect to Hb production in prokaryotes. In order to study the function of Hb in single-celled organisms, we analyzed another strain of Vitreoscilla, i.e., V. stercoraria DW. During our preliminary characterization, through total heme analysis, CO difference spectra, and Western blotting, V. stercoraria DW was found to produce a very small amount of Hb irrespective of changes in the level of aeration (Fig. 2 and Table 2). Spectral analysis of whole cells of both strains indicated that the amount of Hb synthesized by V. stercoraria DW was less than half (during late exponential phase, 8-h-grown culture) of that synthesized by strain C1 (Fig. 2). Under hypoxic conditions, the level of Vitreoscilla Hb increased to 34.6 nmol/g (wet weight) in Vitreoscilla strain C1, which was four times higher than that of V. stercoraria (Table 2). In contrast to Vitreoscilla strain C1, the relative levels of heme and Hb did not change much in V. stercoraria during low-oxygen conditions. However, the growth patterns of the two strains (Table 2), at high and low aeration, were found to be collateral. E. coli was also grown under high and low aeration for comparison. Under microaerobic conditions, Vitreoscilla strains grew to a maximum optical density at 600 nm (OD_{600}) of 1.0. In contrast, E. coli grew poorly under similar microaerobic conditions, where its maximum optical absorption density reached only to 0.4. This indicated that both Vitreoscilla strains are able to cope with oxygen-stressed conditions, and Hb present, in a relatively lower level, in V. stercoraria may be sufficient for survival under low-oxygen conditions. Respiratory activities of these two strains were compared by monitoring the specific oxygen consumption rate in samples withdrawn intermittently from the shake flask cultures. In the well-aerated cultures, there were no significant differences in the specific oxygen consumption rates of these two strains. With less aeration, however, the oxygen utilization rate of V. stercoraria was relatively slow, particularly at the later stages of growth (Table 2).

Purification and characterization of Hb from Vitreoscilla strain C1 and V. stercoraria DW. Earlier studies of Vitreoscilla Hb indicated that it is relatively more autooxidizable than other Hbs and myoglobins, and this property has been correlated with its unique heme pocket. When cell lysates of both strains were compared, V. stercoraria DW exhibited a brownish-pink color, unlike Vitreoscilla strain C1, which normally gives a bright pink color. To check that this is due to the difference in the relative amounts of ferric and ferrous forms of the protein, we compared the rates of autooxidation of V. stercoraria Hb and Vitreoscilla Hb at 37°C in air-equilibrated buffer (Table 3) by simultaneously measuring of oxygen uptake and monitoring the pattern of spectral changes at 414 nm (see Materials and Methods). Results indicated that the rate of Vitreoscilla Hb autooxidation was about one and a half times faster than that of V. stercoraria Hb, indicating that these two proteins may have some differences in their oxygen binding density reached only to 0.4. This indicated that both Vitreoscilla strains are able to cope with oxygen-stressed conditions, and Hb present, in a relatively lower level, in V. stercoraria may be sufficient for survival under low-oxygen conditions. Respiratory activities of these two strains were compared by monitoring the specific oxygen consumption rate in samples withdrawn intermittently from the shake flask cultures. In the well-aerated cultures, there were no significant differences in the specific oxygen consumption rates of these two strains. With less aeration, however, the oxygen utilization rate of V. stercoraria was relatively slow, particularly at the later stages of growth (Table 2).
TABLE 2. Effect of aerobic and microaerobic conditions on growth and total Hb content of Vitreoscilla strain C1 and V. stercoraria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth rate conditions</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>No. of CFU/ml</th>
<th>Total Hb content (nmol/g [wet wt])</th>
<th>Oxygen consumption (μmol of O&lt;sub&gt;2&lt;/sub&gt;/min/10&lt;sup&gt;10&lt;/sup&gt; cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreoscilla strain C1</td>
<td>Aerobic</td>
<td>1.8</td>
<td>5 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>8.2 ± 2</td>
<td>1.6 ± 0.4</td>
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<tr>
<td></td>
<td>Microaerobic</td>
<td>1.0</td>
<td>2 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>34.6 ± 6</td>
<td>1.0 ± 0.35</td>
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<tr>
<td>V. stercoraria DW</td>
<td>Aerobic</td>
<td>1.9</td>
<td>8 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>4.8 ± 2</td>
<td>1.3 ± 0.32</td>
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<tr>
<td></td>
<td>Microaerobic</td>
<td>0.9</td>
<td>1 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6.4 ± 2</td>
<td>0.6 ± 0.25</td>
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<tr>
<td>E. coli</td>
<td>Aerobic</td>
<td>1.2</td>
<td>1 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1 ± 0.15</td>
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<tr>
<td></td>
<td>Microaerobic</td>
<td>0.4</td>
<td>5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.05</td>
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</table>

* For aerobic growth, shake flask cultures were grown under well-aerated conditions (50 ml of culture in a 250-ml baffled flask at 250 rpm) at 37°C, whereas for microaerobic conditions, shake flask cultures were grown under low aeration (150 ml of culture in a 250-ml flask at 100 rpm) at 37°C. Cells were harvested after 8 h of growth. Values given in the table are averages of four to five individual measurements. Hb content and oxygen consumption values are means ± standard deviations. *ND, not determined.

characteristics. However, the CO binding patterns of both species of Hb were more or less similar.

Cloning, expression, and characterization of the Hb-encoding gene (vgb) from *V. stercoraria* DW. To understand the differences between the two species of *Vitreoscilla* Hb at the molecular level, the Hb-encoding gene (vgb) has been cloned in *E. coli* and characterized. Previously reported results for *Vitreoscilla* strain C1 indicate that it yields 2.2-, 8-, and 12-kb vgb gene-carrying genomic fragments after *Hind*III, EcoRI, and *Pst*I digestion, respectively (5). However, in the case of *V. stercoraria* DW, *Hind*III and EcoRI yielded a single 4- and a single 10-kb vgb-positive band, respectively. Similarly, the position of the vgb gene on *Bam*HI- and *Pst*I-restricted fragments was different from that on the C1 strain (Fig. 1). By following the strategy given in Fig. 3A, a 1.1-kb genomic fragment carrying the entire *V. stercoraria* vgb gene along with its regulatory regions was subcloned on pHCH79, resulting in the plasmid construct pMJ1 (see Materials and Methods). *E. coli* cells carrying this plasmid construct exhibited a light red tinge, indicating the presence of Hb inside the cell. The presence of Hb in pMJ1-carrying cells was further checked through CO-difference spectral analysis of the whole cells and Western blotting of cytoplasmic proteins. Both indicated the presence of Hb-like protein in pMJ1-carrying cells, whereas control cells did not give any positive signal through these tests. This finding demonstrates that pMJ1 is able to encode an Hb-like protein in *E. coli*.

In vivo functional state of *V. stercoraria* Hb in *E. coli*. In the actively respiring cells of *Vitreoscilla* strain C1, the predominant form of *Vitreoscilla* Hb is oxygenated Hb, which is converted into the ferrous (reduced) form of Hb when cells are exposed to anaerobic conditions because of depletion of oxygen by respiration. *Vitreoscilla* Hb has a very high autooxidation rate, and a flavoprotein designated as NADH-Met Hb reductase presumably keeps it in the physiologically functional ferrous form. When oxygen was bubbled through the actively growing cells, absorption peaks appeared at 577, 543, and 418 nm, which was very similar to the case found in *Vitreoscilla* strain C1 (Fig. 3B). Bubbling of oxygen through the cells reduced by NADH increased the intensity of these absorption bands (Fig. 3B) in the difference spectra, indicating the presence of both reduced and oxygenated forms of *V. stercoraria* Hb inside the cell. However, the return of the oxygenated form to the reduced form, due to cellular respiration, was relatively slower in the case of *V. stercoraria*. The autooxidation patterns of cloned *Vitreoscilla* Hb and *V. stercoraria* Hb obtained from *E. coli* were more or less similar to those observed in their native host (Table 3). The rate of respiration of *E. coli* cells carrying the vgb gene was slightly higher (1.6 mol/min/10<sup>10</sup> cells) than that of *E. coli* cells carrying the *V. stercoraria* vgb gene (0.9 mol/min/10<sup>10</sup> cells) in late log phase (6-h-grown culture).

Nucleotide sequence analysis of the *V. stercoraria* vgb gene and its flanking regions. In order to characterize the vgb gene at a molecular level, the nucleotide sequence of the entire vgb gene and its flanking region was determined, which is shown in Fig. 4. The 1.1-kb genomic insert, carrying the entire *V. stercoraria* vgb gene on the plasmid pMJ1, was utilized for the sequence analysis with oligomers designed on the basis of the known sequence of the vgb gene (see Materials and Methods). Analysis of the *V. stercoraria* vgb gene sequence and its deduced amino acid sequence indicated several interesting features. The major differences in amino acid sequence between *Vitreoscilla* Hb and *V. stercoraria* Hb are the replacements in the A and E helices at the A7 (Ile to Val), A8 (Asn to Asp), A9 (Ile to Thr), A10 (Ile to Ser), E3 (Glu to Ala), and E16 (Leu to Val) positions. Other changes in *V. stercoraria* Hb are at the C5, F2, HC3, and HC4 positions (Fig. 5A). Most of the changes in amino acids are due to single-base alterations. Besides that, several silent changes within the *V. stercoraria* vgb gene have been observed compared to the *Vitreoscilla* vgb gene (Fig. 4), which reflects the genetic difference between these species.
two species of *Vitreoscilla*. *Vitreoscilla* strain C1 Hb has glutamine in place of the normally conserved histidine at the E7 position, which stabilizes the iron-oxygen complex through hydrogen bonding. The presence of glutamine at the E7 position in *Vitreoscilla* Hb has been correlated with its low oxygen affinity (33). Thus, it was of utmost interest to check the corresponding amino acid in *V. stercoraria* Hb. Indeed, at the E7 position, *V. stercoraria* Hb also showed the occurrence of a glutamine. The other important amino acid which remained unchanged from *Vitreoscilla* Hb was leucine at E11, despite valine being the conserved amino acid in most globins. The invariant residues histidine at F8 and phenylalanine at CD1, conserved throughout the globin phylogeny, were unchanged in *V. stercoraria* Hb. Apart from these changes, the aminoterminal segments of *Vitreoscilla* Hb and *V. stercoraria* Hb exhibit changes in the pattern of the amino acid sequence (Fig. 5A) which significantly altered the hydrophobicity of the aminoterminal region of *V. stercoraria* Hb (Fig. 5B).

**Primary structure of regulatory region.** Sequence comparison of the flanking upstream region of the *V. stercoraria vgb* gene with that of the *Vitreoscilla* strain C1 vgb gene indicated distinct variation in the primary structure and organization of the promoter regions of these two genes (Fig. 4). The analysis of the nucleotide sequence (Fig. 4B) of the flanking region of the *V. stercoraria vgb* gene indicated a good Shine-Dalgarno sequence (AAGGAAGA) and a consensus Pribnow box (TAATAAT) very similar to that present in the vgb promoter. A consensus 235 sequence (TTG** is a good 235 sequence, and there are two such motifs in this region. The spacing between the putative 240 and 235 regions is relatively small (10 to 12 bp). An inverted repeat sequence (CCATAC TGAT GTATGG) has been identified within the promoter region of the vgb gene around the 240 region (Fig. 4), which is absent in the promoter region of the *V. stercoraria vgb* gene. It is interesting to note that the sequences of these two promoters exhibit close similarity up to this 40 region. After that, significant sequence divergence occurs within the upstream regions of these two genes. It is possible that some rearrangement within the promoter region of the *V. stercoraria vgb* gene has occurred in *V. stercoraria* DW which in turn has changed the regulatory characteristics of the *V. stercoraria vgb* gene promoter. The FNR binding site (TTTGA....CAAT..) identified within the upstream region of the vgb gene (13) was not obvious in the *V. stercoraria vgb* gene promoter, which indicated that this gene promoter may be oxygen insensitive, unlike the vgb gene promoter. Scanning of the region of the *V. stercoraria*
Vitreoscilla stercoraria vgb gene promoter further upstream indicated a possible CRP box (TGTGA....AAAA) (Fig. 4) which closely resembled the consensus CRP binding site, TGTGA....CAAA (19). This finding implies that the V. stercoraria vgb gene is primarily under the control of cAMP-CRP regulation, unlike the vgb gene promoter. In contrast to its natural host, V. stercoraria, the V. stercoraria vgb gene expresses strongly in E. coli, which indicates that the regulation mechanisms of the V. stercoraria vgb genes in these two hosts may have some differences.

**Oxygen-independent regulation of the vgb gene in V. stercoraria.** In E. coli, the V. stercoraria vgb gene is expressed constitutively. The relative level of Hb did not change significantly under oxygen-limiting conditions (Table 2). Our findings thus indicate that it is not regulated by oxygen, unlike the earlier-reported vgb gene (7, 17). Analysis of the V. stercoraria vgb promoter sequence revealed a putative CRP binding site (Fig. 4) within this region. To verify whether CRP plays any role in regulating the production of V. stercoraria Hb, a preliminary experiment was done to check the effect of glucose on the cellular level of V. stercoraria Hb. glucose was added to the growth medium to a final concentration of 0.5%, and production of V. stercoraria Hb was compared to that in cells grown in the absence of glucose. There was a distinct red tinge to the pellet in the control, which was white in cells grown in the presence of glucose, indicating that V. stercoraria Hb was not being produced. To confirm this observation, cell lysates from these cultures were electrophoresed and Western blotted with polyclonal antibodies raised against Vitreoscilla Hb. Densitometric scanning of proteins, separated through polyacrylamide gel electrophoresis, indicated a 50 to 60% reduction in the level of V. stercoraria Hb in cells grown in the presence of 0.5% glucose, compared to the control cells (Fig. 6B). Similarly, a CRP-negative mutant of E. coli, MC1000, carrying the plasmid vector pMJ1 produced a 40 to 50% lower level of V. stercoraria Hb compared to the control strain, whereas expression of the V. stercoraria vgb gene in an E. coli mutant lacking FNR was comparable to that of the control. This observation ruled out the possibility of participation of FNR in V. stercoraria Hb production and indicated that besides other factors, CRP plays an important role in the control of V. stercoraria vgb expression. The relevance of CRP regulation in Hb production in V. stercoraria is not obvious at present, and the possibility of another regulatory circuit cannot be ruled out.

**Structural organization of Vitreoscilla Hb and V. stercoraria Hb.** Although Vitreoscilla Hb and V. stercoraria Hb share close similarity in their primary structures, the two exhibit subtle differences in their autooxidation patterns which reflect some alteration in their oxygen binding patterns. Over the length of the 146-residue-long polypeptide, the two Hbs differ by only 11 residues. The amino acid alignment of the two Hbs shows that the residue differences are spread in different helices of the proteins. To look into the relevance of these changes, differences in V. stercoraria Hb were mapped onto the three-dimensional model of Vitreoscilla Hb (Fig. 5C). Among the 11 amino acid replacements in the two Hbs when going from Vitreoscilla Hb to V. stercoraria Hb, Asn8Asp, Lys30Gln, Glu49Ala, Lys79Gln, and Ala93Arg are fully surface exposed. Therefore, because of their solvent exposure and the lack of interactions...
between these side chains and any other atom, these replacements are not expected to make any significant changes in the three-dimensional structure of *V. stercoraria* Hb. The other four changes, namely, Ile7Val, Ile9Thr, Ile10Ser, and Leu62Val, fall in the region where the A and E helices contact each other (Fig. 5C). Significantly, all of these four replacements in amino acid residues reduce the size of the side chain volume considerably. We therefore expect the A and E helices to reorient themselves in order to minimize or eliminate cavities formed because of these mutations. The recently determined crystal structure of *Vitreoscilla* Hb (29) has indicated that the hydrophobic patch of A helix, covering residues Ile7 to Ile10, anchors the N-terminal helix to the EF corner of the globin fold. Therefore, reorientation of the helices is likely to perturb the distal heme binding pocket, which may affect the oxygen affinity of *V. stercoraria* Hb. Thus, our hypothesis points towards perturbation in the distal heme pocket of *V. stercoraria* Hb, due to reorientation of the A and E helices. This change may result in a decrease in the rate of autooxidation of *V. stercoraria* Hb by restricting entry of solvent into the heme pocket or by partially stabilizing the bound oxygen.

**DISCUSSION**

Many microorganisms synthesize globin-like proteins of unknown function (3, 4, 21, 24, 25). It is not yet clear whether proteins belonging to this group have a function as an oxygen transporter, oxygen sensor, or nitrogen compound sensor; have a role in oxidative stress; or have none of these functions. Understanding the pattern of Hb biosynthesis in different microbial systems may unveil the functional relevance of this ubiquitous group of proteins in prokaryotes. Biosynthesis of the *Vitreoscilla* single-domain globinlike protein (Hb) is enhanced dramatically in response to oxygen deprivation (6, 7, 17), which led to the belief that its putative function is to capture oxygen and feed it to terminal oxidases under oxygen-limiting conditions. The recently determined three-dimensional structure of *Vitreoscilla* Hb has shown that it has an unusual distal heme pocket which probably modulates its function in facilitating oxygen diffusion and electron transfer (29). Except for *Vitreoscilla* strain C1, no attempt has been made to characterize Hb from other *Vitreoscilla* strains, which could provide additional information regarding the structure-function relationship of this unique Hb.

In this study, we have examined the pattern of Hb biosynthesis in two closely related species of *Vitreoscilla*. Despite close similarities, *Vitreoscilla* strain C1 and *V. stercoraria* exhibit subtle genetic differences. This work highlights some interesting differences between the physiology of Hb production and functional characteristics of Hb produced in *Vitreoscilla* strain C1 and *V. stercoraria*. The cellular level of Hb increases several fold under hypoxic conditions in *Vitreoscilla* strain C1 (7). The pattern of the vgb gene expression and studies of vgb-xylE
fusion in *E. coli* indicated that biosynthesis of *Vitreoscilla* Hb is regulated at the transcriptional level (6, 7). In contrast to these observations, it was noted that in *V. stercoraria*, production of Hb is not markedly affected by the supply of oxygen. Additionally, *V. stercoraria* Hb exhibited a relatively slow autooxidation pattern compared to *Vitreoscilla* Hb. To understand the differences between these two species of *Vitreoscilla* Hb, we isolated the gene encoding *V. stercoraria* Hb; studies indicated that apart from differences in the upstream regulatory region, the *V. stercoraria* *vgb* gene displayed some changes in the coding region as well. These differences were mapped onto the three-dimensional model of *Vitreoscilla* Hb. Out of eleven amino acid residue replacements in *V. stercoraria* Hb, three amino acid residues (i.e., Ile7Val, Ile9Thr, and Ile10Ser) fall where the N-terminal helix anchors with the EF corner of the heme pocket (Fig. 5C). Also, replacement of a leucine at E16 by a valine in the distal heme pocket of *V. stercoraria* Hb may perturb the conformation of the oxygen binding pocket. Significantly, all of these four changes are at the A-E contact region and may indirectly affect the heme pocket by means of reducing the size of the side chain volume considerably. The autooxidation of Hb with oxygen bound to the heme is thought to be determined by the accessibility of the heme pocket to an exogenous reagent able to induce oxidation. Therefore, the lower autooxidation rate of *V. stercoraria* Hb may be due to lower accessibility of catalysts, such as water or other nucleophiles. It has been observed that the oxygen affinity of various bacterial Hbs varies widely despite significant sequence similarity in the heme binding domain. For example, the dissociation constants for the *Candida norvegensis* and *E. coli* HMP are 2 × 10⁻⁵ and 2 × 10⁻⁶, respectively, compared to 7.2 × 10⁻² in the case of *Vitreoscilla* strain C1 (4, 33). *V. stercoraria* globin has changes within the heme binding pocket at the E3, E11, and F2 positions. It is possible that the changes identified above within *V. stercoraria* Hb might have led to a change in the oxygen binding characteristic of this protein. In contrast to oxygen-regulated biosynthesis of Hb in *Vitreoscilla* strain C1, it was noted that in *V. stercoraria*, production of Hb is not markedly affected by the supply of oxygen. Constitutive production of Hb at a relatively low level suggests that its continual presence in the cell may be required for *V. stercoraria* DW. *Vitreoscilla* species are usually associated with decaying plant material, cow dung, cyanobacterial mats present in waterlogged rice fields, and stagnant ponds (28). These habitats are generally deprived of oxygen availability and remain oxygen deficient for longer periods of time. It is perhaps possible that the natural niche of *V. stercoraria* DW is different from that of *Vitreoscilla* strain C1 such that it never encounters abundant oxygen conditions. The need for an oxygen switch thus becomes less apparent. Alternatively, it may be required for some other physiological function(s), apart from oxygen transport.

In the light of the present findings, questions may arise about the possible relevance of this mode of *V. stercoraria* Hb biosynthesis. The probable explanation is that the cellular function of *V. stercoraria* Hb is different from that of *Vitreoscilla* Hb. *Vitreoscilla* Hb from *Vitreoscilla* strain C1 possibly plays a role as an oxygen transporter. This presumption is based on the following facts. (i) The natural habitat of *Vitreoscilla* strain C1 is oxygen poor, and the presence of Hb helps it to grow well under microaerobic conditions through enhanced production of Hb under low-oxygen conditions. (ii) The rate constant of *Vitreoscilla* Hb for oxygen dissociation is unusually large, which allows faster delivery of oxygen to the respiring membranes. (iii) *Vitreoscilla* Hb is translocated into the periplasmic space, which is well suited to transfer oxygen to the respiring membranes. In contrast, Hb from *V. stercoraria* possibly acts as an oxygen buffer and supplier. Reasons to support this view are as follows. (i) *V. stercoraria* also thrives in an oxygen-poor environment. (ii) The synthesis of *V. stercoraria* Hb is constitutive and low and is not regulated by oxygen. (iii) It does not carry a highly hydrophobic N terminus, and unlike *Vitreoscilla* Hb, its cellular localization is mainly cytoplasmic, suggesting that its presence in close vicinity of respiring membranes may not be required in *V. stercoraria*. (iv) Conformation of the distal heme pocket of *V. stercoraria* Hb differs from that of *Vitreoscilla* Hb, and its autooxidation is slower than that of *Vitreoscilla* Hb. (v) Unlike *Vitreoscilla* strain C1, the respiratory activity of *V. stercoraria* is not very high under hypoxic conditions. Further experiments are under way to generate a *V. stercoraria* Hb knockout strain to get an insight into the functional role of Hb in *V. stercoraria*.

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