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 *Beggiaota* 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 transfer 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 (kₐ), 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 low 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 sterco-raria 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. sterco-raria 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. sterco-raria compared to those in Vitreoscilla strain C1.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** V. sterco-raria was obtained from D. A. Webster (Illinois Institute of Technology, Chicago, Ill.). One of the clonal isolates which consist ofed small round colonies on PYA (1% peptone, 1% yeast extract, 0.02% sodium acetate [pH 7.8]) was selected and designated as V. sterco-raria 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 enzymes, and the Packagene kit were obtained from Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.). DNA sequencing was done with a Sequenase 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-encoding region, a 0.45-kb Afl-I fragment carrying the entire vgb encoding region, a 0.45-kb Afl-I fragment with the 0.45-kb Afl-I fragment of plasmid pUC8:16 (5), carrying a part of the vgb gene, was used as a probe.

**Localization and cloning of the V. sterco-raria vgb gene.** In an attempt to localize an Hb-encoding (vgb) gene on the V. sterco-raria 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. sterco-raria genome is shown in Fig. 1. A genomic library of V. sterco-raria 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 pHC79. It carried a 40-kb genomic fragment on the cosmid vector pHC79. A detailed restriction map of pHC79 and its derivatives is given in Fig. 3A. After subcloning, a 1.1-kb EcoRI-Sall fragment carrying the entire V. sterco-raria vgb gene was separated from the rest of the DNA segment and cloned on pHC79, resulting in the construct pM1.

**Preparation of Vitreoscilla Hb antiserum and Western blot analysis of bacterial 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 the gel. This preparation was used to generate rabbit polyclonal antoglobin antibodies. Western blotting was done according to the standard protocol (30).

**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.

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**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>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>hsdR24(lac-proAB) proAB lacZM15</td>
<td>Pharmacia</td>
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<td></td>
<td>recA1 hsdR17 lacI2 M15 Tn10(tet)</td>
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<td>JRG1728</td>
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<tr>
<td>Vitreoscilla vgb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>strain C1</td>
<td></td>
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</tr>
<tr>
<td>V. sterco-raria vgb</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>DW</td>
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<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-Sall fragment</td>
<td>5</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap’ Te’</td>
<td>New England Biolabs</td>
</tr>
</tbody>
</table>

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

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**FIG. 1.** Autoradiograph of the Southern blot DNA-DNA hybridization of V. sterco-raria with the 0.45-kb Afl-I fragment of plasmid pUC8:16 (5), carrying the vgb structural gene. Lanes: 1, positive control, plasmid pUC8:15 digested with HindIII; 2, HindIII; 3, EcoRI; 4, BamHI; 5, PstI; 6, SalI.

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**VITREOSCILLA STERCORARIA GLOBIN GENE**

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**FIG. 1.** Autoradiograph of the Southern blot DNA-DNA hybridization of V. sterco-raria with the 0.45-kb Afl-I fragment of plasmid pUC8:16 (5), carrying the vgb structural gene. Lanes: 1, positive control, plasmid pUC8:15 digested with HindIII; 2, HindIII; 3, EcoRI; 4, BamHI; 5, PstI; 6, SalI.
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 in the Soret region (22). It can be elicited in aerobic solution by enzymatic reduction with NADH, and when formed in this way, it is relatively stable. The reaction was carried in the presence of Hb (final concentration, A₄₁₄ = 0.5), NADH (1.5 × 10⁻⁴ M), sodium phosphate buffer (0.1 M [pH 7.5]), and cell extract (0.35 mg of protein/ml) obtained from sonicated cell lysate of *Vitreoscilla* strain C1. Conversion of the oxygenated form into the reduced form was monitored by spectral scan (350 to 550 nm) at fixed time intervals, Autooxidation rates were calculated by spectral change at A₄₁₄ at the specified period of time. Time courses were computed at ∆A versus time, where ∆A is defined as the A₄₁₄ at any time minus that obtained at infinite time (final time period). Relative first-order rates for autooxidation were calculated from the analysis of four independent sets of experiments with different concentrations of Hb, and averages of these values were taken as a measure of autooxidation rate.

**Measurement of specific oxygen consumption rate.** The specific oxygen consumption rate was measured with a Yellow Springs Instruments model 55 oxygen monitor in air-saturated 0.1 M potassium phosphate buffer (pH 7.2) at 25°C. One milliliter of cell culture (the total number of cells per ml was simultaneously monitored in air-saturated 0.1 M potassium phosphate buffer (pH 7.2). The resulting pellet was added quantitatively to 4 ml of air-saturated buffer. The change in oxygen concentration of the buffer containing cells was recorded with respect to time.

**Computer modelling of *V. stercoraria* Hb.** Computer modelling and examination of the three-dimensional structure were carried out on a Silicon Graphics Workstation. The starting coordinate set corresponding to the crystal structure of *Vitreoscilla* Hb was kindly provided by M. Bolognesi (29). Differences in *V. stercoraria* Hb were created and analyzed with the program O (12).

**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 (OD₆₀₀) of 1.0. In contrast, *E. coli* grew poorly under similar microaerobic conditions, where its maximum optical 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.
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 *Vitreoscilla 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 24-kb vgb gene-carrying genomic fragments after *Hind*III, *Eco*RI, and *Pst*I digestion, respectively (5). However, in the case of *V. stercoraria* DW, *Hind*III and *Eco*RI 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^{10} cells) than that of *E. coli* cells carrying the *V. stercoraria* vgb gene (0.9 mol/min/10^{10} 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

### Table 2. Effect of aerobic and microaerobic conditions on growth and total Hb content of *Vitreoscilla* strain C1 and *V. stercoraria*<sup>a</sup>

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth 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><em>Vitreoscilla</em> strain C1</td>
<td>Aerobic</td>
<td>1.8</td>
<td>5 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>8.2 ± 2</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Microaerobic</td>
<td>1.0</td>
<td>2 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>34.6 ± 6</td>
<td>1.0 ± 0.35</td>
</tr>
<tr>
<td><em>V. stercoraria</em> DW</td>
<td>Aerobic</td>
<td>1.9</td>
<td>8 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>4.8 ± 2</td>
<td>1.3 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>Microaerobic</td>
<td>0.9</td>
<td>1 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>6.42 ± 2</td>
<td>0.6 ± 0.25</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Aerobic</td>
<td>1.2</td>
<td>1 × 10&lt;sup&gt;6&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 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
<td>0.08 ± 0.05</td>
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</tbody>
</table>

<sup>a</sup> 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.

### Table 3. Relative measurement of oxygen uptake and autooxidation pattern of *Vitreoscilla* Hbs

<table>
<thead>
<tr>
<th>Hb concn (A&lt;sub&gt;410&lt;/sub&gt;)</th>
<th>O&lt;sub&gt;2&lt;/sub&gt; uptake (µmol/min/ml)</th>
<th>First-order autooxidation rate (h&lt;sup&gt;-1&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td><em>Vitreoscilla</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.31</td>
<td>0.61 (native)</td>
<td>120 ± 5.5</td>
</tr>
<tr>
<td>0.46</td>
<td>0.65 (cloned)</td>
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<tr>
<td>0.58</td>
<td>0.79 (cloned)</td>
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<tr>
<td>0.68</td>
<td>0.84 (cloned)</td>
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<td><em>V. stercoraria</em></td>
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<tr>
<td>0.41</td>
<td>0.36 (native)</td>
<td>81 ± 6.5</td>
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<td>0.48</td>
<td>0.39 (cloned)</td>
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<td>0.60</td>
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<tr>
<td>0.80</td>
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<sup>a</sup> Purified preparations of *Vitreoscilla* Hb were obtained from a natural or recombinant *E. coli* strain, carrying cloned Hb genes, by the published procedure (21).

<sup>b</sup> Experimental details are given in Materials and Methods. Values indicate averages of four individual observations. Conversion of the oxygenated into the reduced form was monitored through spectral change at 414 nm at fixed time intervals. The first-order rate designates the rate of conversion of the oxygenated form into the reduced form.
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 (TTgAC) does not exist. However, it has been documented that TTG** is a good 235 sequence, and there are two such motifs in this region. The spacing between the putative 210 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 240 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***FIG. 3. (A) Schematic presentation of the subcloning process of the *V. stercoraria vgb* gene. The relevant restriction sites are indicated. B, BamHI; E, EcoRI; P, PstI; S, SalI. Hb-positive clones were identified through immunoblotting with *Vitreoscilla* Hb (VHb)-specific antibodies. (B) In vivo functional state of *V. stercoraria* Hb (VstHb). E. coli cells carrying recombinant plasmids with *Vitreoscilla* vgb (pUC8:15) and *V. stercoraria* vgb (pMJ1) genes were suspended (20 mg/ml) in 0.1 M potassium phosphate (pH 7.5). After the baseline had been obtained, the cell suspension in the sample cuvette was aerated by agitation in a test tube for 10 s with a vortex mixer, to generate oxygen pressure to an atmospheric level in the sample cuvette. Difference spectra were recorded at different time intervals to check the conversion of the oxygenated form of Hb to deoxy-Hb due to cell respiration. 1, 1 min; 2, 3 min; 3, 6 min.
**Vitreoscilla stercoraria**

In *V. stercoraria*, the vgb gene promoter further upstream indicated a possible CRP box (TGTGA...AAAA) (Fig. 4) which closely resembled the consensus CRP binding site, TGTGA...CACTCA (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 *V. stercoraria*. Analysis of the *V. stercoraria* vgb gene sequence revealed a putative CRP binding site (Fig. 4). Among the 11 amino acid replacements in the two Hbs when going from *V. stercoraria* to *E. coli* gene, 10 of these are located in the 146-residue-long polypeptide, the two Hbs differ by only 11 residues. The amino acid alignment of the two Hbs shows that alterations in their primary structures, the two exhibit subtle similarity in their primary structures, the two exhibit subtle similarity. 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 alterations in their primary structures, the two exhibit subtle similarity.

**Oxygen-independent regulation of the vgb gene in *V. stercoraria***. In *E. coli*, the *V. stercoraria* vgb gene expression is constitutively regulated. 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 region 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 heme group 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 pM1 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. 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 globin-like 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-yxlE

**FIG. 6.** (A) Expression of the *V. stercoraria* vgb gene at different oxygen levels. *E. coli* cells carrying the recombinant plasmids with the *Vitreoscilla* vgb (pUC8:15) and *V. stercoraria* vgb (pMJ1) genes were grown under high- and low-aeration conditions, and their responses were measured through immunoblot analysis. Lanes: 1, *E. coli* (pUC8:15), low aeration; 2, *E. coli* (pUC8:15), high aeration; 3, *E. coli* (pMJ1), low aeration; 4, *E. coli* (pMJ1), high aeration (localization of *V. stercoraria* Hb in *E. coli* cells carrying recombinant plasmid pMJ1 determined through immunoblot analysis); 5, total cytoplasmic proteins; 6, periplasmic fraction. (B) Role of CRP in the regulation of the *V. stercoraria* vgb gene. Comparison of *Vitreoscilla* vgb and *V. stercoraria* vgb gene expression in *E. coli* cells lacking CRP (MC1000 crp mutant) and FNR (JRG 1768 fnr mutant). The total cellular level of Hb was determined through densitometric scanning of the immunoblot, which corresponded well with the results presented above (data not shown).
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 norvegenensis* and *E. coli* HbM are $2 \times 10^{-8}$ and $2 \times 10^{-6}$, respectively, compared to $7.2 \times 10^{-6}$ 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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