Two Distinct Roles for Two Functional Cobaltochelatases (CbiK) in *Desulfovibrio vulgaris* Hildenborough†

Susana A. L. Lobo, Amanda A. Brindley, Célia V. Romão, Helen K. Leech, Martin J. Warren, and Lígia M. Saraiva

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da Republica (EAN), 2780-157 Oeiras, Portugal, and Protein Science Group, Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom

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ABSTRACT: The sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough possesses a large number of porphyrin-containing proteins whose biosynthesis is poorly characterized. In this work, we have studied two putative CbiK cobaltochelatases present in the genome of *D. vulgaris*. The assays revealed that both enzymes insert cobalt and iron into sirohydrochlorin, with specific activities with iron lower than that measured with cobalt. Nevertheless, the two *D. vulgaris* chelatases complement an *E. coli* cysG mutant strain showing that, in vivo, they are able to load iron into sirohydrochlorin. The results showed that the functional cobaltochelatases have distinct roles with one, CbiK<sup>C</sup>, likely to be the enzyme associated with cytoplasmic cobalamin biosynthesis, while the other, CbiK<sup>P</sup>, is periplasmic located and possibly associated with an iron transport system. Finally, the ability of *D. vulgaris* to produce vitamin B<sub>12</sub> was also demonstrated in this work.

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Modified tetrapyrroles such as hemes, siroheme, and cobalamin (vitamin B<sub>12</sub>) are characterized by a large molecular ring structure with a centrally chelated metal ion. This family of compounds share a common pathway until the formation of the first macrocyclic intermediate, uroporphyrinogen III, at which point the pathway branches. Each branch contains a unique chelatase that performs the insertion of a specific metal ion into the modified tetrapyrrole ring (1, 2). In heme biosynthesis, for example, ferrous iron is inserted into the protoheme precursor protoporphyrin IX by the protoporphyrin IX ferrochelatase (3). In the case of vitamin B<sub>12</sub> biosynthesis, cobalt is inserted into the macrocycle by a cobaltochelatase, which is either specific to the aerobic (oxygen-dependent) or anaerobic pathway for cobalamin biosynthesis (2). Along the aerobic pathway, precorrin-2 undergoes several enzymatic modifications that result in the formation of hydrogenobyrinic acid <i>c</i>,<i>c</i>-diamide where cobalt is inserted by the class I ATP-dependent cobaltochelatase CobN-S-T (4, 5). Along the anaerobic pathway, cobalt insertion occurs at an early stage, at the level of sirohydrochlorin (6), by the class II ATP-independent cobaltochelatases CbiX or CbiK (1, 5, 7). The CbiX protein was first identified as a 320 amino acid enzyme, the so-called long form (CbiX<sup>L</sup>), whereas the archael orthologue is constituted by only around 110-145 amino acids, the short form (CbiX<sup>S</sup>). The N-terminal and C-terminal domains of the long form of CbiX<sup>L</sup> share a high degree of amino acid sequence similarity. CbiKs are formed by ~300 amino acid residues and share a low level of similarity with CbiX<sup>L</sup>. Analysis of circular dichroism (CD) spectra suggested that the cobaltochelatases CbiK, CbiX<sup>Long</sup>, and CbiX<sup>Short</sup> may have a similar overall topology (5). The X-ray structure determination of the CbiK from *Salmonella enterica* revealed also that the enzyme is highly similar to the protoporphyrin IX ferrochelatase from *Bacillus subtilis* (8, 9). More recent structural work on CbiX<sup>L</sup> has confirmed that this protein shares the same basic protein architecture as that found in CbiK (10).

Sulfate-reducing bacteria (SRB<sup>†</sup>), belonging to the genus *Desulfovibrio*, are considered to be early organisms on the evolutionary scale and contain a very large number of proteins with modified porphyrins, some of them unusual. Examples of this include the rubredoxin oxygen oxidoreductase from *D. gigas* that contains iron uroporphyrin I (11) and bacterioferritin of *D. vulgaris* (12, 13, 14). The nature of the cobalt—porphyrin macrocycle was identified as Co<sup>III</sup>-syrohydrochlorin (15). Studies in *D. vulgaris* suggested that in these organisms an alternative pathway for the biosynthesis of heme may be operative since coproporphyrinogen III is

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*Corresponding author. Lígia M. Saraiva, Av. da Republica (EAN), 2780-157 Oeiras, Portugal. Phone: +351214469328. Fax: +351-214411277. E-mail: lst@iqub.unl.pt.

<sup>‡</sup>Universidade Nova de Lisboa.

<sup>§</sup>University of Kent.

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<sup>2</sup> Corresponding author. Lígia M. Saraiva, Av. da Republica (EAN), 2780-157 Oeiras, Portugal. Phone: +351214469328. Fax: +351-214411277. E-mail: lst@iqub.unl.pt.

<sup>3</sup> Universidade Nova de Lisboa.

<sup>4</sup> University of Kent.

<sup>5</sup> Abreviations: SRB, sulfate-reducing bacteria; LB, Luria broth medium IPTG, isopropyl-β-D-thiogalactopyranoside; ALA, 5-aminolevulinic acid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; SAM, Sadenosyl-l-methionine; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; MM, minimal medium.
formed not directly from uroporphyrinogen III but from the vitamin B12 precursor, precorrin-2 (16). However, the biosynthesis of porphyrins in Desulfovibrio sp. remains poorly understood.

The genome of D. vulgaris Hildenborough encodes two putative cobaltochelatases, DVU0650 and DVU1365, that share a significant degree of amino acid sequence identity with S. typhimurium CbiK. In order to clarify the role of these proteins and their involvement in modified tetrapyrole biosynthesis in a Desulfovibrio sp., the genes were cloned, the proteins produced recombinantly and the isolated enzymes characterized.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Recombinant Cobaltochelatases. The putative cobaltochelatases encoded by the genes DVU0650 and DVU1365 have 894 bp and 849 bp, respectively. Amplification of the two genes was achieved in PCR reactions using genomic DNA of Desulfovibrio vulgaris Hildenborough and appropriate oligonucleotides specifically designed for each case. A second DNA fragment of the DVU0650 gene was also amplified in order to construct a truncated protein that starts at amino acid 29 (∆28DVU0650). The various DNA fragments were cloned into pET-28a(+) (Novagen) giving pET-28a(+-DVU0650, pET-28a(+-∆28DVU0650, and pET-28a(+-DVU1365 allowing the encoded proteins, DVU0650, ∆28DVU0650 and DVU1365, respectively, to be produced with an 6x-His-tag in the N-terminal region. Sequencing of the PCR products guaranteed the integrity of all gene sequences. To produce DVU0650 and ∆28DVU0650 proteins, the recombinant plasmids were transformed in Escherichia coli BL21Gold(DE3) (Stratagene) and the cells were grown, at 30 °C, in Luria–Bertani (LB) medium containing kanamycin (30 µg/mL) until an OD600 = 0.3. At this point, 200 µM of IPTG (isopropyl-β-d-thiogalactopyranoside) was added and the medium supplemented with 50 µM 5-aminolevulinic acid (ALA) and 100 µM FeSO4. The culture was then grown overnight, at 15 °C. The overexpression of DVU1365 was achieved by transforming the recombinant plasmid in the BL21Gold(DE3) and growing the cells, at 37 °C, in LB medium containing 30 µg/mL kanamycin until an OD600 of 0.8, followed by the addition of 200 µM of IPTG and 100 µM FeSO4. The cells were then grown at 37 °C for 4 h.

Protein Purification and Characterization. Cells expressing DVU0650, ∆28DVU0650, and DVU1365 proteins were harvested, resuspended in 20 mM Tris-HCl buffer at pH 7.5 (buffer A) with 20 µg/mL DNase, and disrupted in a French Press. The soluble fraction was separated from the membranes by ultra centrifugation for 2 h at 160,000 × g. All purification steps were performed at 4 °C, either in aerobic or anaerobic conditions.

DVU0650 protein was purified under aerobic and anaerobic conditions. The fraction isolated from the aerobic purification was used for the biochemical and spectroscopic characterization. For the aerobic purification of DVU0650 protein, the soluble fraction from E. coli cells expressing DVU0650 was applied onto a Chelating Sepharose fast flow column (130 mL) (GE, Healthcare), previously charged with NiCl2 and equilibrated with buffer A containing 400 mM NaCl. Eleven volumes of a linear gradient up to 250 mM imidazole were applied to the column, and the recombinant protein was eluted at 250 mM imidazole. After dialysis against buffer A, the protein fraction was loaded onto a Q-Sepharose High-Performance column (20 mL) (GE, Healthcare), previously equilibrated with buffer A. Ten volumes of a linear gradient up to 400 mM NaCl followed by four volumes of a linear gradient from 400 mM up to 1 M NaCl was applied, and the protein was eluted with ~200 mM NaCl.

DVU0650, ∆28DVU0650, and DVU1365 proteins were purified under anaerobic conditions. The anaerobic purification steps were performed in a Coy model A-2463 anaerobic chamber filled with a gas mixture of 95% argon plus 5% hydrogen. The soluble fraction from the cells expressing DVU0650, ∆28DVU0650, and DVU1365 was applied onto a HiTrap Chelating HP column (GE, Healthcare). The recombinant DVU0650 and ∆28DVU0650 proteins were eluted with 5 volumes of buffer A containing 0.5 M NaCl and 200 mM imidazole, while the DVU1365 protein was eluted with 5 volumes of buffer A plus 250 mM imidazole. The protein fractions containing DVU0650 or ∆28DVU0650 were passed through a PD10 desalting column (GE, Healthcare) in order to exchange the elution buffer to 50 mM Tris-HCl buffer at pH 8 with 100 mM NaCl, while the DVU1365 protein fraction was dialyzed overnight against 50 mM Tris-HCl buffer at pH 8 with 8% glycerol.

The purity of the proteins was analyzed by SDS-PAGE gel (17) and the protein concentration was determined by the bicinchoninic acid method (18) using protein standards from Sigma. Heme content was assayed by the hemochromophyrine method (19), and heme extraction was performed according to the method described by Lubben et al. (20). The protein molecular mass was determined by gel filtration in a Superdex 200 column according to the instructions of the manufacturer (GE, Healthcare), using commercially available standards from GE Healthcare.

Comparisons of the primary amino acid sequences were done using ClustalX (21), and the prediction of sorting signals was achieved using the SOSUI signal program (22, 23).

UV–visible spectra were recorded at room temperature in a Shimadzu UV-1700 spectrophotometer with 4.5 µM of protein, and sodium dithionite was used as a reductant. The redox titration was performed at 25 °C, under continuous agitation and argon atmosphere using 2.8 µM of purified protein and 12 µM of mediators, diluted in 50 mM Tris-HCl at pH 7.5. The redox mediators used allowed to cover the interval range between +340 mV and −225 mV: N,N-dimethyl-p-phenylene-diamine (Eo' = 340 mV), p-benzoquinone (Eo' = 240 mV), 1,2-napthoquinone-4-sulfonic acid (Eo' = 215 mV), 1,2-naphthoquinone (Eo' = 180 mV), trimethylhydroquinone (Eo' = 115 mV), phenazine methosulfate (Eo' = 80 mV), 1,4-napthoquinone (Eo' = 60 mV), duroquinone (Eo' = 5 mV), menadione (Eo' = 0 mV), plumbagin (Eo' = −40 mV), phenazine (Eo' = −125 mV), 2-hydroxy-1,4-napthoquinone (Eo' = −152 mV), and antraquinone sulfonate (Eo' = −225 mV). A silver/silver chloride electrode, previously calibrated in a saturated quinhydrone solution was used. The titration was performed from the oxidized to the reduced state, and an experimental curve was obtained from the potential measured at 560 nm. The experimental data was analyzed using MATLAB (Mathworks, South Natick, MA) for Windows.
Concentrated fractions of DVU0650 (95 μM) were prepared for EPR spectroscopy, and the spectra were acquired at 15 K, on a Bruker ESP 308 spectrometer equipped with an Oxford Instruments continuous flow helium cryostat. The NMR sample was prepared in D2O, and the pH and ionic strength were set using 10 mM phosphate buffer at pH 6.9. 1D 1H NMR spectra (1k scans) were acquired, at 298 K, in a Bruker Avance 500 spectrometer using a QXI probe. The residual water signal was saturated using a selective pulse of 500 ms. The sample was reduced by adding the appropriate volume of a saturated solution of sodium dithionite.

Activity Assays. The chelatase activity assays were performed with proteins purified under either aerobic/anaerobic conditions, and the activity was measured anaerobically following the formation of cobalt-sirohydrochlorin or siroheme, by the decrease of absorbance at 376 nm and using the extinction coefficient 2.4 × 10^6 M^−1 cm^−1 (24). The plasmid pETcoco-2ABCDC (25) harboring the genes encoding for uroporphyrinogen III methyltransferase (CobA) of Methanosarcina barkeri, porphobilinogen synthase (HemB) and precorrin-2 dehydrogenase (SirC) of Methanothermobacter thermautotrophicus, and porphobilinogen (PGB) deaminase (HemC) and uroporphyrinogen III synthase (HemD) of Bacillus megaterium was introduced in E. coli BL21(DE3)pLysS (Novagen), and the proteins were overexpressed in order to produce sirohydrochlorin. To this end, the cells were grown overnight in LB medium containing ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL), and 0.2% (w/v) glucose. The cells were then inoculated in LB medium with the same antibiotics described and grown at 37 °C, until an OD600 ~0.5, followed by the addition of 0.02% (w/v) of 1-arabinose. After 2 h, 400 μM of IPTG was added, and the cells were grown overnight at 24 °C. After breaking the cells in a French Press, the lysate was transferred to the anaerobic chamber where 2 mL of lysate was incubated with 2 mg of SAM (S-adenosyl-l-methionine), 1 mg of ALA, and 1 mg of NAD^+, in a total volume of 6 mL in 50 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl. The pH of the reaction mixture was adjusted to 8 with 2 M KOH. After overnight incubation, at room temperature, the reaction mixture was filtered (0.22 μm filter) and passed through a DEAE-resin (Sigma), and sirohydrochlorin was eluted with 50 mM Tris-HCl buffer at pH 8.0 containing 1 M NaCl. The activity was measured in triplicate using a Shimadzu UV-1203 spectrophotometer. The assays were performed in 1 mL reaction in 50 mM Tris-HCl buffer at pH 8.0 with 100 mM NaCl, with 4.2 μM of sirohydrochlorin, 20 μM of Co^2+ or Fe^2+, and with different amounts of enzyme.

Complementation of E. coli cysG Mutant Strain with D. vulgaris Cobaltochelatases. The genes from pET-28a(+)-DVU0650, pET-28a(+)-DVU1365, and pET-28a(+)-DVU136 were subcloned in pETac, a plasmid under the control of the tac promoter. The resulting plasmids, pETac-DVU0650, pETac-DVU136, and pETac-DVU1365, were transformed into E. coli 302Aa, a strain deleted in the cysG and harboring plasmid pCIS-sirCcobA for expression of the M. thermoautotrophicus sirC and Pseudomonas denitrificans cobA genes. The E. coli cysG mutant strain containing the recombinant plasmids was selected on LB plates supplemented with 100 μg/mL ampicillin and 35 μg/mL chloramphenicol. Strain E. coli 302Aa was also transformed with the plasmid pKK223.2-cysG, which expresses the E. coli CysG, or with an empty pETac plasmid and grown on MM plates in the absence and in the presence of cysteine, in order to provide the positive and negative controls of the experiment, respectively.

Bioassay for Detection of Vitamin B12 in D. vulgaris Cells. Desulfovibrio vulgaris Hildenborough was grown anaerobically at 37 °C for 48 h in sulfate/lactate growth medium supplemented with a trace element solution (26). The cells were collected and the bioassay was performed according to the technique previously described (27).

RESULTS

The analysis of the D. vulgaris Hildenborough genome revealed the presence of two gene loci, DVU0650 and DVU1365, encoding putative cobaltochelatases that share ~30% amino acid sequence identity with CbiK from S. enterica and Porphyromonas gingivalis (28, 29) (Figure 1). In order to study the physiological and biochemical role of these two genes, they were amplified, cloned, and the enzymes produced, purified, and characterized.

D. vulgaris Cobaltochelatase DVU0650 Contains a Heme b. After overproduction in E. coli and isolation, the soluble fraction containing the purified DVU0650 exhibited an intense pink–orange color, which was shown to be due to the presence of a heme cofactor (1 mol/dimer). For convenience, DVU0650 was termed CbiKP (periplasmic form of CbiK).

As-isolated CbiK^P migrated in SDS–PAGE with an apparent molecular mass of 28 kDa (Figure 2A), which could correspond to a protein in which a cleavage of 28 residues had occurred. In fact, the removal of a signal peptide was confirmed by N-terminal sequencing, which showed that the mature protein starts at residue 29. Gel exclusion chromatography of purified CbiKP suggests that the protein exists in solution as a homotetramer (data not shown).

A UV–visible spectrum of the oxidized form of the protein exhibited a Soret band at 414 nm and a broadband in solution as a homotetramer (data not shown). The absorption spectrum did not show the fingerprint for a diamagnetic heme b, confirming the presence of a heme b type (19). To characterize the heme b present in DVU0650, a redox titration was performed, and a midpoint redox potential of ~130 mV was determined (Figure 2C). NMR spectroscopy was used to infer the nature of the heme ligands through the analysis of the redox spectrum. To elucidate further the type of heme present, pyridine hemochromes was performed in the total protein and in its isolated cofactor. In both cases, the redox spectrum presented a β band with an absorption maxima of 555 nm confirming the presence of a heme b type (19).
g-values of 2.94, 2.26, and 1.53, indicative of the presence of a low spin heme group (Figure 2D).

CbiKP (DVU0650) Is a Sirohydrochlorin Cobaltochelatase. The DVU0650 enzyme purified under aerobic conditions had activity values very similar to those obtained with the protein purified anaerobically. A truncated protein that lacks the signal peptide was also produced (see Experimental Procedures). This protein, named Δ28CbiKP, exhibited the same molecular mass profile observed for CbiKP on both SDS-PAGE gel and gel exclusion chromatography (data not shown). However, no heme content was determined in the Δ28CbiKP protein. The chelatase activity could be then assessed for two forms of the enzyme, one containing the heme cofactor and another lacking the chromophore, allowing us to infer the influence of the heme in the enzymatic activity of CbiKP.

The chelatase activity assays revealed that both CbiKP and Δ28CbiKP were able to insert cobalt and iron into sirohydrochlorin and that the activities were not affected by the presence of the heme since both proteins had very similar specific activities. These results also showed that the two forms of the enzymes have a slightly higher activity with cobalt than with iron (Table 1).

DVU1365 Is a Second Sirohydrochlorin Cobaltochelatase. As mentioned above, D. vulgaris DVU1365 encodes a second putative CbiK cobaltochelatase that shares 39% amino
acid sequence identity with CbiKP (DVU0650) (Figure 1) but lacks an N-terminal transit peptide. Consequently, this form of CbiK was termed CbiK C (for cytosolic). The gene was cloned, and the enzyme was overproduced in E. coli.

In contrast to CbiKP, no cofactor was detected in the purified CbiKC. The purified CbiK C migrates on a denaturating gel with a molecular mass of approximately 31 kDa. The chelatase activity was assayed with both cobalt and iron in the same conditions described before. CbiKC has an approximate order of magnitude lower activity than CbiKP with a specific activity of 4 nmol·min⁻¹·mg⁻¹ with cobalt and 1.5 nmol·min⁻¹·mg⁻¹ with iron (Table 1).

∆28CbiKP and CbiKc Complement an E. coli Sirohydrochlorin Ferrochelatase Mutant Strain. Previously, it has been shown that cobaltochelatases can complement a sirohydrochlorin ferrochelatase deficient strain of E. coli. This is due to the lack of metal ion specificity displayed by these chelatases and their ability to insert either ferrous or cobalt ions into sirohydrochlorin (10, 29). In order to investigate whether the D. vulgaris CbiKP and CbiKc were able to complement the sirohydrochlorin ferrochelatase deficiency of an E. coli strain, the genes were cloned into a pETac plasmid and introduced in E. coli 302∆a that harbored pCIQsirCcobA (i.e., a strain that is able to make sirohydrochlorin but not siroheme). To overcome the potential problem of CbiKP being exported to the periplasm in E. coli, the complementation was performed with the truncated version. Both CbiKc and ∆28CbiKP cobaltochelatases were found to complement the mutant strain as observed by growth of the transformed strains on minimal medium in the absence of cysteine. Thus, both enzymes are able to act as sirohydrochlorin ferrochelatases in the biosynthesis of siroheme.

D. vulgaris Produces Vitamin B12. D. vulgaris cells were grown on a defined medium in the absence of any exogenous cobalamin. After growth, the cells were harvested and analyzed for the level of cobalamin that was produced. By bioassay, the strain was found to produce cobalamin to a level of about 10 nmol/L of culture.

DISCUSSION

In this work, we characterized two putative CbiK cobaltochelatases present in the genome of D. vulgaris encoded in the loci DVU0650 and DVU1365. Production of the recombinant DVU0650 (CbiKP) revealed that the protein was processed to a form that lacks the first 28 amino acid residues in accordance with the predicted prokaryotic leader sequence. A leader sequence in cobaltochelatases has so far only been reported for the P. gingivalis enzyme (28); however, a search in the protein database revealed that other prokaryotic CbiKs are also predicted to contain signal peptide sequences (Figure 1).

Table 1: Sirohydrochlorin Cobalt and Iron Chelatase Specific Activity Measured for DVU0650, ∆28DVU0650, and DVU1365 Enzymes

<table>
<thead>
<tr>
<th>protein</th>
<th>Co²⁺</th>
<th>Fe²⁺</th>
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</thead>
<tbody>
<tr>
<td>DVU0650</td>
<td>22 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>∆28DVU0650</td>
<td>25 ± 2</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>DVU1365</td>
<td>4 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
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* The activity values are presented with the standard deviation.
The presence of redox centers, whose role remains unclear, has been previously reported in some prokaryotic chelatases. A recent study revealed that a [4Fe-4S]^{2+/-1} center exists in the CbiX^2 cobaltchelatases from *Bacillus megaterium* and *Synechocystis* PCC6803 (6) whereas several bacterial protoporphyrin ferrochelatases contain [2Fe-2S]^{2+/-1} clusters (32, 33). However, the presence of a heme in a chelatase enzyme is so far unique. Our study showed that in *D. vulgaris* CbiK^p cobaltchelatase the heme is not required for the fully enzyme activity since CbiK^p and Δ28CbiK^p were able to insert cobalt and iron into sirohydrochlorin and the absence of the heme did not affect the activity of metal insertion.

Insertion of iron into sirohydrochlorin, yielding siroheme, has been reported for other cobaltchelatases but in a lower ratio compared to that of cobalt insertion (5, 29). The *D. vulgaris* CbiK^p and CbiK^c cobaltchelatases were also found to have specific activities with iron lower than that measured with cobalt. Nevertheless, the two *D. vulgaris* chelatases were able to complement the *E. coli* cysG mutant strain showing that in *vivo*, they can both insert iron into sirohydrochlorin, thereby rescuing the cell sirohydrochlorin ferrochelatase deficient phenotype.

The presence of two putative genes encoding cobaltchelatases is also found in other genomes, including *D. desulfuricans* G20 (gene locus Dde2181 and Dde3103), *Clostridium acetobutylicum* (gene locus CAC1373 and CAC0583), *Methanospirillum hungatiae* (gene locus Mhmu0266 and Mhmu0668), *Methanobrevibacter smithii* (gene locus Msml1280 and Msml1281) and in the Methanogenic archaean RC-1 (gene locus RCIX2006 and RCIX98). The fact that in *D. vulgaris* one of cobaltchelatases is periplasmic and the other is cytoplasmic suggests that the two chelatases may contribute to different processes.

In *P. gingivalis*, an outer membrane hemin-binding protein, termed IhtB, is proposed to be involved in iron transport and has ~38% sequence identity with the CbiK from *S. enterica* (34). The *ihtB* gene is located in a gene cluster *ihtABCDE*, where the other genes are predicted to encode iron transporters of the ABC type (34). The protein was shown to be an outer membrane hemin-binding protein and it was suggested that the protein may be involved in the removal of iron from heme prior to uptake by *P. gingivalis* (34). The *P. gingivalis* protein was also shown to act as a functional cobaltchelatase in both *in vivo* and *in vitro* studies, although the organism does not appear to have a complete cobalamin biosynthetic pathway (28). Moreover, in *Dichelobacter nodosus* cbiK was shown to be under the control of the ferric uptake regulator (Fur) as was the gene for an orthologue of the periplasmic iron binding protein YfeA, itself a component of an ABC transporter system that is involved in iron uptake (35). It is interesting to note that the DVU0650 gene is also located in a putative operon, predicted by the Softberry FGENESB program (36), which contains genes for iron transport proteins (Figure 3). Cumulatively, this information provides a case for *D. vulgaris* CbiK^p being part of an iron or heme acquisition system. The presence of the protein in the periplasm means that it cannot participate in cobalamin biosynthesis as this process takes place in the cytosol. Thus, *D. vulgaris* has two distinct cbiK genes, which encode proteins that can both act as cobaltchelatases. The CbiK^c is likely to be the enzyme associated with cobalamin biosynthesis as this protein is produced in the cytoplasm without any transit peptide. CbiK^p is moved to the periplasm and is likely to be associated with the iron transporter system. The actual role of CbiK^p in iron transport will require further investigation.

Tetrapyrrole biosynthesis and modification in sulfate reducing bacteria are still poorly understood at the biochemical and molecular level. The production of guanylylcobamide and hypoxyanthylcobamide by *D. vulgaris* was reported a few years ago, and the addition of 5,6-dimethylbenzimidazole to *D. vulgaris* culture led to the production of cyanocobalamin (vitamin B_{12}) (26). Furthermore, analysis of the *D.
vulgatis Hildenborough genome shows the presence of all the genes required for the anaerobic production of vitamin B12 (Table 2), with the exception of precorrin-6x reductase (cblIcokK), which within the anaerobic metal-reducing δ-proteobacteria is only found in the genome of Desulfovibrio sp (37). Although the requirement of vitamin B12 and/or related corrinoids in Desulfovibrio for growth is still unclear, the ability of D. vulgat is to produce vitamin B12 was demonstrated in this work.

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