CHAPTER SEVEN

Sulfate-Reducing Bacteria Reveal a New Branch of Tetrapyrrole Metabolism

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Abstract

Sulfate-reducing microorganisms are a diverse group of bacteria and archaea that occupy important environmental niches and have potential for significant biotechnological impact. *Desulfovibrio*, the most studied genus among the sulfate-reducing microorganisms, contains proteins with a wide variety of tetrapyrrole-derived cofactors, including some unique derivatives such as uroporphyrin I and coproporphyrin III. Herein, we review tetrapyrrole metabolism in *Desulfovibrio* spp., including the production of sirohaem and cobalamin, and compare and contrast the biochemical properties of the enzymes involved in these biosynthetic pathways. Furthermore, we describe a novel pathway used by *Desulfovibrio* to synthesize haem *b*, which provides a previously unrecognized link between haem, sirohaem, and haem d_1. Finally, the organization and regulation of genes involved in the tetrapyrrole biosynthetic pathway is discussed.

ABBREVIATIONS

ALA δ-aminolevulinic acid
COPRO III coproporphyrin III

Advances in Microbial Physiology, Volume 61
ISSN 0065-2911
http://dx.doi.org/10.1016/B978-0-12-394423-8.00007-X

COPROGEN III coproporphyrinogen III
DDSH 12,18-didecarboxysirohaem
HMB hydroxymethylbilane
PBG porphobilinogen
PC-2 precorrin-2
PROTO IX protoporphyrin IX
PROTOGEN IX protoporphyrinogen IX
SH sirohaem
SHC sirohydrochlorin
SRM sulfate-reducing microorganisms
URO I uroporphyrin I
UROGEN III uroporphyrinogen III

1. INTRODUCTION TO *DESULFOVIBRIO* SPECIES

Sulfate-reducing microorganisms (SRM) include both bacteria and archaea that are able to perform the dissimilatory reduction of sulfate. SRM are divided into five bacterial and two archaeal lineages according to the analysis of their 16S rRNA sequences. The bacterial lineages include the mesophilic Deltaproteobacteria, where most of the SRM belong (including the genera *Desulfovibrio*, *Desulfobacterium*, *Desulfobacter*, and *Desulfolobus*); the Gram-positive *Bacillus/Clostridium* group of the phylum Firmicutes with several spore formers (e.g., *Desulfotomaculum*, *Desulfosporosinus*, and *Desulfosporomusa genera*); and the thermophilic Gram-negative bacteria with the genera Thermodesulfobacteria (*Thermodesulfobacterium* genus), Thermodesulfobiaceae (*Thermodesulfobium* genus), and Nitrospirae (e.g., *Thermodesulfovibrio* genus). The two groups of sulfate reducers from the Archaea domain belong to Euryarchaeota (e.g., *Archaeoglobus*) and Crenarchaeota (e.g., *Caldivirga* and *Thermocladium*) (Thauer, Stackebrandt, & Hamilton, 2007).

Although defined by their ability to reduce sulfate, SRM are quite versatile and are able to use several other terminal electron acceptors for anaerobic respiration, such as sulfur, fumarate, dimethylsulfoxide, Mn (IV), and Fe(III) (Thauer et al., 2007). In spite of initially being considered strict anaerobes, SRM have been found to live close to oxygenated niches and a number of studies have demonstrated the tolerance of the *Desulfovibrio* spp. to oxygen (Frund & Cohen, 1992; Lobo, Melo, Carita, Teixeira, & Saraiva, 2007; Minz et al., 1999; Mogensen, Kjeldsen, & Ingvorsen, 2005; Okabe, Ito, & Satoh, 2003). The presence of detoxifiers such as superoxide dismutases and reductases, catalases, and peroxidases within

their proteomes enables them to cope with oxygen. The physiological adaptations of *Desulfovibrio* go as far as having membrane-bound terminal oxygen reductases, which has led to the proposal that these microorganisms can actually respire oxygen (Dolla, Donald, Kurtz, Teixeira, & Voordouw, 2007).

As SRM are mainly found in sulfate-rich anoxic habitats like marine and freshwater sediments, they have been considered environmentally important microorganisms where they display both favorable and unfavorable roles. Thus, although they are involved in the bioremediation of aromatic and chlorinated hydrocarbons (e.g., benzene, chloroethenes, and nitroaromatic compounds) and toxic metals such as U(VI) and Cr(VI) in contaminated soils, and are able to enhance the recovery of precious metals from waste streams (e.g., platinum, palladium, and gold), SRM are also responsible for the biocorrosion of petroleum pumping equipment, storage tanks, and pipelines (Hockin & Gadd, 2007). Additionally, SRM are part of the normal human intestinal flora where *Bilophila wadsworthia* and *Desulfovibrio piger* are the dominant species (Jia et al., 2012). Nevertheless, it has been proposed that there is a correlation between the presence of some species of *Desulfovibrio* and diseases such as autism and inflammatory bowel diseases (Finegold, 2011; Jia et al., 2012; Loubinoux, Bronowicki, Pereira, Mougenel, & Faou, 2002).

Within the SRM, the genus *Desulfovibrio* has been widely studied and shown to contain a diverse range of single and multihaem proteins, often with the haem group found together with other cofactors. Several *Desulfovibrio* haem proteins are involved in sulfate respiration including a variety of haem *c* containing proteins with 1 (cytochrome c_{553}), 4 (cytochrome c_3), 9 (nine-haem cytochrome *c*), and 16 (dodecahaem cytochrome *c*) haem groups covalently bound to a single polypeptide chain via thioether bridges to cysteine residues (Fig. 7.1; Pereira & Xavier, 2005). Both the usual haem *c* binding motif (CXXCH) and a modified version (CXXXXCH) are found, the latter specifically in one of the cytochrome c_3 from *Desulfovibrio vulgaris* Hildenborough (DVU3171). The histidine of the motif forms one of the iron axial ligands and, when present, the second ligand is taken either by a methionine or a further histidine residue (Pereira & Xavier, 2005).

Proteins with combinations of different haem types also exist as part of several respiratory complexes and enzymes, such as the quinol oxygen reductases of the *bd* family, which contain a haem *d* (Fig. 7.1) together with two types of haems *b* (b_{558} and b_{595}) (Fig. 7.1; Lemos et al., 2001), and the haem–copper oxygen reductase proposed to contain haems *c*, *o*, *b*, and o_3 types (Lamrabet et al., 2011; Lobo, Almeida, Carita, Teixeira, & Saraiva, 2008).

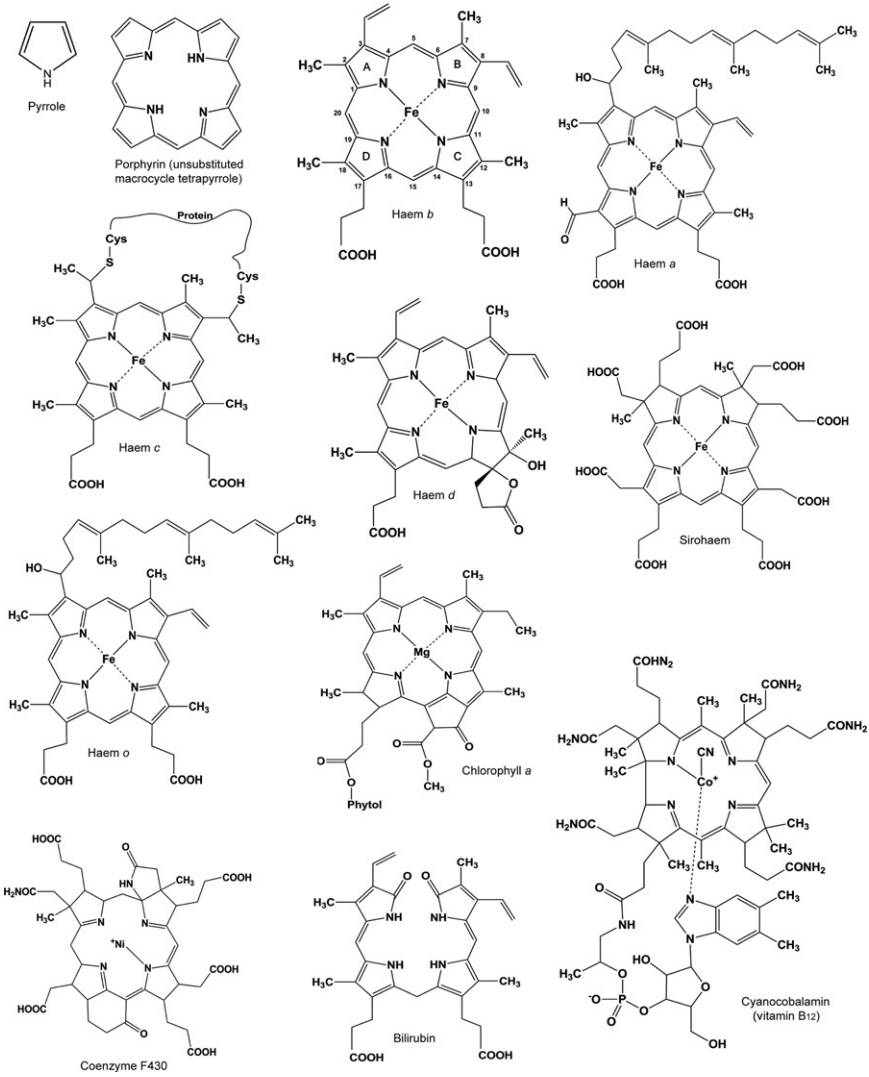


Figure 7.1 Tetrapyrrole framework (pyrrole and porphyrin) and examples of modified tetrapyrrole structures found in nature (haem *b*, haem *a*, haem *c*, haem *d*, sirohaem, haem *o*, chlorophyll *a*, cyanocobalamin (vitamin B_{12}), coenzyme F_{430}, and bilirubin.

Proteins with unique tetrapyrrole derivatives have also been isolated from *Desulfovibrio* spp. Iron-uroporphyrin I (URO I) (Fig. 7.2) is present in *D. gigas* rubredoxin:oxygen oxidoreductase of *D. gigas* (Timkovich, Burkhalter, Xavier, Chen, & LeGall, 1994), and iron-coproporphyrin III (COPRO III) (Fig. 7.2) is the cofactor of bacterioferritin of *D. desulfuricans*

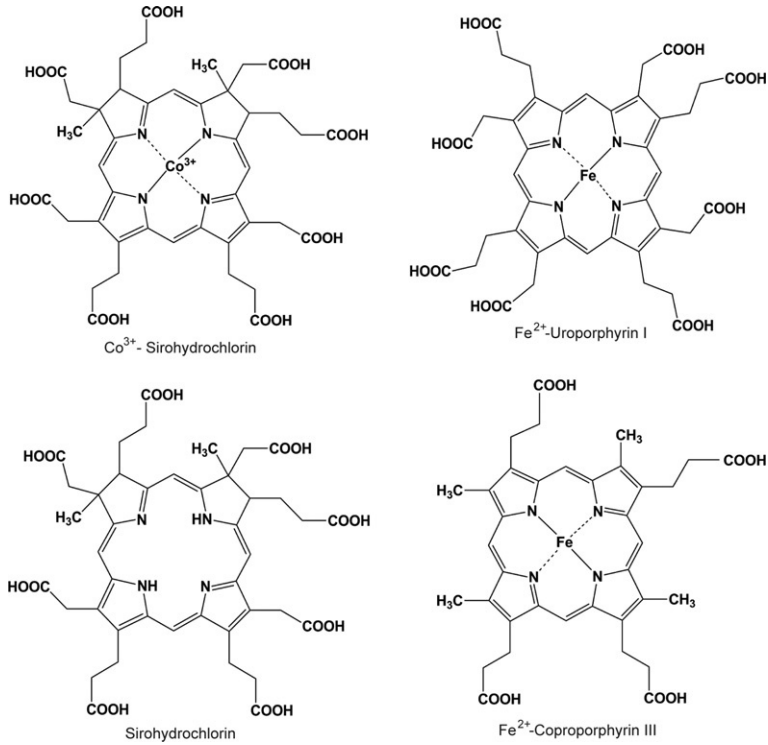


Figure 7.2 Unusual modified tetrapyrroles found in *Desulfovibrio* species.

ATCC 27774 (Romão et al., 2000). Sirohaem (SH) (Fig. 7.2) is also present in SRM as a cofactor of the assimilatory and dissimilatory sulfite and nitrite reductases, which are key metabolic enzymes in these organisms. The sulfite reductase that performs the six electron reduction of sulfite to sulfide has a unique combination of SH/iron–sulfur centers. As well as the normal SH-$[4Fe–4S]^{2+/1+}$ center, the *D. vulgaris* enzyme contains a demetalated form of SH, that is, sirohydrochlorin (SHC) (Fig. 7.2), closely located to the $[4Fe–4S]^{2+/1+}$ cluster but not coupled to it (Oliveira et al., 2008).

Proteins containing cobalt-isobacteriochlorins, namely Co^{3+}-SHC (Fig. 7.2) or a derivate of this tetrapyrrole, are present in *Desulfovibrio* spp. (Battersby & Sheng, 1982). Moreover, corrinoids such as guanylcobamide and hypoxanthylcobamide were reported as being produced by *D. vulgaris*, although the addition of 5,6-DMB to these cells generates the normal form of cobalamin (the biological form of vitamin B_{12}), through the incorporation of this unusual base into the lower nucleotide loop (Fig. 7.1; Guimarães,

Weber, Klaiber, Vogler, & Renz, 1994). Moreover, *D. desulfuricans* LS methylates mercury via methylcobalamin (Choi & Bartha, 1993).

Currently, several completed sequenced *Desulfovibrio* genomes are available (http://www.jgi.doe.gov/), and their analysis reveals the presence of genes involved in tetrapyrrole synthesis. In this review, we provide an overview of tetrapyrrole biosynthesis in *Desulfovibrio* and of the alternative haem *b* biosynthesis pathway present in these bacteria.

2. TETRAPYRROLE BIOSYNTHESIS

Modified tetrapyrroles are essential pigments of life because of their involvement in key biological processes such as electron transfer, light harvesting, respiration, catalysis, and oxygen binding. These macrocycles are formed from four pyrrole rings (Fig. 7.1) linked through methylene bridges, generating a structure that is able to chelate specific metal ions and can have a variety of substitutions on the ring carbons. The resulting metalloprosthetic groups include haems (Fe), SH (Fe), chlorophylls (Mg), bacteriochlorophylls (Mg), cobalamin (Co), coenzyme F_{430} (Ni), and haem d_1 (Fe) (Fig. 7.1; Battersby, 2000).

Tetrapyrroles are generated along a branched biosynthetic pathway that starts with the formation of the universal tetrapyrrole precursor, δ-aminolevulinic acid (ALA). This intermediate, a linear five-carbon aminoketone, is converted via the general tetrapyrrole pathway in just three steps into the first macrocyclic intermediate, uroporphyrinogen III (UROGEN III). Subsequent branching of the pathway allows the formation of haem *b*, haem d_1, SH, cobalamin, chlorophyll, or cofactor F_{430} (Fig. 7.3). Haem *b* is the precursor of other types of haems such as haem *c*, *a*, and *o* (Fig. 7.3; Gibson, Laver, & Neuberger, 1958; Heinemann, Jahn, & Jahn, 2008).

In proteobacteria, fungi, yeast, and animals, ALA is formed by the condensation of succinyl-coenzyme A and glycine via the Shemin pathway (also called the C_4-pathway) in a one-step reaction performed by a pyridoxal-5′-phosphate (vitamin B_6)-dependent enzyme named ALA synthase, which is encoded by *hemA* (Fig. 7.3; Gibson et al., 1958). Analysis of the *Desulfovibrio* genomes strongly suggests that, as in plants, algae, and the majority of prokaryotes, ALA is formed by the C_5-pathway. This starts with the ligation of a glutamate molecule to a $tRNA^{Glu}$, to form glutamyl-$tRNA^{Glu}$ via the enzyme glutamyl-tRNA reductase, encoded by the *gltX* gene. The resulting compound is reduced by a NADPH-dependent glutamyl-tRNA reductase (*gltR* gene) yielding glutamate 1-semialdehyde. Finally, transamination of this

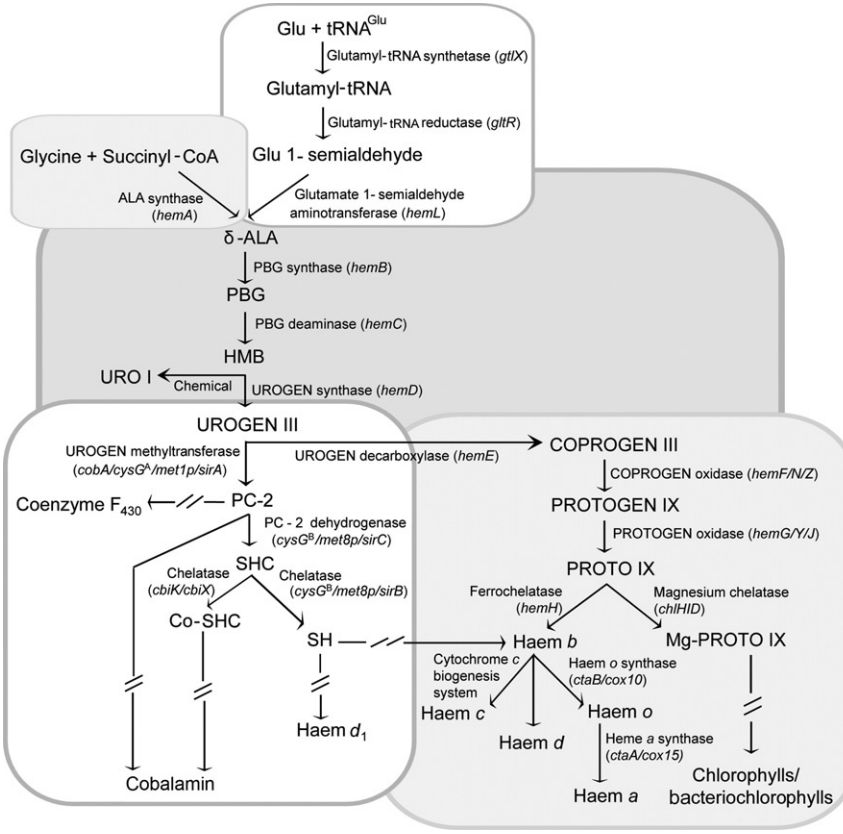


Figure 7.3 General tetrapyrrole biosynthetic pathway. δ-Aminolevulinic acid (δ-ALA), porphobilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen III (UROGEN III), uroporphyrin I (URO I), precorrin-2 (PC-2), sirohydrochlorin (SHC), cobalt-sirohydrochlorin (Co-SHC), sirohaem (SH), coproporphyrinogen III (COPROGEN III), protoporphyrinogen IX (PROTOGEN IX), protoporphyrin IX (PROTO IX), and magnesium-protoporphyrin IX (Mg-PROTO IX).

reaction product, by glutamate 1-semialdehyde aminotransferase (*hemL* gene), leads to the generation of ALA (Figs. 7.3 and 7.4; Heidelberg et al., 2004; Jahn, Verkamp, & Soll, 1992).

The step that promotes the asymmetric condensation of two ALA molecules into the monopyrrole porphobilinogen (PBG) is performed by PBG synthase, an enzyme encoded by *hemB* (Jaffe, 2004). In *D. vulgaris*, the PBG synthase HemB contains a catalytically important zinc, which is bound through a conserved cysteine-rich sequence $C_{121}XC_{123}X_7C_{131}$, and an allosteric magnesium site (R11 and E238) (Jaffe, 2003; Lobo, Brindley,

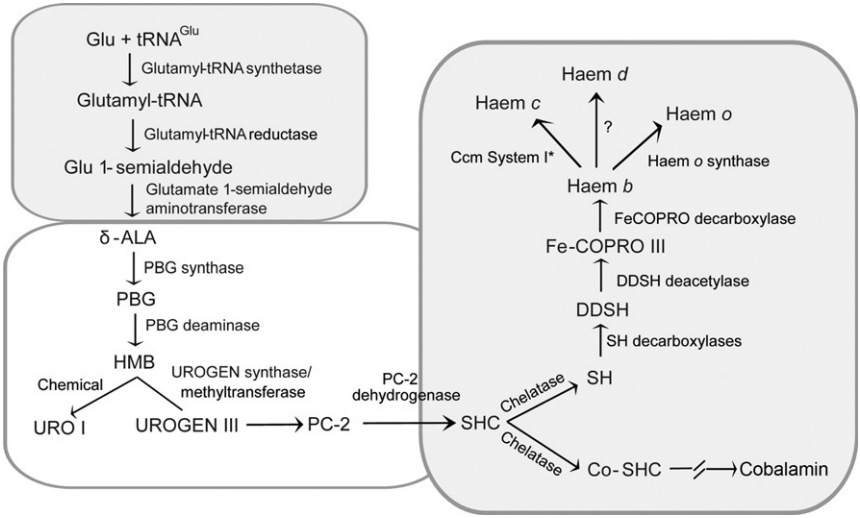


Figure 7.4 Tetrapyrrole biosynthesis in *Desulfovibrio*. δ-Aminolevulinic acid (δ-ALA), porphobilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen III (UROGEN III), uroporphyrin I (URO I), precorrin-2 (PC-2), sirohydrochlorin (SHC), cobalt-sirohydrochlorin (Co-SHC), sirohaem (SH), 12,18-didecarboxysirohaem (DDSH), and Fe-coproporphyrin III (Fe-COPRO III).

Warren, & Saraiva, 2009). In contrast to the majority of bacterial HemB enzymes that are octamers, *D. vulgaris* HemB is active as a homohexamer (226 kDa) with a specific activity for zinc of 45 μmol of PBG h^{-1} mg^{-1} and K_M (ALA) of 0.05 mM, which are within the range of values of canonical bacterial PBG synthases (Lobo et al., 2009).

The next enzyme in the pathway, PBG deaminase (encoded by *hemC*), catalyzes the polymerization of four molecules of PBG into a linear tetrapyrrole called hydroxymethylbilane (HMB) (Jordan & Berry, 1981). As with all PBG deaminases, the *D. vulgaris* enzyme (34 kDa) contains a covalently bound dipyrromethane cofactor at the active site (Lobo et al., 2009). The cofactor acts as the point of polypyrrole chain assembly, forming stable enzyme–substrate complexes as it progresses through its catalytic cycle. These intermediate complexes represent enzyme with one (ES1), two (ES2), and three (ES3) PBG-derived pyrroles attached to the cofactor. The binding of the final fourth (ES4) pyrrole to this chain results in hydrolytic cleavage between the cofactor and first pyrrole unit, generating HMB and holoenzyme. The holoenzyme is formed from the attachment of HMB to a conserved cysteine in the apoenzyme, which results in holoenzyme in the ES2 form. The UV–visible spectrum of the oxidized HemC displays two weak

bands around 410 and 500 nm attributed to the generation of an inactive oxidized dipyrromethane (dipyrromethene) cofactor (Jordan, Thomas, & Warren, 1988; Jordan & Warren, 1987; Jordan et al., 1988; Lobo et al., 2009). The *D. vulgaris* HemC has an activity of 20 μmol h^{-1} mg^{-1} and a K_M (PBG) of 214 μM. While the specific activity is similar to other bacterial HemC enzymes, the K_M is higher than the range of values usually observed (19–89 μM) (Lobo et al., 2009).

HMB is very unstable and chemically cyclizes to form the uroporphyrinogen I isomer, which will spontaneously oxidize to form URO I (Figs. 7.3 and 7.4). Interestingly, *D. gigas* rubredoxin:oxygen oxidoreductase contains an iron–URO I complex as prosthetic group, although it does not appear to be required for the oxygen reduction activity of the enzyme (Gomes et al., 1997; Timkovich et al., 1994).

In the general tetrapyrrole pathway, HMB is cyclized to UROGEN III by UROGEN III synthase, an enzyme encoded by the *hemD* gene. This reaction involves not only cyclization but also the inversion of ring D of the bilane to generate the unsymmetrical type III isomer. For the majority of organisms that perform tetrapyrrole biosynthesis, UROGEN III is the last common intermediate of the pathway. At this point, the pathway branches such that decarboxylation of UROGEN III leads to the formation of haems and chlorophylls, whereas C-methylation directs intermediates to the synthesis of SH, haem d_1, coenzyme F_{430}, and cobalamin (Fig. 7.3; Dailey, 2002; Heinemann et al., 2008; Shoolingin-Jordan, 2003).

In most other organisms that operate a canonical haem biosynthetic pathway, haem synthesis is mediated by the decarboxylation of the peripheral side chains of UROGEN III. This reaction is catalyzed by uroporphyrinogen decarboxylase (HemE) and generates coproporphyrinogen III (COPROGEN III) (Fig. 7.3). This intermediate subsequently undergoes oxidative decarboxylation to give protoporphyrinogen IX (PROTOGEN IX), in a reaction catalyzed by coproporphyrinogen oxidase (HemN, HemF, or HemZ) (Heinemann et al., 2008; Shoolingin-Jordan, 2003; Fig. 7.3). Oxidation of PROTOGEN IX by the removal of six protons and six electrons generates the porphyrin, which acts as the substrate for the ferrochelatase (HemH) to give haem (Fig. 7.3) (Dailey, 2002). Interestingly, no orthologues of any of these enzymes have been found in the *Desulfovibrio* spp., suggesting that these organisms make haem via an alternative pathway. This is discussed later in the review. The absence of an uroporphyrinogen decarboxylase in *D. vulgaris* resulted in the search for other known uroporphyrinogen metabolizing enzymes. Interestingly, what was found was that the

UROGEN III synthase and UROGEN III methyltransferase are fused in a single enzyme, which was named CobA/HemD (55 kDa). The individual enzyme activities could be separated by dissecting the *cobA/hemD* gene, thereby producing two distinct proteins representing a uroporphyrinogen methyltransferase (CobA: N-terminal) and a uroporphyrinogen synthase (HemD: C-terminal). Expression of either the full-length fusion protein (CobA/HemD) or just the uroporphyrinogen methyltransferase (CobA domain) in a mutant *Escherichia coli* strain unable to generate precorrin-2 (PC-2) demonstrated that both enzyme variants perform the *in vivo* transmethylation of UROGEN III to yield PC-2 (Fig. 7.4). Furthermore, the cell growth deficiency of an *E. coli hemD* strain could also be overcome by the expression of either the *D. vulgaris* CobA/HemD or the HemD domain protein variants (Lobo et al., 2009). The recombinant *D. vulgaris* CobA/HemD has a methyltransferase activity of 3 nmol min^{-1} mg^{-1} and a K_M for UROGEN III of 0.4 μM, and in contrast to the *Bacillus megaterium* and *Pseudomonas denitrificans* CobAs, the enzyme does not display substrate inhibition by UROGEN III (Blanche, Debussche, Thibaut, Crouzet, & Cameron, 1989; Lobo et al., 2009; Robin et al., 1991). The presence of a bifunctional UROGEN III synthase/methyltransferase gene occurs in all available *Desulfovibrio* genomes and in other bacteria such as the obligate anaerobic and fermentative bacteria *Lactobacillus reuteri*, *Selenomonas ruminantium*, and *Clostridium josui* (Anderson, Entsch, & McKay, 2001; Fujino, Fujino, Karita, Sakka, & Ohmiya, 1995; Taranto, Vera, Hugenholtz, De Valdez, & Sesma, 2003). The presence of a fused multifunctional enzyme is likely to promote the direct transformation of HMB into PC-2, thereby reducing the level of UROGEN III as a free intermediate. Consistent with this idea is the observation that an *in vitro* incubation of purified recombinant *D. vulgaris* HemB, HemC, and CobA/HemD together with ALA and SAM resulted in the generation of PC-2 in high yield, that is, the full reconstitution of the common pathway was achieved (Lobo et al., 2009).

3. PRODUCTION OF SH

In general, PC-2 or its oxidized relative SHC are intermediates of the synthesis of cobalamin, SH, and haem d_1 (Fig. 7.3). The synthesis of SH from UROGEN III involves three different reaction steps: (i) methylation of UROGEN III to generate PC-2; (ii) dehydrogenation of PC-2 to form SHC; and (iii) chelation of iron into SHC to give SH (Stroupe, Leech, Daniels,

Warren, & Getzoff, 2003). Depending on the organisms, these steps are achieved by the action of different enzymes (Fig. 7.3). In *B. megaterium*, for example, SH is synthesized in three independent reactions catalyzed by SirA (SAM-dependent UROGEN III methyltransferase), SirC (NAD^+-dependent PC-2 dehydrogenase), and SirB (SHC ferrochelatase) that are encoded by the *sirABC* operon (Raux et al., 2003). In the case of *E. coli* and *Salmonella typhimurium*, the conversion of UROGEN III into SH is catalyzed by one single multifunctional enzyme, the SH synthase (CysG). The SAM-dependent methyltransferase activity is associated with the C-terminal domain of CysG ($CysG^A$), and the NAD^+-dependent dehydrogenation and ferrochelation activities are linked to the N-terminal domain ($CysG^B$) (Stroupe et al., 2003; Warren et al., 1994). In yeast two independent enzymes are required for SH synthesis, namely Met1p (SAM-dependent methyltransferase) and Met8p (NAD^+-dependent dehydrogenase/ferrochelatase) (Raux, McVeigh, Peters, Leustek, & Warren, 1999).

D. vulgaris contains a $CysG^B$-like protein that shares 17%, 21%, and 26% sequence identity with *Saccharomyces cerevisiae* Met8p, *B. megaterium* SirC, and N-terminal part of the *E. coli* CysG, respectively. The N-terminal region of *D. vulgaris* $CysG^B$ contains the sequence $GxGxxGx_{10}G$, a motif that represents a consensus NAD^+ binding site, which is present in the bacterial CysG and Met8p homologues. *In vitro* studies have now shown that the *D. vulgaris* $CysG^B$ exhibits a NAD^+-dependent PC-2 dehydrogenase activity of $\sim$700 nmol min^{-1} mg^{-1}, with a K_M for NAD^+ of 70 μM. However, the enzyme is not able to perform the insertion of iron or cobalt into SHC, indicating that it is a monofunctional dehydrogenase similar to SirC (Fig. 7.4; Lobo et al., 2009).

4. *D. VULGARIS* CBIK COBALTOCHELATASES

Previous research had shown that the monofunctional chelatases associated with iron insertion for haem synthesis (HemH) and the cobalt inserting enzymes (CbiK, $CbiX^L$, $CBiX^S$) linked to the anaerobic pathway for cobalamin synthesis are structurally related. These enzymes would appear to have evolved from a $CbiX^S$ homologue. Two molecules of $CbiX^S$ are required to form an active chelatase, where the active site is generated at the interface between the domains. $CbiX^L$, CbiK, and HemH represent fusions of two $CbiX^S$-type proteins, where the main catalytic groups are found either in the N-terminal or in C-terminal domain (Brindley, Raux, Leech, Schubert, & Warren, 2003; Pisarchik, Petri, & Schmidt-Dannert, 2007).

D. vulgaris does not appear to have a unique SHC ferrochelatase as it contains two distinct CbiK SHC cobaltochelatases, one cytoplasmic, and the other periplasmic, named $CbiK^C$ and $CbiK^P$, respectively (Lobo et al., 2008). However, a lack of metal ion specificity displayed by these cobaltochelatases as iron is also chelated into SHC both *in vitro* and *in vivo* by the two *D. vulgaris* CbiK enzymes (Brindley et al., 2003; Lobo et al., 2008). The occurrence of two *cbiK* genes is not restricted to *Desulfovibrio* spp. as they are also present in the genomes of other members of the deltaproteobacteria group including *Desulfobulbus*, *Desulfatibacillum*, and *Desulfobacterium*.

D. vulgaris $CbiK^C$ exists in solution as a mixture of tetramers and dimers and chelates both cobalt and iron into SHC with a specific activity of 4 nmol min^{-1} mg^{-1} and 1.5 nmol min^{-1} mg^{-1}, respectively. Moreover, the enzyme is also able to insert iron into SHC *in vivo* as expression of the *D. vulgaris* $CbiK^C$ in an *E. coli* strain deficient in SHC ferrochelatase activity restores the wild-type phenotype (Lobo et al., 2008).

There are a number of significant differences between $CbiK^P$ and $CbiK^C$ apart from their spatial location. *D. vulgaris* $CbiK^P$ is a tetramer that harbors one haem *b* per dimer with a mid-point redox potential of −130 mV. The UV–visible spectrum of the oxidized protein has a Soret band at 414 nm and a broad band between 515 and 580 nm; upon reduction with sodium dithionite, the Soret band shifts to 424 nm with concomitant appearance of two bands at 530 and 560 nm, consistent with the presence of haem *b*. The haem coordination was analyzed by NMR and EPR, indicates a bis-histidinyl coordination, which into a low-spin paramagnetic haem protein. In particular, the EPR spectrum of the as-isolated $CbiK^P$ exhibits *g* values of 2.94, 2.26, and 1.53 characteristic of a low-spin haem group (Lobo et al., 2008).

Subsequent structural studies confirmed the coordination of the haem by two symmetrically coordinated histidines (His96) with each haem located in between two monomers in a hydrophobic pocket. The haems are apart from each other (distance between the two iron atoms of c.a. 32 Å) and from the SHC binding site (Fig. 7.5) (Romão et al., 2011). Apparently, the haem groups are not required for the tetrameric structure or for the chelation of cobalt or iron into SHC as the chelatase activities of both haem-loaded and apo-$CbiK^P$ proteins are similar (22 nmol min^{-1} mg^{-1} of protein for cobalt insertion and 13 nmol min^{-1} mg^{-1} of protein for iron incorporation) (Lobo et al., 2008). Structural studies of the *D. vulgaris* Hildenborough cobalt-loaded $CbiK^P$ showed that the metal binds *via* residues His154,

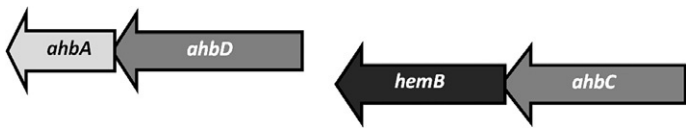
Glu184, and His216 and that cobalt incorporation into CbiK^P promotes rearrangement of the protein ligands, namely, by shifting the imidazole ring of His154 in relation to its initial position in the apoprotein (Romão et al., 2011). The overall topology of the protein shares similarity with the *Salmonella enterica* CbiK, *Archaeoglobus fulgidus* CbiX^S, and the human and *Bacillus subtilis* protoporphyrin IX (PROTO IX) ferrochelatase HemH, particularly on what concerns the conservation of the cobalt ligand (Al-Karadaghi, Hansson, Nikonov, Jonsson, & Hederstedt, 1997; Romão et al., 2011; Schubert, Raux, Wilson, & Warren, 1999; Yin et al., 2006).

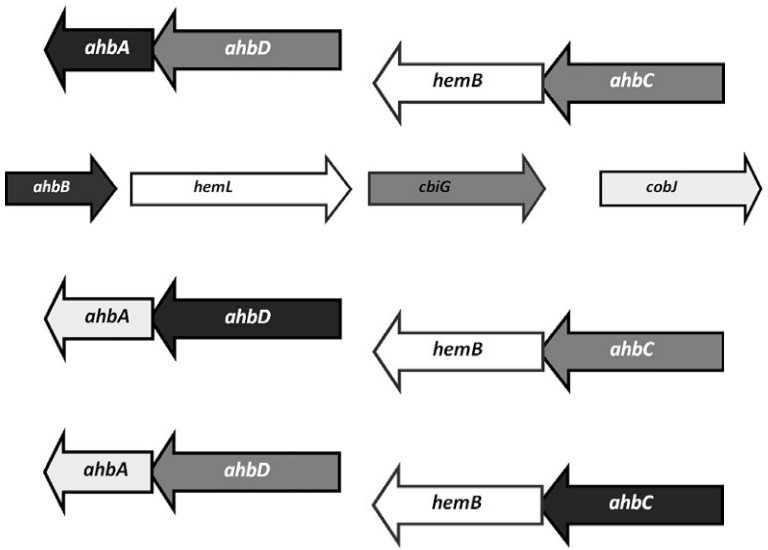
The periplasmic location of *D. vulgaris* CbiK^P rules out a role in the cytoplasmic synthesis of cobalamin. In fact, complementation with CbiK^P of an *E. coli* strain deficient in SHC ferrochelatase activity was only possible when *D. vulgaris* CbiK^P was expressed lacking the leader periplasmic sequence, that is, by retaining the protein in the cytoplasm (Lobo et al., 2008). Considering that the *D. vulgaris cbiK*^{P} is part of an operon formed by gene products that share sequence similarity to those encoding iron transport proteins, namely, permeases, Fe(III) siderophores, and periplasmic iron binding proteins, CbiK^P is proposed to be involved in haem/iron transport in *Desulfovibrio* (Table 7.1; Heidelberg et al., 2004; Lobo et al., 2008). With respect to this idea, it is interesting to note that the CbiK orthologues of *Porphyromonas gingivalis* and *Dichelobacter nodosus* are also considered to be involved in iron and haem binding/uptake (Dashper et al., 2000; Parker, Kennan, Myers, Paulsen, & Rood, 2005; Roper, et al. 2000). The *P. gingivalis cbiK* encodes an outer membrane hemin-binding protein, functional as a cobaltochelatase, whose gene is also located in a gene cluster containing genes encoding iron transporters of the ABC type (Dashper et al., 2000). In the case of *D. nodosus*, *cbiK* forms an operon with a gene coding for an orthologue of the periplasmic iron-binding protein YfeA, itself a component of an ABC transporter system involved in iron uptake (Parker et al., 2005).

5. PRODUCTION OF COBALAMIN

The *de novo* synthesis of cobalamin requires around 30 enzymes and is restricted to some archaea and bacteria, for example, the archaeon *A. fulgidus*, and a few bacterial species of the genera *Bacillus*, *Clostridium*, *Pseudomonas*, *Mycobacterium*, and *Salmonella*. The greater complexity of the biosynthetic pathway for cobalamin reflects the fact that the corrin ring is much more decorated than the porphyrin ring found in haems, has

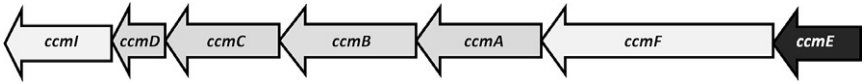
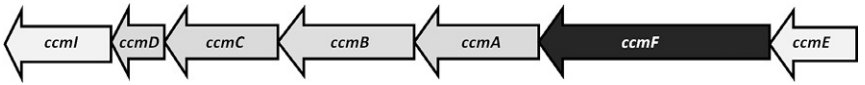
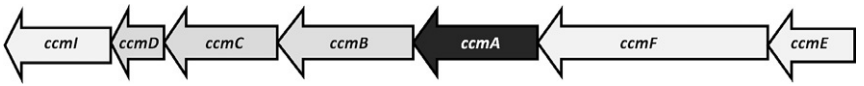
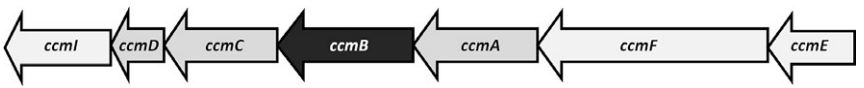
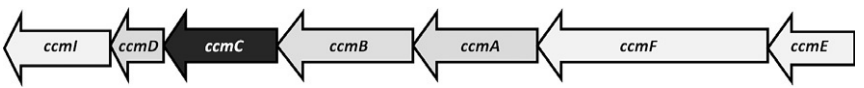
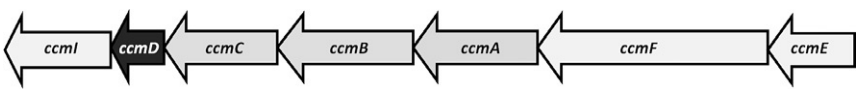
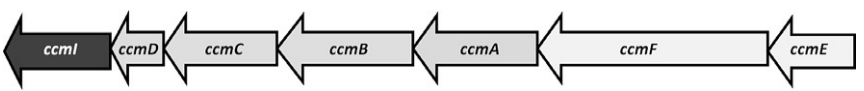
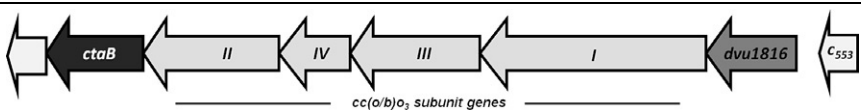
Table 7.1 *Desulfovibrio vulgaris* Hildenborough modified tetrapyrrole biosynthesis related genes

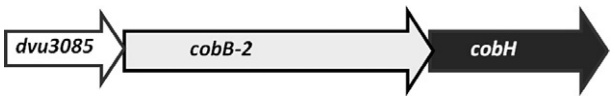
Tetrapyrrole biosynthesis	Gene locus	Gene	Protein	Genomic context
C5 pathway for ALA generation	DVU1693	*gltX-1*	Glutamyl-tRNA synthetase	
	DVU1461	*gltR*	Glutamyl-tRNA reductase	
	DVU3168	*hemL*	Glutamate-1-semialdehyde-2,1-aminomutase	
Generation of sirohydrochlorin from ALA	DVU0856	*hemB*	Delta-aminolevulinic acid dehydratase	
	DVU1890	*hemC*	Porphobilinogen deaminase	
	DVU0734	*cobA/ hemD*	Uroporphyrinogen III methyltransferase/synthase	
	DVU1463	*sirC*	Precorrin-2 dehydrogenase	

Cobalt and iron insertion into sirohydrochlorin	DVU0650	*cbiK*^{P}	Periplasmic cobaltochelatase CbiK^{P}	
	DVU1365	*cbiK*^{C}	Cytoplasmic cobaltochelatase cbiK^{C}	
Haem *b* synthesis from sirohaem	DVU0854	*ahbA*	Sirohaem decarboxylase	
	DVU3167	*ahbB*	Sirohaem decarboxylase	
	DVU0855	*ahbD*	SAM-dependent Fe-coproporphyrin III decarboxylase	
	DVU0857	*ahbC*	SAM-dependent 12,18-didecarboxysirohaem deacetylase	

Continued

Table 7.1 *Desulfovibrio vulgaris* Hildenborough modified tetrapyrrole biosynthesis related genes—cont'd

Tetrapyrrole biosynthesis	Gene locus	Gene	Protein	Genomic context
Haem *c* biogenesis System I*	DVU1051	*ccmE*	Cytochrome *c*-type biogenesis protein CcmE	
	DVU1050	*ccmF*	Cytochrome *c*-type biogenesis protein CcmF	
	DVU1049	*ccmA*	Cytochrome *c*-type biogenesis protein CcmA	
	DVU1048	ccmB	Cytochrome *c*-type biogenesis protein CcmB	
	DVU1047	*ccmC*	Cytochrome *c*-type biogenesis protein CcmC	
	DVU1046	*ccmD*	Cytochrome *c*-type biogenesis protein CcmD	
	DVU1045	*ccmI*	Cytochrome *c*-type biogenesis protein CcmI	
Haem *o* synthesis from haem *b*	DVU1811	*ctaB*	Protohaem IX farnesyltransferase	

Synthesis of cobalamin from cobalt-sirohydrochlorin	DVU0646	*cobI*	Precorrin-2 methyltransferase CobI	
	DVU2750	*cbiD*	Cobalamin biosynthesis protein CbiD	
	DVU3169	*cbiG*	Cobalamin biosynthesis protein CbiG	
	DVU3170	*cobJ*	Precorrin-3b C17-methyltransferase CobJ	
	DVU2748	*cobM*	Precorrin-4 C11-methyltransferase CobM	
	DVU2749	*cobL*	Precorrin-6y methylase CobL	
	DVU3087	*cobH*	Precorrin-8X methylmutase CobH	

Continued

Table 7.1 *Desulfovibrio vulgaris* Hildenborough modified tetrapyrrole biosynthesis related genes—cont'd

Tetrapyrrole biosynthesis	Gene locus	Gene	Protein	Genomic context
	DVU3086	*cobB-2*	Cobyrinic acid a,c-diamide synthase CobB-2	
	DVU0405	*cobB-1*	Cobyrinic acid a,c-diamide synthase CobB-1	
	DVU1403	*cobO*	Cob(I)alamin adenosyltransferase CobO	
	DVU0816	*cobQ*	Cobyric acid synthase CobQ	
	DVU2237	*cobD*	Cobalamin biosynthesis protein CobD	
	DVU1007	*cobU/ cobP*	Cobinamide kinase/ cobinamide phosphate guanylyltransferase CobU	
	DVU0914	*cobS/ cobY*	Cobalamin 5′-phosphate synthase/cobalamin synthase CobS	
	DVU3279	*cobT*	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase CobT	

undergone ring contraction through elimination of the C-20 position, and provides both upper and lower axial ligands for the centrally chelated cobalt ion (Martens, Barg, Warren, & Jahn, 2002; Warren, Raux, Schubert, & Escalante-Semerena, 2002). Adenosylcobalamin and methylcobalamin are the two major biological forms of cobalamin found in nature, representing coenzyme and cofactor forms, respectively. Methylcobalamin is involved in intramolecular methyl transfer while adenosylcobalamin participates in radical-based chemistry including the reduction of ribonucleotide triphosphate to 2′-deoxyribonucleotide triphosphate and carbon skeleton rearrangements of metabolites. B_{12}-dependent enzymes include methionine synthase, ribonucleotide reductase, and enzymes involved in fermentation processes such as methylmalonyl CoA mutase and diol dehydratases (Banerjee & Ragsdale, 2003). The *D. vulgaris* genome encodes at least three putative cobalamin-binding enzymes, including a methionine synthase MetH (encoded by the gene locus DVU1585) and two proteins with a B_{12}-binding domain-like, associated with a radical SAM domain (DVU3019 and DVU3016), but whose functions remain unclear (Heidelberg et al., 2004).

There are two different pathways for the biosynthesis of the corrin ring of cobalamin that are termed the aerobic (oxygen-dependent) and the anaerobic routes. The cobalamin biosynthetic genes in *D. vulgaris* suggest that it operates an anaerobic pathway, which would be consistent with the natural environmental habitat of the organism. The anaerobic pathway starts from SHC, where the intermediate is guided toward cobalamin biosynthesis by the insertion of cobalt via a cobaltochelatase to yield cobalt-SHC (Figs. 7.3 and 7.4; (Martens et al., 2002; Warren et al., 2002).

According to the location of the two *D. vulgaris* CbiK cobaltochelatases, it is likely that cytoplasmic $CbiK^C$ is the one involved in the formation of vitamin B_{12}. Genomic analysis reveals that all the enzymes required for the anaerobic production of vitamin B_{12} are encoded by genes present in *Desulfovibrio* (Table 7.1). An exception is the precorrin-6x reductase (*cbiJ*/*cobK*) that within the anaerobic metal-reducing δ-proteobacteria only exists in the genome of *Desulfuromonas* spp. (Lobo et al., 2008; Rodionov, Dubchak, Arkin, Alm, & Gelfand, 2004). *D. vulgaris*, which is missing CbiJ, has been shown to produce vitamin B_{12} (around 10 nmol/l of culture) (Guimarães et al., 1994; Lobo et al., 2008) indicating that either CbiJ is not required or that another enzyme has evolved to take its place.

6. ALTERNATIVE HAEM *B* BIOSYNTHETIC PATHWAY

As previously mentioned, for most organisms that make haem *b*, the synthesis branches from the central pathway at UROGEN III after decarboxylation of the acetic acid side chains to form COPROGEN III in a reaction catalyzed by UROGEN III decarboxylase (HemE) (Heinemann et al., 2008). COPROGEN III is acted upon a coproporhyrinogen oxidase (HemN, HemF, or HemZ) to form protoporhyrinogen IX, which is further oxidized to PROTO IX by protoporphyrinogen III oxidase (HemY or HemG). Finally, the insertion of a ferrous iron into PROTO IX performed by the PROTO IX ferrochelatase enzyme (HemH) generates haem *b* (Fig. 7.3; (Dailey, 2002; Heinemann et al., 2008).

In *Desulfovibrio*, the synthesis of haem *b* occurs via an alternative pathway that starts with decarboxylation of SH, followed by two SAM-dependent reactions in a pathway that has only recently been elucidated (Figs. 7.4 and 7.5; Bali et al., 2011). The first evidence for this alternative haem synthesis arose from studies performed in the 1990s, on the cytochrome c_3 of *D. vulgaris*. Isolation of the cytochrome c_3 from cultures grown in the presence of labeled methionine revealed that the methyl groups of the C2 and C7 of the haem *c* originated from SAM-derived methyl groups and not from ALA (Akutsu, Park, & Sano, 1993). Subsequent experiments identified a novel intermediate compound, the 12,18-didecarboxysirohydrochlorin, a decarboxylated form of SHC, supporting the existence of a different tetrapyrrole pathway in these organisms in which haem would be made via PC-2 (Ishida et al., 1998; Matthews et al., 1998).

Several other lines of evidence are also consistent with an alternative pathway. These include the observations that (i) several *Desulfovibrio* genomes lack genes encoding the canonical haem biosynthetic enzymes known to produce haem *b* from UROGEN III; (ii) PC-2 is an intermediate for haem biosynthesis in the archaeon *Methanosarcina barkeri*, which also lacks genes coding for enzymes orthologues of the late haem biosynthesis pathway (Buchenau, Kahnt, Heinemann, Jahn, & Thauer, 2006; Storbeck et al., 2010); and (iii) a fused UROGEN III methyltransferase/synthase is operative in *Desulfovibrio* implying that UROGEN III is not released as a free intermediate, reinforced the hypothesis that PC-2, SHC, or SH rather than UROGEN III are the branch-point intermediates of this different route (Lobo et al., 2009; Storbeck et al., 2010). The confirmation of this alternative pathway emerged from experiments where incubation of *D. vulgaris* cell lysates with SHC and

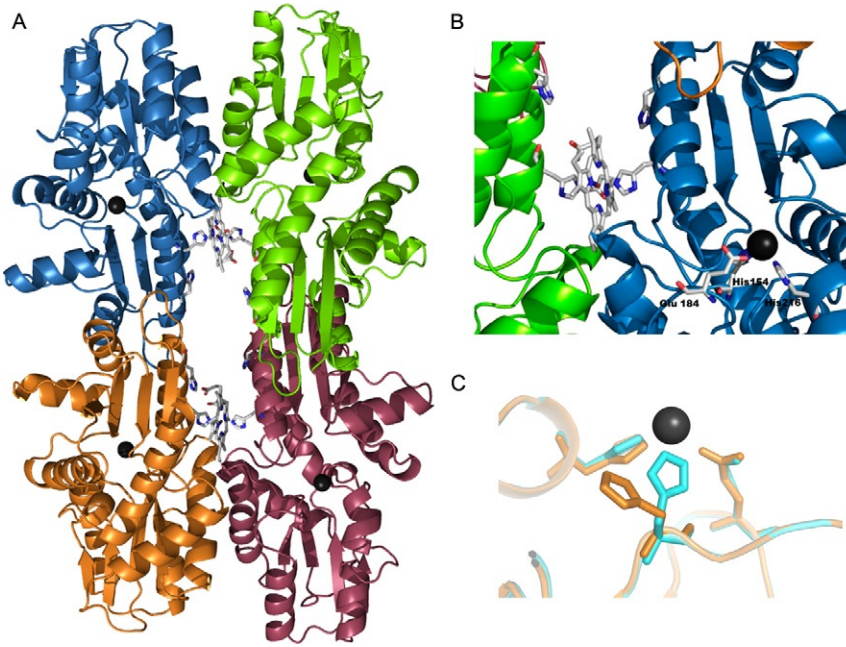


Figure 7.5 (A) *D. vulgaris* CbiK^{P} tetrameric structure. The four monomers are represented in different colors, namely, magenta, green, blue, and orange. The haems *b* are located in between dimers and coordinated by His96 of each monomer. Cobalt is represented as a black sphere in each monomer. (B) Closer view of the haem pocket and of the cobalt ligands His154, Glu184, and His216. (C) Detailed view of the cobalt binding site, in which a shift of the His154 residue can be observed. *Adapted from Romão et al. (2011).*

SH allowed identifying intermediates such as monodecarboxysirohaem, didecarboxysirohaem (DDSH), Fe-COPRO III, and monovinyl Fe-COPRO III. The presence of these intermediates is consistent with decarboxylation of the C2 and C7 acetic acid side chains of SH, and sequential decarboxylation of the C3 and C8 propionic acid chains of Fe-COPRO III to yield haem *b* (Bali et al., 2011).

A scan of the genomes of *Desulfovibrio* and Archaea allowed the identification of a number of *nir*-like genes encoding enzymes designated for the synthesis of haem d_1 (*nirD*, *nirH*, *nirJ-1*, and *nirJ-2*), the cofactor of the cd_1 nitrite reductase, an enzyme that is apparently absent from *Desulfovibrio* and Archaea. This led to the hypothesis that in these microorganisms, these gene products could be involved in generation of haem *b* from SH and suggested a possible link between the alternative haem pathway and the

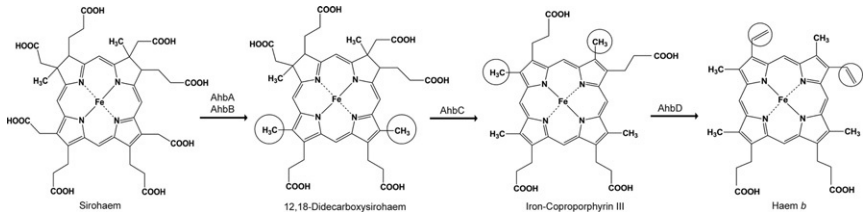


Figure 7.6 Alternative haem *b* biosynthesis in *Desulfovibrio* spp. *Adapted from Bali et al. (2011).*

pathway for haem d_1 construction (Bali et al., 2011; Storbeck et al., 2010). Moreover, three of these genes were clustered together (*nirD*, *nirJ1*, and *nirJ2*) and two of the genes encoded proteins with SAM-radical motifs. Experiments using *D. vulgaris* and *D. desulfuricans* recombinant Nir-like proteins demonstrated that, in fact, NirD, H, J1, and J2 participate in these reactions. To distinguish these from other Nir proteins, NirD, H, J1, and J2 were renamed AhbA, B, C, and D (Alternative haem biosynthetic proteins), respectively. *Desulfovibrio* AhbA and B were shown to catalyze the removal of the carboxyl groups from the acetic acid chains attached to C12 and C18 of SH- generating 12,18-DDSH. This intermediate was then shown to be transformed into Fe-COPRO III by the action of AhbC from the archaeon *M. barkeri* in a SAM-radical-dependent reaction, removing the acetic acid side chains at C2 and C7 of the substrate. Fe-COPRO III is subsequently modified by *Desulfovibrio* AhbD, which converts the two propionate side chains attached to C3 and C8 into vinyl groups, giving rise to haem *b* (Fig. 7.6). The transformation by AhbD is another radical SAM-dependent reaction (Bali et al., 2011). This novel haem synthesis pathway has implications for pathway evolution and links haem, SH, and d_1 haem in a way not previously recognized.

7. BIOSYNTHESIS OF HAEM *B* DERIVATIVES

The covalent binding of haem *c* to the polypeptide chain via two thioether bonds between the two vinyl groups of haem *b* and the sulfur atoms of two cysteine residue thiols is a posttranslational reaction catalyzed by a specialized set of proteins that are specific of groups of organisms (Simon & Hederstedt, 2011; Stevens et al., 2011). In the case of *Desulfovibrio*, this attachment is performed by the cytochrome *c* maturation (Ccm) process, which is also named System I and is employed by archaea, other

Gram-negative bacteria and plant mitochondria for the posttranslational haem processing (Goddard et al., 2010). The extensively studied Ccm system of *E. coli* comprises eight genes of the *ccmABCDEFGH* operon. These genes encode proteins associated with the cytoplasmic membrane that function in the periplasm (Thony-Meyer, Fischer, Kunzler, Ritz, & Hennecke, 1995). Genomic and biochemical studies performed on the Ccm proteins of *D. desulfuricans* have shown that, in contrast to *E. coli*, the *ccm* operon of *Desulfovibrio* differs in three aspects: (i) lacks the genes *ccmG* and *ccmH*, (ii) contains a *ccmI* gene encoding a protein homologous to the C-terminal domain of *E. coli CcmH*, and (iii) the *ccmE* gene encodes a CcmE protein variant with a cysteine replacing the essential histidine residue of the *E. coli* CcmE that binds haem covalently. In spite of these differences, the *D. desulfuricans* G20 *ccmEFABCD* genes alone were shown to be sufficient for cytochrome *c* biogenesis in *E. coli*. As a consequence of this genetic variation but similar functionality, the *Desulfovibrio* machinery was renamed System I★ (Goddard et al., 2010).

Haem *o* is synthesized by the protohaem IX farnesyltransferase, also known as haem *o* synthase, a membrane-bound enzyme that transfers a farnesyl group from farnesyl diphosphate to the 2-vinyl group at the C2 of the ferrous form of haem *b* (Figs. 7.3 and 7.4; Mogi, 2003). As mentioned previously, haem *o* occurs in the novel $cc(o/b)o_3$ haem–copper oxygen reductase of *D. vulgaris*, therefore, playing an important role in respiration (Lamrabet et al., 2011).

Haem *a*, another cofactor of the terminal enzymes of the aerobic respiratory chain, is synthesized via haem *o* by conversion of the C8 methyl to a formyl group via the action of the membrane-bound enzyme monooxygenase haem *a* synthase (Fig. 7.3). The conversion of haem *o* to haem *a* is proposed to occur in three steps involving the formation of two hydroxylated haem *o* intermediates (Hederstedt, 2012). However, the gene encoding the haem *a* synthase is absent in all *Desulfovibrio* genomes; accordingly, there are no reports of the presence of haem *a* in these organisms (Lamrabet et al., 2011).

Haem *d* is found in the widespread quinol oxygen reductases of the *bd*-type and in some haem-containing catalases, such as that in *Penicillium vitale* catalase and the *E. coli* hydroperoxidase II (HPII or KatE) (Murshudov et al., 1996). The formation of haem *d* from haem *b* requires conversion of a propionic acid side chain to a hydroxylated spirolactone (Hansson & von Wachenfeldt, 1993), and it is suggested that haem *d* from catalase HPII is formed by the enzyme itself, using hydrogen peroxide as a substrate

(Loewen et al., 1993; Obinger, Maj, Nicholls, & Loewen, 1997). In *Desulfovibrio*, haem *d* is also produced as a *bd*-type oxygen oxidoreductase was purified from *D. gigas* (Lemos et al., 2001).

8. ORGANIZATION AND REGULATION OF TETRAPYRROLE BIOSYNTHESIS-RELATED GENES

Analysis of the available genomes has shown that the organization of the *Desulfovibrio* tetrapyrrole biosynthetic related genes in is quite diverse but consistent among the several species. In *D. vulgaris* Hildenborough, genes like *gltX*, *hemC*, *cobA/hemD*, *cbiK*^{C} genes, *cob-1*, *cobO*, *cobQ*, *cobD*, *cobU*, *cobS*, and *CobT* are isolated in the genome or are associated with gene clusters unrelated to tetrapyrrole biosynthesis. In contrast, the *gltR* gene, encoding glutamyl reductase, is closely located to *sirC* gene, and *gltR* seems to form a putative operon with three genes, one of which (*resC/ccsA*) encodes a protein related to the cytochrome *c* assembly System II. The alternative haem biosynthetic genes *ahbA*, *ahbC*, *ahbD*, and *hemB* are clustered (Table 7.1), while the *ahbB* is adjacent to the *hemL* gene and to two cobalamin-related genes (Table 7.1). The gene encoding the protohaem IX farnesyltransferase (CtaB) is located in an apparent operon that includes genes for the putative *cc*(*o*/*b*)o_3 cytochrome *c* oxygen oxidoreductase. The genes encoding the cytochrome *c* biogenesis System I* are also predicted to form an operon (*ccmEFABCD*) (Table 7.1). Moreover, *cbiL* clusters with *cbiK*^{P} and three genes encoding iron transport-related proteins (Table 7.1).

The factors that influence the expression of genes involved in tetrapyrrole biosynthesis in *Desulfovibrio* spp. remain largely unknown. In other microorganisms, the expression of some tetrapyrrole biosynthetic genes, including *hemA*, *hemCD*, and *hemH*, has been shown to respond to variations of iron, oxygen, and haem content (McNicholas, Javor, Darie, & Gunsalus, 1997). However, several transcriptomic studies undertaken in *Desulfovibrio* spp. grown under similar conditions did not show a comparable induction. Although data regarding expression and regulation of the *cbiK*^{P} and *cbiK*^{C} genes of *Desulfovibrio* are not yet available, the *D. vulgaris cbiK*^{P} gene is reported to undergo a slight induction upon exposure of the *D. vulgaris fur* mutant to iron, suggesting it may be controlled by the ferric uptake regulator Fur (Bender et al., 2007). Interestingly, control by Fur was also observed for the *cbiK* gene of *D. nodosus* (Parker et al., 2005). Further work is then needed to access the transcription factors that control the expression of the genes of the tetrapyrrole biosynthesis in *Desulfovibrio* species.

ACKNOWLEDGMENTS

This work was supported by Project Grants PEst-OE/EQB/LA0004/2011, PTDC/BIA-PRO/098224/2008 (LMS) from Fundação para a Ciência e Tecnologia (FCT). SL is recipient of FCT grant SFRH/BPD/63944/2009.

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