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Functional characterization of the early steps of tetrapyrrole biosynthesis and modification in *Desulfovibrio vulgaris* Hildenborough

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Abstract

The biosynthesis of the tetrapyrrole framework has been investigated in the sulphate reducing bacterium *Desulfovibrio vulgaris* Hildenborough by characterization of the enzymes required for the transformation of 5-aminolevulinic acid into sirohydrochlorin. Porphobilinogen synthase (HemB) was found to be a zinc-dependent enzyme that exists in its native state as a homohexamer. Porphobilinogen deaminase (HemC) was shown to contain the dipyrromethane cofactor. Uroporphyrinogen III synthase is found fused with a uroporphyrinogen III methyltransferase (HemD-CobA). Both activities could be demonstrated in this amalgamated protein and the individual enzyme activities were separated by dissecting the relevant gene to allow the production of two distinct proteins. A gene annotated on the genome as a bifunctional precorrin-2 dehydrogenase/sirohydrochlorin ferrochelatase was in fact shown to act only as a dehydrogenase and is simply capable of synthesizing sirohydrochlorin rather than sirohaem. Genome analysis also reveals a lack of any uroporphyrinogen III decarboxylase, an enzyme necessary for the classical route to haem synthesis. However, the genome does encode some predicted haem d_1 biosynthetic enzymes even though the bacterium does not contain the cd_1 nitrite reductase. We suggest that sirohydrochlorin acts as a substrate for haem synthesis using a novel pathway that involves homologues of the d_1 biogenesis system. This explains why the uroporphyrinogen III synthase is found fused with the methyltransferase, bypassing the need for uroporphyrinogen III decarboxylase activity.

Short (page heading) title: Tetrapyrrole biosynthesis in *D. vulgaris*

Keywords: sulphate reducing bacteria, tetrapyrrole biosynthesis, precorrin-2, sirohydrochlorin

Abbreviations: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; SAM, S-adenosyl-L-methionine; NAD^+ , nicotinamide adenine dinucleotide; SRB, sulphate reducing bacteria; IPTG, isopropyl- β -D-thiogalactopyranoside.

Introduction

Sulphate reducing bacteria (SRB) are environmentally important microorganisms that are found in a wide range of habitats but are reliant on being able to reduce sulphate in a dissimilatory manner to produce sulphide. To assist them in their metabolic challenge SRB employ a rich repertoire of redox groups including porphyrins and related modified tetrapyrroles. Genomic analysis suggests that SRB are able to make at least three types of modified tetrapyrroles, including haem, sirohaem and adenosylcobalamin, the coenzyme form of vitamin B₁₂.

Modified tetrapyrroles have a similar structural architecture reflecting the fact that they are all derived from a common macromolecular intermediate, uroporphyrinogen III. Thus all modified tetrapyrroles are made along a branched biosynthetic pathway, as outlined in Figure 1. The pathway starts with the generation of 5-aminolevulinic acid¹ (ALA), the first common intermediate of tetrapyrrole biosynthesis, which is synthesized either from succinyl-coenzyme A and glycine (in the case of some proteobacteria, fungi, yeast and animals) or from a tRNA-bound glutamate (in plants, algae, archaea and eubacteria) [1, 2]. The next step involves the asymmetric condensation of two ALA molecules to form the monopyrrole porphobilinogen (PBG), in a Knorr-type condensation reaction catalysed by the enzyme porphobilinogen synthase (also called 5-aminolevulinic acid dehydratase), which is encoded by the *hemB* gene [3]. Porphobilinogen synthases are distinguished according to their requirement in the active site for zinc or magnesium atoms and the need for magnesium in an allosteric binding site. The only known porphobilinogen synthases where these metal binding sites are absent are from the bacterial genus *Rhodobacter* [4]. *R. capsulatus* porphobilinogen synthase was shown not to require any metal ion for catalysis, having the added peculiarity of forming a hexameric structure [5], unlike most other studied porphobilinogen synthases that form octamers (eg.[6]).

Four molecules of the pyrrole PBG are subsequently polymerised into the linear tetrapyrrole hydroxymethylbilane (HMB) by the action of porphobilinogen deaminase, which is coded by *hemC*. The enzyme forms stable enzyme-intermediate complexes with one, two and three PBG-derived pyrrole molecules. A feature of this enzyme is the presence of a covalently bound dipyrromethane cofactor where the growing poly-pyrrole chain is assembled [7, 8]. The HMB intermediate is further modified and cyclised by uroporphyrinogen III synthase (encoded by the *hemD* gene) to yield uroporphyrinogen III [9]. In the absence of uroporphyrinogen III synthase, HMB cyclises spontaneously to yield the uroporphyrinogen I isomer [9]. The synthase is able to invert the terminal pyrrole unit of HMB, ring D, and cyclise the macromolecule to give the asymmetric and only biologically relevant type III isomer. In many organisms uroporphyrinogen III represents the first branch point in the pathway, where the action of enzymes such as HemE, F (N) and G results in the formation of protoporphyrin IX, a precursor of modified tetrapyrroles such as haem and chlorophyll. Alternatively, uroporphyrinogen III can undergo an *S*-adenosyl-L-methionine (SAM) dependent-transmethylation at positions 2 and 7 (by CysG, Met1p or CobA depending on the organism) to generate precorrin-2, a highly unstable yellow dipyrrocorphin [10, 11]. Precorrin-2 is further modified by a dehydrogenase (CysG, Met8p or SirC enzymes) to yield sirohydrochlorin. This isobacteriochlorin can be chelated with either ferrous iron to form sirohaem by the action of sirohydrochlorin ferrochelatase (CysG, Met8p or SirB enzymes) or with cobalt to generate cobalt-sirohydrochlorin, an intermediate that is directed along the cobalamin biosynthetic pathway. Thus, as with uroporphyrinogen, sirohydrochlorin also represents an important branch point in the biosynthesis of modified tetrapyrroles.

In *E. coli* and *Salmonella enterica* the transformation of uroporphyrinogen III into sirohaem is performed by a single multifunctional enzyme, sirohaem synthase (CysG). The SAM-dependent methyltransferase activity is associated with the C-terminal domain of GysG (CysG^A) and the NAD⁺-dependent dehydrogenation and ferrochelatase activities are linked to the N-terminal domain (CysG^B) [12, 13]. In yeast, two independent enzymes, Met1p (SAM-dependent methyltransferase) and Met8p (NAD⁺-dependent dehydrogenase/ferrochelatase) are required for the sirohaem synthesis [14]. A third alternative is found in *Bacillus megaterium*, where the three reactions are catalyzed independently, by SirA (SAM-dependent uroporphyrinogen III methyltransferase), SirC (NAD⁺-dependent precorrin-2 dehydrogenase) and SirB (sirohydrochlorin ferrochelatase) [15].

Fused enzyme systems are not just restricted to the sirohaem branch of the pathway. In some bacterial species, such as *Selenomonas ruminantium* [16], *Lactobacillus reuteri* [17] and *Clostridium josui* [18], the uroporphyrinogen III methyltransferase and uroporphyrinogen III synthase activity is performed by one single protein, since the *cobA* and *hemD* genes are linked together. The biochemical logic and metabolic importance of such fusions is not fully understood.

The sulphate reducers of the *Desulfovibrio* genus have many different haemproteins [19-21] that play essential roles in the metabolism of the organism. Although tetrapyrrole biosynthesis has not been studied in any molecular detail in the SRB, earlier research on haem synthesis in *D. vulgaris* had uncovered one of the most interesting discoveries in tetrapyrrole biosynthesis of recent times as it demonstrated that haem is made by a novel route utilizing precorrin-2 or sirohydrochlorin as an intermediate [22]. This new pathway is likely also to be the major route for haem synthesis in the archaea. A follow-up study in *D. vulgaris* identified 12,18-didecarboxysirohydrochlorin (or its reduced form) as a possible intermediate but no specific enzymes/genes were linked with the metabolic activity. The authors also suggested that this novel pathway proceeded via coproporphyrinogen III, but this was not rigorously shown [23]. Despite the novelty of this intriguing pathway, no genomic or biochemical investigation into tetrapyrrole synthesis in *D. vulgaris* has been undertaken even though the genome has been fully sequenced. In this paper we searched the *D. vulgaris* Hildenborough genome [24] and noted it contains genes encoding enzymes for the transformation of ALA into sirohydrochlorin via uroporphyrinogen III. Consistent with the presence of a novel haem biosynthetic pathway, the organism appears to be missing the majority of genes that encode the classical enzymes required for the transformation of uroporphyrinogen III into haem. In this paper we report the characterization of the early enzymes of tetrapyrrole assembly and modification in *D. vulgaris* and suggest enzymes that may be involved in the novel haem pathway.

Materials and Methods

Cloning and expression of recombinant proteins

D. vulgaris Hildenborough genes encoding putative porphobilinogen synthase (*hemB*), porphobilinogen deaminase (*hemC*), uroporphyrinogen III methyltransferase/synthase (*cobA/hemD*), sirohaem synthase (*cysG^B*) were PCR amplified from genomic DNA and specific oligonucleotides (Table S1, supplementary material). Two additional oligonucleotides were designed for amplification of truncated forms of *cobA/hemD* to generate *hemD* or *cobA* encoding regions. The individual DNA fragments were cloned into pET vectors (Novagen) (Table S1), which produced proteins with a 6x-His-

Tag tail in the N-terminal or C-terminal, depending on the vector utilized. The genes *cobA/hemD*, *cobA* and *cysG^B* were also subcloned into pETac [25] for use in complementation studies. The integrity of the gene sequence for all cloned fragments was confirmed by DNA sequencing.

Plasmids pET-28a(+)*cysG^B* and pET-22b(+)*cobA/hemD* were transformed into *E. coli* BL21Gold(DE3) cells (Stratagene) and plasmids pET-14b(+)*hemB*, pET-14b(+)*hemC*, pET-14b(+)*cobA* and pET-14b(+)*hemD* into *E. coli* BL21Star(DE3)(pLysS) cells (Invitrogen). In order to over express the proteins, the *E. coli* cells were grown at 37 °C, in Luria-Bertani (LB) medium with appropriate antibiotics, until an OD600 between 0.5 and 0.8 was reached. Induction of the genes was achieved by addition of 200-400 µM of IPTG and by growing the cells for 4h at 37 °C, for *cobA/hemD* and *cysG^B* genes, or overnight at 19 °C for *hemB*, *hemC*, *cobA* and *hemD* genes. The truncated forms of *cobA/hemD* are designated *cobA^d* and *hemD^d* (where “d” stands for domain).

Protein purification

Cells expressing HemB, HemC, CobA/HemD, CobA^d (Met1-His246), HemD^d (Lys247-Lys503) and CysG^B proteins were harvested and resuspended in 20 mM Tris-HCl buffer, pH 8 containing 500 mM NaCl and 10 mM imidazole (buffer A); for the purification of CysG^B all buffers contained 5 % (v/v) glycerol. All cells were lysed by sonication and centrifuged for 20 minutes at ~30000 x g. The soluble fraction was loaded on to a NiCl₂ charged chelating Sepharose resin (4 ml) (GE, Healthcare) equilibrated with buffer A. After washing with 5-10 volumes of buffer A containing 50 and 100 mM imidazole, all proteins were eluted with 2-5 volumes of buffer A with 500 mM imidazole, except CysG^B which was eluted with 100 mM imidazole. Protein fractions were pooled together after detection with Bio-Rad protein assay (BioRad), concentrated with Millipore Ultra Centrifugal Filters with a 10-kDa membrane and buffer exchanged, by means of a PD10 column (GE Healthcare), into buffer B (50 mM Tris-HCl pH 8), with the exception of CobA^d protein, which was buffer exchanged into buffer B containing 500 mM NaCl and 200 mM imidazole. The purity of the proteins was assessed by SDS-PAGE [26] and protein concentration was determined by the bicinchoninic acid method [27] using protein standards from Sigma. The native molecular mass of the various proteins was determined by gel filtration chromatography on either a Superdex 200 or 300 HR 10/30 column (GE, Healthcare), previously equilibrated with 20 mM Tris-HCl, pH 7.5.

The detection of the dipyrromethane cofactor of *D. vulgaris* porphobilinogen deaminase was determined by incubating 0.5 ml of the enzyme (0.2 mg) with an equivalent volume of Ehrlich's reagent [28] and by following the UV-visible spectral changes over 20 min.

Enzymatic assays

The various activity measurements were performed in a Hewlett Packard 8452A photodiode array spectrophotometer, a Shimadzu UV-1700 spectrophotometer or a BMG Labtech Flurostar Optima plate reader. All activities were assayed in buffer B, with the exception of porphobilinogen synthase which was assayed in 100 mM Tris-HCl pH 8.

Porphobilinogen synthase. HemB (0.5-5 µg) was pre-incubated for 5 min at 37 °C, and the reaction started by addition of ALA (5 mM), in a final volume of 0.5 ml. The reaction mixture was incubated for a further 5 min at 37 °C. The reaction was stopped by the addition of 500 µl of 10% (w/v) TCA in 100 mM HgCl₂. The mixture was centrifuged for 5

min at 9700 x g and an equal volume of a modified Ehrlich's reagent [28] was added to the supernatant. After a 15 min incubation, at room temperature, the absorbance at 555 nm was measured ($\epsilon_{555} = 6.02 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) [28].

Porphobilinogen deaminase. The HemC activity was determined essentially as described in [29], using 10-30 μg of enzyme and 200 nmol of PBG, and a $\epsilon_{405} = 5.48 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of enzyme is the amount of enzyme necessary to catalyze the utilization of 1 μmol of PBG in one hour.

Uroporphyrinogen III methyltransferase. Uroporphyrinogen III methyltransferase activity was followed by coupling it to precorrin-2 dehydrogenase (SirC) activity, allowing the transformation of uroporphyrinogen III into sirohydrochlorin ($\epsilon_{376} = 2.4 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) to be monitored. The substrate, uroporphyrinogen III, was generated anaerobically by incubating HemC and HemD from *B. megaterium* with 2 mg of PBG. The reaction mixture was prepared in an anaerobic chamber (Belle Technology, <2ppm O_2), in a final volume of 250 μl with NAD (100 μM), uroporphyrinogen III (2 μM), *B. megaterium* SirC (20 μg) and with different amounts of *D. vulgaris* bi-functional CobA/HemD (0.5-10 μg); the reaction was started by addition of SAM (100 μM).

Sirohaem synthase (CysG^B). Precorrin-2 dehydrogenase and sirohydrochlorin chelatase activities were assayed anaerobically following the formation of sirohydrochlorin or cobalt-sirohydrochlorin and sirohaem, respectively ($\epsilon_{376} = 2.4 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) [30]. The substrates, precorrin-2 and sirohydrochlorin, were generated anaerobically as described previously [30]. The dehydrogenase assay was performed in a 1 ml reaction, at 30 °C, with precorrin-2 (2 - 3 μM), NAD^+ (800 μM) and different amounts of *D. vulgaris* CysG^B (0.5-10 μg). The chelatase activity was measured with 4.2 μM of sirohydrochlorin, 20 μM of Co^{2+} or Fe^{2+} and varying the amount of enzyme (10-50 μg).

In vitro generation of precorrin-2 with D. vulgaris enzymes

HemB, HemC and CobA/HemD enzymes of *D. vulgaris* were tested simultaneously and individually in a linked assay for the *in vitro* generation of precorrin-2 [31]. The combination of enzymes and substrates used for each assay is described in Table 1. All the reaction mixtures were performed in 50 mM Tris-HCl pH 8, in a final volume of 3 ml and contained between 0.05-5 mg of each enzyme, SAM (1 mg) and ALA (0.5 mg) or PBG (0.2 mg). The reaction was incubated for 2 h at room temperature, and after colour development UV-visible spectra were recorded. *B. megaterium* SirC (0.1 mg) was added to the assays to generate sirohydrochlorin, as a further confirmation of precorrin-2 production (Table 1).

Complementation of E. coli cysG and E. coli hemD mutant strains

The pETac plasmids containing the genes *cobA/hemD*, *cobA* and *cysG^B* were transformed into *E. coli cysG* mutant strain 302 Δ a (cysteine auxotrophic) (Table 2). *E. coli cysG* transformants were selected on LB plates, with 100 $\mu\text{g}/\text{ml}$ ampicillin and 35 $\mu\text{g}/\text{ml}$ chloramphenicol, and the mutant phenotype rescue tested on minimal medium plates with the appropriate antibiotics, in the absence and presence of cysteine. The plasmids pET14b(+)*cobA/hemD* and pET14b(+)*hemD* were introduced in *E. coli* SASZ31 (a *hemD* gene mutant strain) and selected on LB plates, with 100 $\mu\text{g}/\text{ml}$ ampicillin by incubation at 37 °C, for approximately 20 h. The mutant strains were re-streaked on LB plates and incubated at 37 °C for a shorter time period (~12 h) to evaluate cell growth.

Results and Discussion

The genome of *D. vulgaris* [25] was searched using the basic local alignment search tool (BLAST) of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and DNA sequences encoding known bacterial enzymes involved in the early steps of tetrapyrrole biosynthesis and modification, namely HemB, HemC, CobA/HemD and CysG. Four gene-derived amino acid sequences that have a high degree of sequence identity with these enzymes from different organisms, such as *E. coli* and *B. subtilis*, were identified (Table 3). To study the *D. vulgaris* proteins the corresponding genes were cloned, the recombinant proteins produced and biochemically characterized.

D. vulgaris porphobilinogen synthase is active as a hexamer

A comparison of the gene deduced amino acid sequence of *D. vulgaris* porphobilinogen synthase with the porphobilinogen synthases of other organisms showed that *D. vulgaris* HemB contains the conserved cysteine rich sequence for Zn²⁺ binding (C121, C123 and C131, *D. vulgaris* HemB residue numbering), the determinant residues for allosteric magnesium binding (R11 and E238, *D. vulgaris* HemB residue numbering) [4] and the amino acid residues known to be involved in the binding of the substrate and reaction mechanism [32-34] (Figure 2). The recombinant *D. vulgaris* HemB migrates by SDS-PAGE analysis with an apparent molecular mass of ~36 kDa whereas gel filtration studies indicated the enzyme has a native mass of 226 kDa. This suggests that *D. vulgaris* exists as a homo hexamer.

The specific activity of *D. vulgaris* HemB was determined in the presence of zinc to be 45 μmol of porphobilinogen $\text{h}^{-1}\text{mg}^{-1}$ with a K_M value for ALA of 0.05 mM (Table 4). This activity is within the values measured for other bacterial porphobilinogen synthases such as *E. coli* [6], also assayed in the presence of zinc, and has a similar K_M .

Porphobilinogen deaminase of *D. vulgaris* contains the dipyrromethane cofactor in its active site

The purified recombinant *D. vulgaris* HemC migrates by SDS-PAGE with an apparent molecular mass of about 38 kDa, which is in close agreement with the predicted mass of 34 kDa. The UV-visible spectrum of the oxidized protein, at pH 8, contains two weak bands around 410 nm and 500 nm (data not shown), similar to the spectrum reported for the *E. coli* HemC enzyme [7]. To investigate the presence of the dipyrromethane cofactor, the enzyme was mixed with Ehrlich's reagent, and the reaction followed by UV-visible spectroscopy over a 20 min period. A change in absorbance from 566 nm to 495 nm (Figure 3) was observed, consistent with the presence of the dipyrromethane cofactor at the catalytic site of *D. vulgaris* HemC [7]. The specific activity of enzyme was measured as being 20 $\mu\text{mol h}^{-1}\text{mg}^{-1}$, which is similar to the activity of *E. coli* HemC enzyme (43 $\mu\text{mol h}^{-1}\text{mg}^{-1}$) [29]. *D. vulgaris* HemC has a K_M of 214 μM for porphobilinogen, which is higher than the range of values measured for HemC of *E. coli*, *Rhodopseudomonas sphaeroides*, *Clostridium josui* and *Chlorella regularis* (19-89 μM) [18, 29, 35, 36].

In D. vulgaris the uroporphyrinogen III synthase and methyltransferase activities are performed by a single enzyme

A BLAST search with the locus DVU0734 revealed that the N-terminal domain of the protein (amino acids 1-246) exhibits significant amino acid sequence similarity with the uroporphyrinogen III methyltransferase enzymes (CobA), whereas the C-terminal region of

DVU0734 (amino acids 247-503) has similarity with uroporphyrinogen III synthases (HemD). This suggests that in *D. vulgaris* the two activities are fused into a single multifunctional enzyme, which was named CobA/HemD. An amino acid sequence alignment of *D. vulgaris* DVU0734 with other separate bacterial CobA and HemD enzymes, allowed us to predict accurately the point of fusion between the two functional domains. Consequently, three proteins were recombinantly produced: the whole CobA/HemD protein, the truncated form corresponding to the N-terminal region (named CobA^d), and a second shorter protein corresponding to the C-terminal domain, (named HemD^d).

The initial characterization of DVU0734 was done by complementation. In *E. coli* the multifunctional enzyme CysG synthesizes sirohaem from uroporphyrinogen III. A deletion of this gene produces a mutant strain that is not able to produce sirohaem and thus cannot synthesize cysteine. The mutant phenotype can be rescued by complementation with the expression of genes from other organisms, which encode enzymes that catalyze the reactions performed by CysG (SAM-dependent methyltransferase of uroporphyrinogen III, NAD⁺-dependent dehydrogenation of precorrin-2 and ferrochelation of sirohydrochlorin) [25, 31, 37]. To test if the bi-functional CobA/HemD and CobA^d of *D. vulgaris* have uroporphyrinogen III methyltransferase activity *in vivo*, they were used to rescue the *E. coli* *cysG* mutant strain by co-expression with the bi-functional MET8 of yeast, which encodes a protein with precorrin-2 NAD⁺-dependent dehydrogenation and sirohydrochlorin ferrochelation activities (Table 2). The *E. coli* *cysG* strain expressing MET8 of *Saccharomyces cerevisiae* and *cobA/hemD* or *cobA^d* of *D. vulgaris* is able to grow in the absence of cysteine, demonstrating that the *D. vulgaris* enzyme variants are able to perform the *in vivo* transmethylation of uroporphyrinogen III to yield precorrin-2.

The *in vivo* uroporphyrinogen III synthase activity of the *D. vulgaris* CobA/HemD and HemD^d variants was investigated by complementation of an *E. coli* *hemD* strain (SASZ31) (see [38]). This strain grows very poorly in LB medium, forming mini colonies, and thus complementation is observed only when the bacterium is transformed with a gene that allows the organism to form normal-sized colonies. Transformation of the mutant strain with plasmids harbouring *D. vulgaris* *cobA/hemD* or *hemD^d* resulted in fast growing strains, demonstrating that the *D. vulgaris* *cobA/hemD* eliminates the cell growth deficiency and indicating that the encoded protein is functioning *in vivo*. Furthermore, *E. coli* cells producing the *D. vulgaris* CobA/HemD exhibited a reddish pigmentation, which is due to the accumulation of sirohydrochlorin and trimethylpyrrocorphin and is consistent with increased uroporphyrinogen III methyltransferase activity [13, 39].

The *in vitro* activity of the recombinantly produced and purified enzyme variants was also studied. After purification, the CobA/HemD, which has a molecular mass of ~55 kDa, was tested for uroporphyrinogen III synthase and methyltransferase activity. A specific activity of 3 nmol.min⁻¹.mg⁻¹ was determined for the uroporphyrinogen III methyltransferase activity of the fused enzyme, with a K_M for uroporphyrinogen III of 0.4 μM (Table 4). This is within the range of values observed previously, e.g. the *Pseudomonas denitrificans* CobA (1 μM) [40]. In contrast to the *B. megaterium* and *P. denitrificans* CobAs [10, 40], the *D. vulgaris* CobA/HemD did not display substrate inhibition with uroporphyrinogen III, a characteristic that had previously been observed with an archaeal uroporphyrinogen III methyltransferase [41]. The uroporphyrinogen III synthase activity of the CobA/HemD fusion protein was evaluated in a linked assay. Here, all the enzymes required to transform ALA into precorrin-2, except for the uroporphyrinogen III synthase, are mixed together. When ALA and SAM are added to this incubation the reaction stalls with the accumulation

of HMB. If uroporphyrinogen III synthase is added, the reaction proceeds to the synthesis of precorrin-2 and the development of a characteristic yellow colour. When the CobA/HemD fusion was supplied to this assay (Table 1), the generation of precorrin-2 was confirmed with the appearance of a characteristic UV-visible spectrum and the development of the typical yellow chromophore of the compound. The further addition of the *B. megaterium* SirC enzyme, which oxidizes precorrin-2, led to the formation of sirohydrochlorin providing further evidence for the formation of precorrin-2 in the reaction mixture (data not shown).

The activities of the two truncated proteins were investigated separately in an *in vitro* linked assay. This revealed that the CobA^d had uroporphyrinogen III methyltransferase activity but not uroporphyrinogen III synthase activity, while HemD^d was found to possess uroporphyrinogen III synthase activity (Table 1). During the course of these studies a mutant variant, F446A, was serendipitously obtained in HemD^d and this variant showed no measurable uroporphyrinogen III synthase activity. The function of F446 in HemD enzymes is unknown, and although is not strictly conserved this residue seems to play an essential role. However, more data will be required to confirm the catalytic role of F446.

The D. vulgaris CysG^B has precorrin-2 dehydrogenase activity

The *D. vulgaris* CysG^B has 17, 21 and 26 % sequence identity with *Saccharomyces cerevisiae* MET8, *B. megaterium* SirC and to the N-terminal part of the *E. coli* CysG, respectively (Table 3). Although this is a relatively low value of amino acid identity, it is in the same range of identity observed between the first 223 amino acids of *E. coli* CysG and MET8 (16 %). A common feature of all of these proteins is the presence of a consensus NAD⁺ binding motif at the start of the N-terminal region [42]. This motif on the *D. vulgaris* CysG^B (GxGxxGx₁₀G) is identical to the one observed in MET8 [14] and similar to the one found in the N-terminal domain of the *B. megaterium* SirC (GxGxxAx₁₀G) and *E. coli* CysG (GxGxxAx₃Ax₆G).

CysG^B of *D. vulgaris* was assayed for NAD⁺-dependent precorrin-2 dehydrogenase activity by following the appearance of sirohydrochlorin, and was found to have a specific activity of ~700 nmol.min⁻¹.mg⁻¹ and a *K_M* for NAD⁺ of 70 μM (Table 4). The specific activity of *D. vulgaris* CysG^B is significantly higher than the NAD⁺-dependent precorrin-2 dehydrogenase activity reported for the *B. megaterium* SirC (60 nmol.min⁻¹.mg⁻¹) [15]. However, the *D. vulgaris* enzyme is not able to perform the insertion of iron or cobalt into sirohydrochlorin, a result that is consistent with complementation studies, where the corresponding gene was unable to rescue the *E. coli cysG* mutant strain phenotype (Table 2). Cumulatively, these results show that the putative CysG^B of *D. vulgaris*, although annotated in the *D. vulgaris* genome as an orthologue of the yeast bi-functional enzyme Met8p [31], is in fact a functional SirC enzyme, a single NAD⁺-dependent precorrin-2 dehydrogenase that does not possess any chelatase activity [15].

D. vulgaris enzymes generate precorrin-2 from ALA in vitro

An *in vitro* incubation with purified recombinant *D. vulgaris* HemB, HemC and CobA/HemD together with ALA and SAM resulted in the generation of precorrin-2 (Figure 4 and Table 1). The presence of the yellow dipyrrocorphin was confirmed by the loading of purified *B. megaterium* SirC and NAD⁺ to the incubation, an addition that generated the isobacteriochlorin, sirohydrochlorin (Figure 4).

Conclusion

D. vulgaris makes sirohydrochlorin

In the work reported herein, the biochemical and functional characterization of several enzymes that catalyze the early steps of the modified tetrapyrrole biosynthetic pathway in *D. vulgaris* was undertaken. Thus, the *D. vulgaris* porphobilinogen synthase (HemB) was produced recombinantly and shown to be a zinc-dependent enzyme that appears to have a native hexameric structure, in contrast to the majority of bacterial HemB enzymes that are octamers. For porphobilinogen deaminase (HemC), the presence of the dipyrromethane cofactor, which covalently binds the growing poly-pyrrole chain, further supports the universal nature of this essential prosthetic group.

The recombinant *D. vulgaris* CobA/HemD was shown to be active both *in vivo* and *in vitro*, and represents the first such characterisation of a bi-functional uroporphyrinogen III synthase/methyltransferase. This suggests that, in this organism, uroporphyrinogen III is not released as a free intermediate. Rather, uroporphyrinogen III seems to be directed towards the formation of precorrin-2. This highly unstable intermediate is acted upon by a SirC-like protein, which, in the presence of NAD⁺, transforms it into sirohydrochlorin. It is known that sirohydrochlorin acts as an intermediate for B₁₂ biosynthesis, since it is chelated with cobalt to generate cobalt-sirohydrochlorin by CbiK [37]. It also plays a role in sirohaem synthesis where ferrous chelation generates this prosthetic group, which is required by sulfite reductase and which is also found in *D. vulgaris*. However, it is also likely that sirohydrochlorin acts as an intermediate in the biosynthesis of haem.

D. vulgaris does not contain HemE, N, G or H

One of the interesting features of the *D. vulgaris* genome is the absence of the genes that encode enzymes for the classical synthesis of haem from uroporphyrinogen III. Thus, there are no homologues to *hemE*, *hemN*, *hemG* or *hemH*, which encode the enzymes uroporphyrinogen III decarboxylase, coproporphyrinogen III oxidase, protoporphyrinogen IX oxidase and protoporphyrin IX ferrochelatase, respectively. The absence of these genes is consistent with the presence of a novel pathway for the transformation of precorrin-2 or sirohydrochlorin into haem. Previous work by Sano and co-workers had shown that haem must be made via precorrin-2, since the two methyl groups found on rings A and B of the final porphyrin product are derived from methionine and not from the C2 position of ALA [22]. However, in their follow-up work, it was reported that an incubation of crude cell extracts of *D. vulgaris* with uroporphyrinogen III resulted in the appearance of sirohydrochlorin, 12,18-didecarboxysirohydrochlorin, coproporphyrin III and protoporphyrin IX. However, in this study only the structure of the novel 12,18-didecarboxysirohydrochlorin was fully characterised [23].

If coproporphyrinogen III is an intermediate, then it could be acted upon by an anaerobic coproporphyrinogen oxidase such as HemN. Interestingly, the *D. vulgaris* genome does encode an orthologue of the radical SAM-reliant coproporphyrinogen III oxidase. The HemN proteins are characterised by the presence of an Fe-S redox group, which is constituted by 4 cysteines within the conserved motif (CxxxCxxCxC). However, in the *D. vulgaris* homologue, one of the cysteines is missing, suggesting that this *D. vulgaris* protein is not a HemN. Proteins missing this conserved motif have been shown not to be involved in haem biosynthesis [43]. Thus, if coproporphyrinogen III is an intermediate, it is not clear how it is converted into protoporphyrin IX as there are no recognized enzymes present to undertake the required reactions.

Possible proteins involved in the novel haem synthesis pathway

A scan of the genome reveals a number of genes encoding enzymes that are designated for the synthesis of haem d_1 (*nirD* and *nirJ*), the cofactor of the nitrite reductase *cd₁*. However, *D. vulgaris* does not seem to have *cd₁*. These haem d_1 synthetic homologues are also found in some genomes of methanogens and other bacteria that do not have *cd₁*. In the methanogens, it has also been shown that haem is also made via a dimethylated uroporphyrinogen III derivative such as precorrin-2 or sirohydrochlorin [44]. Significantly, there are some mechanistic similarities between the pathways for haem d_1 synthesis and haem construction via precorrin-2. In haem d_1 synthesis the two propionic acid side chains attached at C3 and C8 of the macrocycle are lost and are replaced by oxygen. To achieve haem synthesis from precorrin-2/sirohydrochlorin, the acetic acid side chains at C2 and C7 have to be removed. Both processes may involve radical chemistry and thus could be mediated via a radical SAM enzyme system. It is therefore of interest to note that NirJ is proposed to be a radical SAM protein. We thus propose that the *D. vulgaris nirJ* and *nirD* genes are involved in the transformation of either sirohydrochlorin or sirohaem into haem.

In summary, the research described in this paper outlines how the basic tetrapyrrole framework is synthesized up to the first branch point, which we believe to be sirohydrochlorin. Genomic analysis thus suggests that cobalamin and sirohaem are made by classical previously described routes but that haem is synthesized along a novel route using homologues of the haem d_1 biosynthetic apparatus. The presence of a bifunctional CobA/HemD is consistent with uroporphyrinogen III not being a branch point in this bifurcated pathway and may represent a useful marker for an alternative haem biosynthetic route. More research is required to elucidate this surrogate synthesis. It is not clear why some of the *Desulfovibrio* or archaea have an alternative haem biosynthetic pathway. Possibly, the pathway evolved as a method of producing the more oxidized porphyrin ring system under anaerobic conditions at a time when the major tetrapyrroles were sirohaem and cobalamin. Then as molecular oxygen became more abundant so the haem biosynthesis pathway involving several oxidative steps in the synthesis of protoporphyrin evolved and became the more common route.

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Table 1 Enzymes and substrates utilized in the linked *in vitro* assays for anaerobic generation of precorrin-2

<i>D. vulgaris</i> enzyme	Other enzymes and substrates present in the assay*	Precorrin-2 generation
HemB	<i>B. megaterium</i> HemC and HemD, <i>P. denitrificans</i> CobA, ALA, SAM	Yes
HemC	<i>B. megaterium</i> HemD, <i>P. denitrificans</i> CobA, PBG, SAM	Yes
HemD ^d	<i>B. megaterium</i> HemC, <i>P. denitrificans</i> CobA, PBG, SAM	Yes
F446S-HemD ^d	<i>B. megaterium</i> HemC, <i>P. denitrificans</i> CobA, PBG, SAM	No
CobA ^d	<i>B. megaterium</i> HemC and HemD, PBG, SAM	Yes
CobA/HemD	<i>B. megaterium</i> HemC, PBG, SAM	Yes
HemB, HemC, CobA/HemD	ALA, SAM	Yes
None	<i>B. megaterium</i> HemC and HemD, <i>P. denitrificans</i> CobA, PBG, SAM (Positive control)	Yes

**Pseudomonas (P.) denitrificans* CobA and *Bacillus (B.) megaterium* HemC and HemD were used in the assays where *D. vulgaris* enzymes were tested individually.

Table 2 Strains and plasmids used in complementation studies together with *D. vulgaris* enzymes

<i>D. vulgaris</i> enzyme	Strain	Plasmids	Plasmid description	References
CobA/HemD*	<i>E. coli</i> 302Δa	pETac- <i>cobA/hemD</i>	<i>D. vulgaris cobA/hemD</i> cloned in pETac	This study
		pCIQ- <i>met8p</i>	<i>Sa. met8p</i> cloned in pCIQ	This study
CobA ^d *	<i>E. coli</i> 302Δa	pETac- <i>cobA</i> pCIQ- <i>met8p</i>	<i>D. vulgaris cobA</i> cloned in pETac <i>Sa. cerevisiae met8p</i> cloned in pCIQ	This study This study
		<i>E. coli</i> 302Δa (Positive control)	pETac- <i>PdcobA</i> pCIQ- <i>met8p</i>	<i>P. denitrificans cobA</i> cloned in pETac <i>Sa. cerevisiae met8p</i> cloned in pCIQ
CobA/HemD [§]	<i>E. coli</i> SASZ31	pET14b(+)- <i>cobA/hemD</i>	<i>D. vulgaris cobA/hemD</i> cloned in pET14b(+)	This study
HemD ^d §	<i>E. coli</i> SASZ31	pET14b(+) <i>hemD</i>	<i>D. vulgaris hemD</i> cloned in pET14b(+)	This study
		<i>E. coli</i> SASZ31 (Positive control)	pET14b(+) <i>EchemD</i>	<i>E. coli hemD</i> cloned in pET14b(+)
CysG ^B ¶	<i>E. coli</i> 302Δa	pETac- <i>cysG^B</i>	<i>D. vulgaris cysG^B</i> cloned in pETac	This study
		pCIQ- <i>PdcobA</i>	<i>P. denitrificans cobA</i> cloned in pCIQ	[37]
	<i>E. coli</i> 302Δa (Positive control)	pETac- <i>met8p</i> pCIQ- <i>PdcobA</i>	<i>Sa. cerevisiae met8p</i> cloned in pETac <i>P. denitrificans cobA</i> cloned in pCIQ	[30] [37]
CysG ^B Δ	<i>E. coli</i> 302Δa	pETac- <i>cysG^B</i>	<i>D. vulgaris cysG^B</i> cloned in pETac	This study
		pCIQ- <i>sirCcobA</i>	<i>M. thermoautotrophicus sirC</i> and <i>P. denitrificans cobA</i> cloned in pETac	[37]

<i>E. coli</i> 302Δa (Positive control)	pETac- <i>met8p</i> pCIQ- <i>sirCcobA</i>	<i>Sa. cerevisiae met8p</i> cloned in pETac <i>M. thermoautotrophicus sirC</i> and <i>P. denitrificans cobA</i> cloned in pCIQ	[30] [37]
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* CobA activity tested with CobA/HemD protein and CobA^d.

§ HemD activity tested in the presence of CobA/HemD protein and HemD^d.

¶ CysG^B protein was tested for precorrin-2 dehydrogenase and sirohydrochlorin ferrochelatase activity.

Δ CysG^B protein was tested for sirohydrochlorin ferrochelatase activity.

Pseudomonas (P.); *Saccharomyces (Sa)*; *Methanothermobacter (M.)*.

Table 3 Amino acid sequence identities and similarities of *D. vulgaris* enzymes with orthologs

	Identity (%)	Similarity (%)
<i>D. vulgaris</i> HemB		
<i>E. coli</i>	47	66
<i>Br. japonicum</i>	42	60
<i>P. aeruginosa</i>	44	62
<i>Pi. sativum</i>	41	54
<i>D. vulgaris</i> HemC		
<i>E. coli</i>	52	63
<i>S. enterica</i>	51	63
<i>R. capsulatus</i>	40	54
<i>C. josui</i>	33	50
<i>D. vulgaris</i> CobA/HemD		
<i>Se. ruminantium</i>	52	63
<i>L. reuteri</i>	29	47
<i>C. josui</i>	36	56
<i>Li. innocua</i>	35	53
<i>D. vulgaris</i> CobA ^d		
<i>P. denitrificans</i>	40	56
<i>Br. megaterium</i>	48	67
<i>Pa. denitrificans</i>	34	47
<i>M. thermoautotrophicus</i>	42	62
<i>D. vulgaris</i> HemD ^d		
<i>E. coli</i>	23	33
<i>S. enterica</i>	22	33
<i>B. megaterium</i>	27	47
<i>Pa. denitrificans</i>	16	27
<i>D. vulgaris</i> SirC		
<i>E. coli</i> CysG	12	20

<i>E. coli</i> CysG N-terminal	26	42
<i>Sa. cerevisiae</i> MET8	17	31
<i>B. megaterium</i> SirC	21	41

NCBI accession numbers in parenthesis: HemB proteins: *D. vulgaris* (AAS95336), *E. coli* (NP_414903), *Bradyrhizobium* (*Br.*) *japonicum* (P45622), *Pseudomonas* (*P.*) *aeruginosa* (AAG08628), *Pisum* (*Pi.*) *sativum* (AAA33640); HemC proteins: *E. coli* (AAA67601), *Salmonella* (*S.*) *enterica* (CAD09382), *Rhodobacter* (*R.*) *capsulatus* (AAG50298), *Clostridium* (*C.*) *josui*, (BAA05861); CobA/HemD proteins: *Selenomonas* (*Se.*) *rumantium* (AAK00606), *Lactobacillus* (*L.*) *reuteri* (AAX14527), *C. josui* (BAA05862), *Listeria innocua* (NP_470501); CobA proteins: *P. denitrificans* (AAA25773), *Bacillus* (*B.*) *megaterium* (AAA22317), *Paracoccus* (*Pa.*) *denitrificans* (AAA93119), *Methanothermobacter* (*M.*) *thermautotrophicus* (NP_275310); HemD proteins: *E. coli* (NP_418248), *B. megaterium* (CAD48147), *Pa. denitrificans* (YP_915785), *S. enterica* (AAL22782); *E. coli* CysG and CysG N-terminal (NP_417827 and residues 1-223 of NP_417827), *Saccharomyces* (*Sa.*) *cerevisiae* MET8 (NP_009772), *B. megaterium* SirC (CAD48923). The amino acid sequence alignments were performed in Clustal W2 [45].

Table 4 Kinetic parameters of *D. vulgaris* enzymes studied in this work

<i>D. vulgaris</i> enzyme	Specific Activity	K_M (substrate)
HemB	45*	0.05 mM (ALA)
HemC	20*	214 μ M (PBG)
CobA/HemD	3 [§]	0.4 μ M (uroporphyrinogen III)
SirC	700 ^{§§}	70 μ M (NAD ⁺)

* μ mol. h⁻¹. mg⁻¹; [§]nmol. min⁻¹. mg⁻¹

Figure Legends

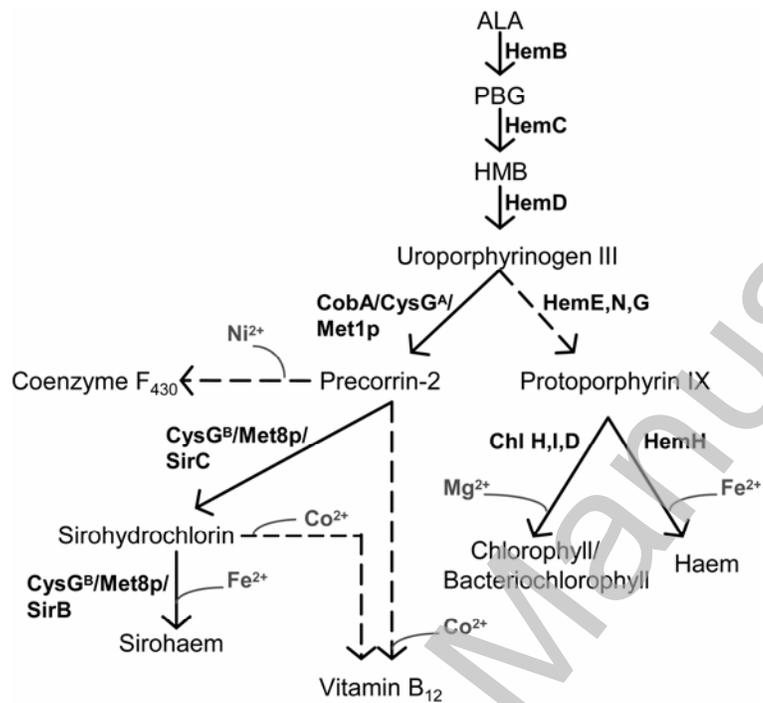
Figure 1 Tetrapyrrole metabolic pathway. Adapted from [46].

Figure 2 Amino acid sequence alignment of porphobilinogen synthases from *D. vulgaris* (AAS95336), *E. coli* (NP_414903), *Bradyrhizobium (Br.) japonicum* (P45622), *Pseudomonas (P.) aeruginosa* (AAG08628) and *Pisum (Pi.) sativum* (AAA33640) (adapted from [32]) (NCBI accession numbers in parenthesis). Strictly conserved residues are shaded. The symbols represent functionally relevant residues in the *E. coli* enzyme [32, 33] which are also conserved in *D. vulgaris* porphobilinogen synthase: (#) cysteine residues that bind zinc; (*) residues involved in allosteric magnesium binding; (“) arginine residues involved in binding A-side ALA; (—) residues of the lid region that covers the active site; (&”) lysine residue required for correct function of the enzyme; (&) lysine residue that forms the Schiff base with P-side ALA; (\$) residues involved in binding of P-side ALA.

Figure 3 *D. vulgaris* porphobilinogen deaminase spectral features. Reaction with Ehrlic's reagent assessed by UV-visible. The spectra were recorded at the indicated times.

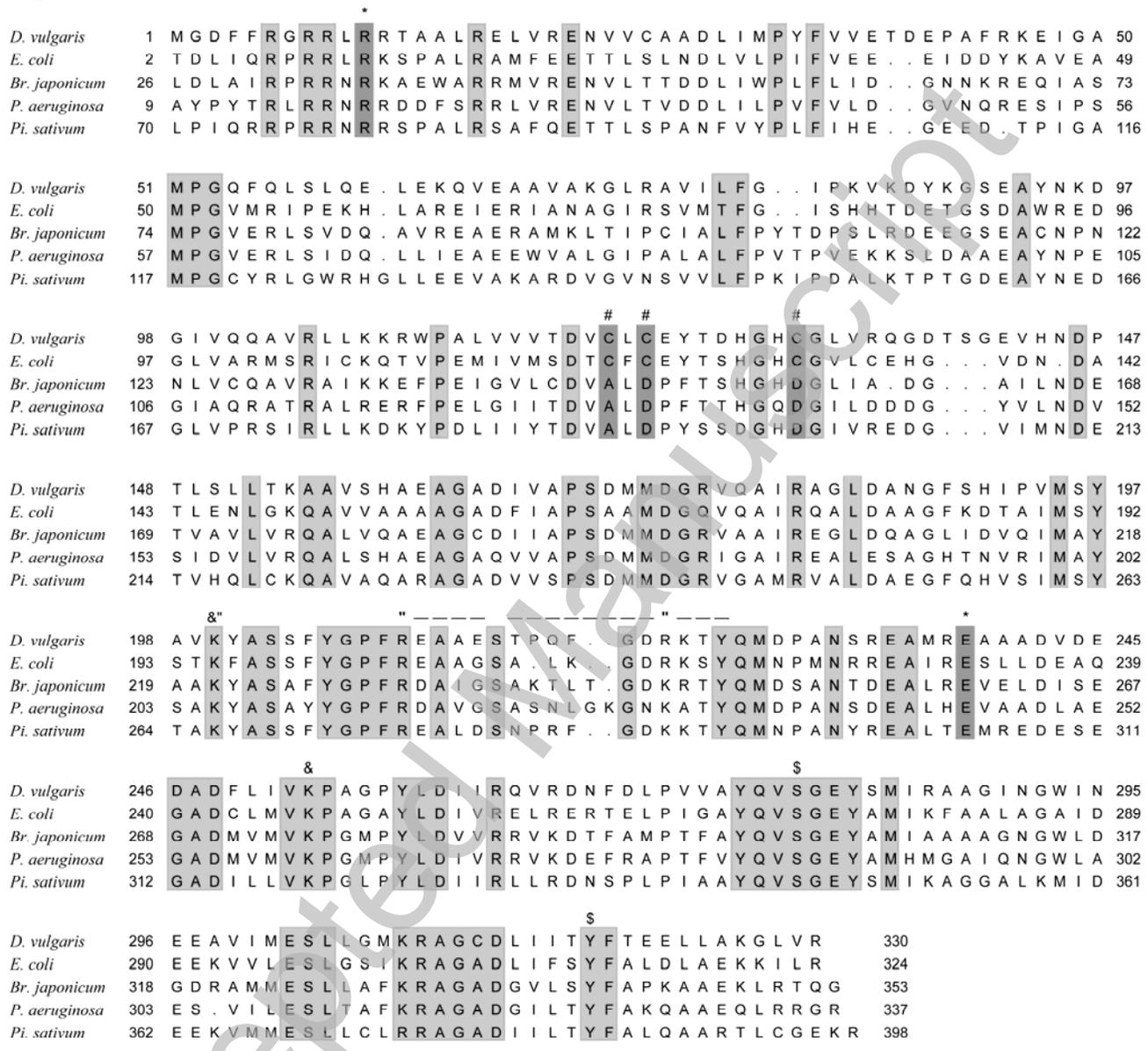
Figure 4 UV-visible spectra of precorrin-2 (A) and sirohydrochlorin (B) generated in the linked assay performed with *D. vulgaris* recombinant enzymes.

Figure 1



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Figure 2

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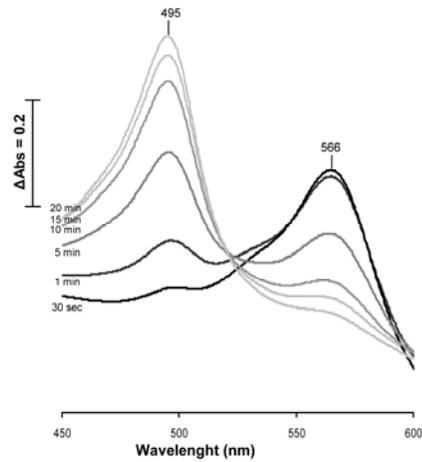
Figure 3

Figure 4