Chapter 13
The Tetrapyrrole Biosynthetic Pathway and Its Regulation in *Rhodobacter capsulatus*

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Abstract The purple anoxygenic photosynthetic bacterium *Rhodobacter capsulatus* is capable of growing in aerobic or anaerobic conditions, in the dark or using light, etc. Achieving versatile metabolic adaptations from respiration to photosynthesis requires the use of tetrapyrroles such as heme and bacteriochlorophyll, in order to carry oxygen, to transfer electrons, and to harvest light energy. A third tetrapyrrole, cobalamin (vitamin B_{12}), is synthesized and used as a cofactor for many enzymes. Heme, bacteriochlorophyll, and vitamin B_{12} constitute three major end products of the tetrapyrrole biosynthetic pathway in purple bacteria. Their respective synthesis involves a plethora of enzymes, several that have been characterized and several that are uncharacterized, as described in this review. To respond to changes in metabolic requirements, the pathway undergoes complex regulation to direct the flow of tetrapyrrole intermediates into a specific branch(s) at the expense of other branches of the pathway. Transcriptional regulation of the tetrapyrrole synthesizing enzymes by redox conditions and pathway intermediates is reviewed. In addition, we discuss the involvement of several transcription factors (RegA, CrtJ, FnrL, AerR, HbrL, Irr) as well as the role of riboswitches. Finally, the interdependence of the tetrapyrrole branches on each other synthesis is discussed.

13.1 Introduction

Cyclic tetrapyrroles encompass porphyrins, such as heme, chlorophyll and bacteriochlorophyll (Bchl), and porphynoids, which are more reduced. The porphynoid class consists of the corrinoids (cobalamin), siroheme, heme d_1, and coenzyme F_{430} (Frankenberg et al. 2003). Many species are capable of synthesizing numerous tetrapyrrole end products. One of the best studied organisms is *Rhodobacter capsulatus*, an anoxygenic photosynthetic bacterium of the α-proteobacteria subfamily. It is capable of growing under a variety of different environmental conditions such

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as dark aerobic, dark and light anaerobic. This versatile metabolism requires the capability of synthesizing the tetrapyrroles heme and Bchl that are needed for respiratory or photosynthetic growth. Heme is needed for electron transfer during both respiration and photosynthesis while Bchl is the major pigment involved in collecting and converting light energy into biochemical energy during photosynthesis. In addition to synthesizing heme and Bchl, *R. capsulatus* also synthesizes cobalamin [vitamin B_{12} (vitB_{12})]. VitB_{12} and its derivatives are the most complex tetrapyrrole synthesized in nature and are involved in various cellular functions such as DNA repair and methionine synthesis (Martens et al. 2002). The synthesis of siroheme in *R. capsulatus* strain has been reported as well (Olmo-Mira et al. 2006).

The four tetrapyrrole end products synthesized by *R. capsulatus* are derived from different branches that split off from a common tetrapyrrole biosynthetic pathway "core" (Fig. 13.1). A common trunk of the pathway used by all three branches involves a multistep conversion of δ-aminolevulinic acid (δ-ALA) into uroporphyrinogen III (uro'gen III). At uro'gen III, the "corrin branch" splits off leading to the synthesis of cobalamin. Uro'gen III is also the potential substrate for siroheme synthesis. The central core of the pathway continues to synthesize intermediates up to protoporphyrin IX (proto IX) that are common to both the heme and Bchl branches. At proto IX, the heme and Bchl branches differentiate either through the insertion of Fe to form heme or the insertion of Mg to form Mg–proto IX which is the first committed step in the Mg branch that leads to the production of Bchl.

The biosynthesis of heme, vitB_{12}, and Bchl is tightly controlled in order to achieve an adequate Bchl/heme/vitB_{12} ratio to meet the metabolic requirements of the cell. Heme and cobalamin levels appear to be maintained at rather stable levels while the amount of Bchl swings dramatically depending on the presence or absence of environmental oxygen. Moreover, a number of the intermediates in each of the three branches are potentially toxic as they can generate reactive singlet oxygen as a byproduct of light absorption. Tuning the relative amount of Bchl, heme, and vitB_{12} as a function of the metabolic requirements, while coping with the potential toxicity of intermediate products, requires this pathway to be highly regulated. Early work on the regulation of pigment synthesis in purple bacteria was initiated in the 1950s by Cohen-Bazire (Cohen-Bazire et al. 1957). More recently it has been demonstrated that the tetrapyrrole biosynthetic pathway in purple bacteria is highly complex and its regulation extremely sophisticated. This review is centered on the model species *R. capsulatus* and attempts to identify the actors involved in this pathway, both synthesizing enzymes and regulators.

13.2 Topology of Genes Involved in Synthesis of Tetrapyrroles

Examination of the complete genome of *R. capsulatus* has enabled the identification of the genes involved in each branch of tetrapyrrole synthesis (cf. annotations available at http://www.ergo-light.com and http://onco.img.cas.cz/rhodo/results/index.html). While genes involved in the vitB_{12} and Bchl synthesis

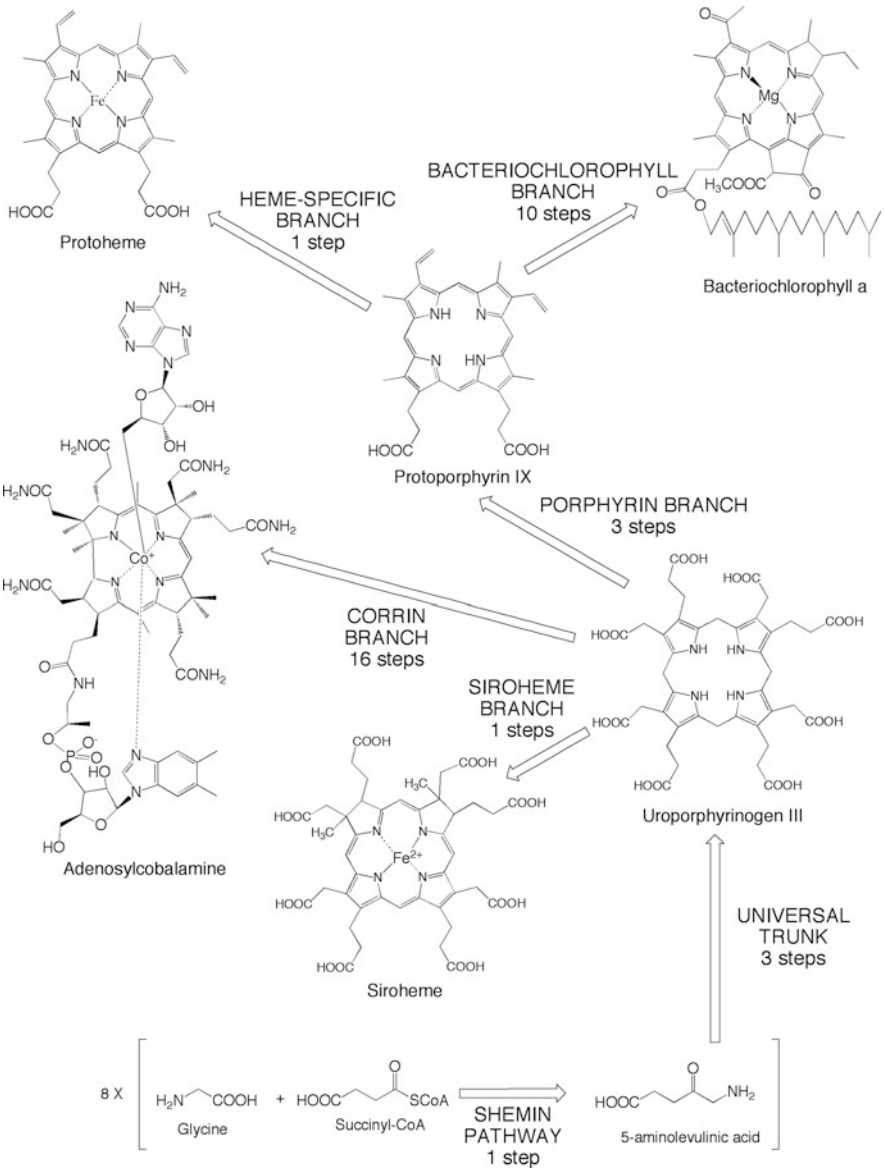

Fig. 13.1 Overview of the tetrapyrrole biosynthetic pathway with its substrates, major intermediates, and products. The number of enzyme reactions for each branch is indicated

are found in large operons, genes encoding the enzymes involved in heme synthesis are scattered over the genome (Young et al. 1989; McGoldrick et al. 2002; Warren et al. 2002; Smart et al. 2004). This feature is not unusual, though a few organisms show an operon organization for some of their *hem* genes (Avissar and Moberg

1995). The one exception in *R. capsulatus* is the *hemC* and *hemE* genes that are divergently transcribed and separated by 111 bp (Smart et al. 2004).

13.3 The Universal Trunk: From δ-ALA to Urog'en III

13.3.1 Feeding the Universal Trunk: The Synthesis of δ-ALA

The biosynthetic pathway to produce uro'gen III from δ-ALA is highly conserved in nature and is therefore considered universal. However, there are two different pathways to synthesize δ-ALA, which is the precursor of all known tetrapyrroles. One is the C-5 pathway where two enzymatic reactions transform glutamyl-tRNA^{Glu} into δ-ALA. The other is the C-4 or Shemin pathway that involves the condensation of glycine and succinyl-CoA to produce δ-ALA, CO_2, and CoA (Avissar and Moberg 1995; Frankenberg et al. 2003). As a typical representative of the α-proteobacteria subfamily, *R. capsulatus* synthesizes δ-ALA using the C-4 pathway. This reaction is performed by δ-ALA synthase (EC 2.3.1.37, ALAS), encoded by the *hemA* gene, previously identified and cloned in 1988 (Biel et al. 1988). *R. capsulatus* ALAS is a piridoxal-5′-phosphate-dependent homodimer, consisting of two 44 kDa subunits. It shows 49% identity and 70% similarity with the human erythroid-specific ALAS. The structure, solved in 2005 by Astner et al., provided insight on mutations that generate dysfunctional forms of the human eALAS (Astner et al. 2005). Moreover, despite a very low primary sequence identity of 18%, ALAS shows remarkable three-dimensional homology with the glutamate-1-semialdehyde-2,1-aminomutase (GSAM) of *Thermosynechococcus elongatus*. The GSAM is the last enzyme of the C-5 pathway and, as such, synthesizes δ-ALA. It is likely that the C-4 pathway originated from the evolution of GSAM into ALAS, followed by the loss of the C-5 pathway in common ancestors of α-proteobacteria and of early mitochondria (Schulze et al. 2006). ORF encoding a putative GSAM, *hemL*, was found in the genome of *R. capsulatus*. In prokaryotes, the occurrence of two uncoupled δ-ALA biosynthetic pathways was shown in *Streptomyces nodosus* subsp. *asukaensis*, where the C-5 pathway produces δ-ALA for heme synthesis, while the C-4 pathway generates δ-ALA targeted to secondary metabolism (Petricek et al. 2006). In *R. capsulatus* there is no evidence for an alternative δ-ALA synthesis pathway as the first enzyme of the C-5 pathway, glutamyl-tRNA reductase, is not found in the genome. Furthermore, the annotation of this ORF as a *hemL* gene has to be confirmed experimentally as GSAM-encoding genes share a great deal of similarity with genes coding for other types of aminotranferases (Panek and O'Brian 2002). Interestingly, when a δ-ALA-requiring mutant containing a single insertion was characterized, it was found that a low level (10%) of the ALAS activity of the parental strain remained (Wright et al. 1987). Finally, the synthesis of δ-ALA appears as one of the crucial regulation points of the tetrapyrrole biosynthetic pathway, the transcription of *hemA* being up-regulated under low oxygen tension (Wright et al. 1991; Smart et al. 2004) and down-regulated in the presence of excess of exogenous heme (Smart and Bauer 2006).

13.3.2 Entering the Universal Trunk: Synthesis of Porphobilinogen

The first common step in tetrapyrrole synthesis is achieved by porphobilinogen synthase (EC 4.2.1.24, PBGS), or δ-ALA dehydratase, that converts two δ-ALA molecules into a monopyrrole porphobilinogen. In *R. capsulatus*, *hemB* encodes a 35.8 kDa protein which presents unusual characteristics among the PBGS studied so far. Indeed, most PBGS are known to be homooctameric metalloenzymes presenting a high variability regarding their cations requirement in terms of catalysis (Zn^{2+}-, Mg^{2+}-, or K^{+}- binding site at the active site) and activity regulation, most of them containing an extra allosteric Mg^{2+}-binding site. The biochemical characterization of *R. capsulatus* revealed that its enzyme is a homohexamer clearly independent of cations (Zn^{2+}, Mg^{2+}, K^{+}) or chelators (EDTA, 1,10-phenathroline). *R. capsulatus* PBGS seems to switch between a highly active homohexameric form and a less active homodimeric form (Nandi and Shemin 1973; Bollivar et al. 2004). Regarding metal dependence, this feature was partially predicted from the primary sequence. Indeed, among 130 PBGS sequences representing the entire tree of life, only the ones from the *Rhodobacter* genus, namely *R. capsulatus* and *R. sphaeroides*, lack the cysteine-rich motif involved in Zn binding at the active site and the conserved arginine and glutamate residues that coordinate the allosteric Mg. It is postulated that PBGS containing Zn at the active site evolved to other configurations (metal free, Mg- and/or K-containing active site) during the set up of the photosynthetic process and the early synthesis of Bchl. Indeed, Zn is a competitor of Mg in the metallation step of proto IX (Jaffe 2003; Frère et al. 2005). If the PBGSs of the *Rhodobacter* genus fit well in this model regarding their active site, the evolutionary pressure(s) that allowed them to totally lose metal content, including the Mg in the allosteric site, remain(s) unknown. Moreover, despite porphobilinogen accumulation under low oxygen tension in *R. capsulatus*, the activity of PBGS is unregulated by oxygen, proto IX, and hemin in *R. capsulatus*. In addition, no or only a slight increase in *hemB* transcription was observed upon decreasing the oxygen tension (Biel et al. 2002; Smart et al. 2004). However, the conversion of δ-ALA into porphobilinogen seems to be a major control point in the tetrapyrrole biosynthesis pathway (Biel 1992). This may be achieved by controlling the level of porphobilinogen through the availability of the substrate of PBGS, i.e., δ-ALA. Indeed, the vast majority of the δ-ALA synthesized is transformed into aminohydroxyvalerate by an oxygen-dependent δ-ALA dehydrogenase (Biel et al. 2002). Lastly, although not occurring in a manner as dramatic as for *hemA*, *hemC*, or *hemE*, it was shown that the transcription of *hemB* is repressed in the presence of exogenous heme (Smart and Bauer 2006).

13.3.3 Reaching the First Crossroad: Synthesis of the Tetrapyrrole Ring, Uro'gen III

The transformation of monopyrrole porphobilinogen into the cyclic tetrapyrrole uro'gen III is catalyzed by the enzymes porphobilinogen deaminase (EC 4.2.1.24, PBGD) and uro'gen III synthase (4.2.1.75, UROS) encoded by *hemC* and *hemD*,

respectively. During the first step, PBGD polymerizes four molecules of porphobilinogen into a linear tetrapyrrole, pre-uro'gen or 1-hydroxymethylbilane, and four NH_3. The main product, pre-uro'gen, is very unstable and, if exposed to solvent, will be converted chemically into a toxic cyclic porphyrin, uro'gen I. To avoid toxicity pre-uro'gen is passed to UROS that presumably interacts with PBGD to limit exposure of the cell to free pre-uro'gen (Shoolingin-Jordan 1995). UROS catalyzes the formation of uro'gen III, by circularizing the linear tetrapyrrole while inverting ring D. An asymmetric ring D is a signature of biologically functional porphyrins.

PBGD was purified from *R. capsulatus* and the N-terminal sequence was determined in order to identify the corresponding gene, *hemC*. The latter was also expressed heterologously in *Escherichia coli*, confirming that this ORF is sufficient to produce an active form of PBGD (Biel et al. 2002). No biochemical characterization of the *R. capsulatus* enzyme has been performed so far, but analysis of the sequence indicates that *hemC* corresponds to a 34.1 kDa protein with 47% homology with PBGDs from *E. coli* and *Pseudomonas aeruginosa* and 43% with the *Bacillus subtilis* homolog. In addition, previous characterizations of PBGDs highlighted the use of a unique cofactor, dipyrromethane which consists of two porphobilinogen molecules. This cofactor acts as a primer during the polymerization of a hexapyrrole, which finally releases the tetrapyrrolic reaction product and the intact cofactor. *R. capsulatus* PBGD contains the conserved cysteine residue that is involved in cofactor binding. More work has been done regarding the genetics. Indeed, characterization of a *hemC* mutant confirmed that it was incapable of synthesizing either siroheme or vitB_{12} and grew only in media supplemented with cysteine and methionine, in addition to hemin (Biel et al. 2002). As well, *hemC* expression was shown to be regulated by oxygen, with a fivefold increase of the transcription level when changing from aerobic to anaerobic growth conditions (Smart et al. 2004). Finally, the transcription of *hemC* was repressed two-thirds upon the addition of exogenous hemin (Smart and Bauer 2006).

UROS is only very poorly characterized unclear as it has never been purified from *R. capsulatus*. A high variability in the primary sequences of UROSs has made it difficult to identify the *hemD* gene in the *R. capsulatus* genome. In addition, the lack of genetic organization such as a *hemCD* operon or a *hem* gene cluster, as in *E. coli* or *B. subtilis,* renders the task even harder (Avissar and Moberg 1995). Recently, an ORF in the *R. capsulatus* genome was annotated as encoding a UROS but because *hemD* genes have been miss-annotated in many other organisms, experimental work has to be undertaken to confirm this annotation (Panek and O'Brian 2002).

13.4 The Porphyrin Branch: From Uro'gen III to Heme

13.4.1 The Synthesis of Coproporphyrinogen III and Its Puzzling Secretion

The first step after the uro'gen III crossroad consists in the synthesis of coproporphyrinogen III (copro'gen III). This activity is performed by uro'gen III decarboxylase (EC 4.1.1.37, UROD) which catalyzes the sequential decarboxylation of the

four acetate side chains into methyl groups. The reaction is ordered, starting with the ring D and then decarboxylating rings A, B, and finally ring C. Numerous UROD-encoding genes from various biological sources have been identified showing a rather high level of conservation and presumably a common mechanism of action (Frankenberg et al. 2003; Heinemann et al. 2008). The *R. capsulatus* gene encoding UROD, *hemE*, was identified in 1995 (Ineichen and Biel 1995). The deduced protein sequence shows more than 34% identity with previously characterized UROD and contains a signature sequence of this class of enzyme (PXWXMRQAGR) at the amino-terminal. No biochemical work has been performed with *R. capsulatus* UROD but a bit is known regarding the genetics and its regulation. An interesting feature of *hemE* is its linkage to *hemC*, while the other *hem* genes are scattered on the genome. These two genes are divergently transcribed and separated by an intergenic region of 111 bp. Furthermore, the transcription of *hemE* is strongly dependent on redox conditions, as seen using a *hemE::lacZ* fusion. Expression of *hemE* increases by 112- and 52-fold under semi-aerobic and anaerobic conditions, respectively (Smart et al. 2004). As with *hemC*, the transcription level of *hemE* is decreased by two-thirds in the presence of exogenous heme (Smart and Bauer 2006).

An interesting phenomenon about this step of the pathway is that the cells excrete copious amounts of the product of the reaction, copro'gen III under certain growth conditions. Cooper (1956, 1963) highlighted this feature in *R. capsulatus,* using cells grown under iron starvation or with excess exogenous methionine. More recently, various *R. capsulatus* mutants, either unable to synthesize Mg–protoporphyrin monomethyl ester or lacking *c*-type cytochromes, were studied and showed a similar phenotype. Moreover, it was discovered that coproporphyrin is excreted as a complex with a 66 kDa protein (Biel and Biel 1990; Biel 1991) that turned out to be the major outer membrane porin (Bollivar and Bauer 1992).

13.4.2 Reaching the Second Crossroad: The Synthesis of Protoporphyrin IX

The antepenultimate and penultimate steps of heme synthesis are achieved by copro'gen III oxidase (EC 1.3.99.22, CPO) and proto'gen IX oxidase (EC 1.3.3.4, PPO), respectively. These steps lead to the formation of protoporphyrin IX which is a precursor for the synthesis of heme and Bchl. Oxygen-dependent and oxygen-independent CPOs conventionally named HemF and HemN (or HemZ), respectively, are known to catalyze this reaction. HemF is prevalent in eukaryotes and in a few bacterial groups (primarily cyanobacteria and proteobacteria) while HemN is widely distributed among prokaryotes. These two forms of CPOs share no obvious primary sequence homology indicating that they arose from distinct evolutionary processes. Both enzymes convert copro'gen III into proto'gen IX by decarboxylating the propionate side chains of rings A and B consecutively to yield vinyl groups (Panek and O'Brian 2002; Frankenberg et al. 2003; Heinemann et al. 2008). Careful examination of the genome of *R. capsulatus* revealed no HemF-encoding gene but three ORFs that may code for putative HemNs. This is in contrast to the genome of the phylogenetically related species *R. sphaeroides* that contains one

hemF and three *hemN* genes (locus tags in img.jgi.doe.gov: RSP_0682, RSP_0699, RSP_1224, RSP_0317).

HemN contains a [4Fe–4S] cluster for catalysis and requires *S*-adenosyl-L-methionine (SAM) as a cofactor or co-substrate. As such, this enzyme belongs to a family of "radical SAM" enzymes that carry the signature sequence CXXXCXXC, where the cysteine residues are involved in the coordination of the Fe atoms (Layer et al. 2005). This motif is present in the deduced protein sequences of the three putative CPOs. Occurrence of multiple *hemN* genes in one organism has been reported and several bacterial oxygen-independent CPOs have been shown to be expressed and active in the presence of oxygen (Rompf et al. 1998; Schobert and Jahn 2002). The expression of *hemN1*, designated *hemZ* in the cited articles, was studied as a function of aeration, which showed only a moderate influence. Indeed, a roughly twofold variation was recorded, with a maximum transcription level under semi-aerobic conditions (Smart et al. 2004). In addition, the presence of exogenous heme under these growth conditions decreased the expression of the gene by twofold factor, bringing it back to its basal level of transcription (Smart and Bauer 2006). Finally, in silico analysis of α-proteobacterial genomes revealed an "iron-rhodobox" in the promoter region of the *hemN2* gene. Thus, *hemN2* is predicted to be part of the iron regulon (Rodionov et al. 2006).

The next step in the pathway is the aromatization of proto'gen IX by removal of six electrons to yield proto IX. This is performed by proto'gen IX oxidase (PPO) that exists as two forms, oxygen-dependent (HemY) and oxygen-independent (HemG). Most of our knowledge about PPOs comes from studies of eukaryotic enzymes that are oxygen dependent and inhibited by diphenyl ether herbicides in plants (Dailey 2002; Heinemann et al. 2008). In prokaryotes, the situation is very unclear as the PPO-encoding gene is unidentifiable in many genomes and, so far, *hemG* seems to be limited to six genera within the γ-proteobacteria group (Panek and O'Brian 2002; Frankenberg et al. 2003). As is the case with many other heme-synthesizing prokaryotes, a PPO-encoding gene has not been identified in the genome of either *R. capsulatus* or *R. sphaeroides* (cf. img.jgi.doe.gov). Even if the oxidation of proto'gen IX into proto IX can occur chemically, an enzyme-mediated catalysis is most probably required. Indeed, on the one hand, *hemY* and *hemG* mutants have been shown to be heme deficient in *B. subtilis* and *E. coli*, respectively (Panek and O'Brian 2002). On the other hand, this oxidation has to occur under anaerobic conditions as well, when the need for Bchl peaks. So there must be either an unknown PPO-encoding gene or an already known gene carrying out such an activity. The ERGO annotation of the *R. capsulatus* genome recently indicated an ORF encoding a "NAD (FAD) utilizing dehydrogenase with a possible PPO activity." This has yet to be experimentally confirmed.

13.4.3 Delivering the Final Product: The Synthesis of Heme

The synthesis of heme from proto IX is performed by the enzyme ferrochelatase (EC 4.99.1.1, FC) that chelates ferrous iron and inserts it into the center of the tetrapyrrole ring. The core of the enzyme is highly conserved between bacteria,

plants, and mammals, although bacterial FCs do not contain [2Fe–2S] clusters (Frankenberg et al. 2003; Heinemann et al. 2008). Interestingly, FC and the anaerobic cobalt chelatase CbiK are both class 2 metal ion chelatases that exhibit a similar three-dimensional structure (Schubert et al. 2002). Recently, Masoumi et al. confirmed in vivo what Koch et al. predicted in silico that PPO and FC form a stable PPO–FC complex (Koch et al. 2004; Masoumi et al. 2008). Such a configuration may ensure the channeling of the potentially toxic proto IX to FC. Finally, it has been suggested that this complex might include the CPO to form a tripartite association CPO–PPO–FC (Dailey 2002; Koch et al. 2004).

In *R. capsulatus,* the FC-encoding gene, *hemH*, was described and cloned in the 1990s with the deduced protein sequence exhibiting 41% (59%) and 21% (44%) identity (similarity) with *E. coli* and *B. subtilis* FCs (Kanazireva and Biel 1996). Moreover, homologous expression of a second copy of *hemH* causes a dramatic reduction of the tetrapyrrole pool, with 2.3-fold and 5-fold decreases of the porphyrin and the Bchl concentrations, respectively. In addition, the ALAS activity is also decreased 2.3-fold under these conditions (Kanazireva and Biel 1995). This phenotype is a signature of negative feedback by heme operating on the entire pathway, as has been studied more recently (Smart and Bauer 2006). The transcription of *hemH* was analyzed as well, highlighting a unique responsive behavior to oxygen among the other *R. capsulatus hem* genes. Indeed, while the expression level is rather constant between aerobic and anaerobic conditions, it is threefold lower under semi-aerobic conditions (Smart et al. 2004). Finally, in semi-aerobic conditions, *hemH* transcription was found to be independent of the exogenously added heme (Smart and Bauer 2006).

13.4.4 Regulation of hem Gene Expression

13.4.4.1 RegA

The RegA–RegB system is a global regulator in *R. capsulatus* that has been shown to regulate synthesis of numerous cellular processes as a function of redox conditions (reviewed in Elsen et al. 2004; Wu and Bauer 2008). As shown in Fig. 13.2, studies of the transcription of various *hem* genes in a *regA* mutant strain highlighted that *hem* genes are part of the RegA–RegB regulon (Smart et al. 2004). Indeed, the most significant effect was observed on *hemE* and *hemH* where transcription was activated 5- to 10-fold by RegA. A strong activating role of RegA was also observed with *hemA* but only in semi-aerobic and anaerobic conditions. Moderate influences were reported on *hemC* and *hemZ*. Finally, the transcription of *hemB* does not seem to be regulated by RegA (Smart et al. 2004). Overall, the dramatic transcriptional activation of *hemE* and *hemH* highlights the crucial intervention of the RegA–RegB system at key steps of the tetrapyrrole synthetic pathway. Activating the transcription of these genes ensures a constant stock of mRNA for a quick synthesis of UROD and FC when the flow of tetrapyrrole synthesis needs to be directed toward the porphyrin branch rather than the corrin branch and when heme needs to be produced at the expense of Bchl. Finally, the RegA-induced increase

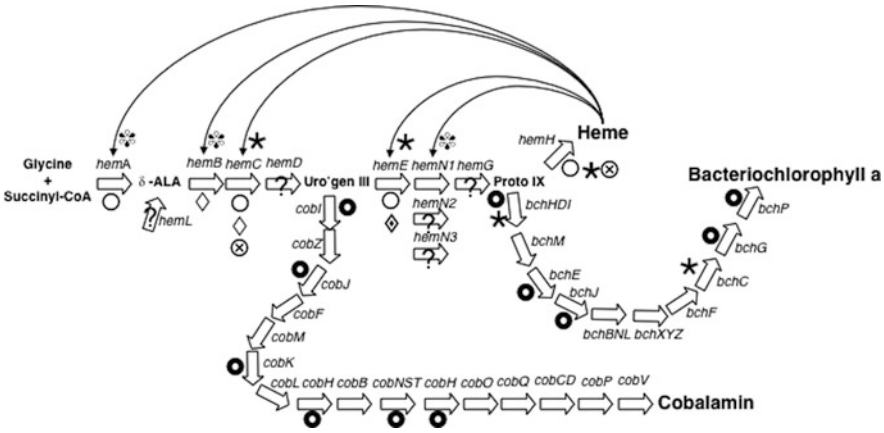

Fig. 13.2 Overview of the transcriptional regulation of the tetrapyrrole biosynthesis pathway. Transcription factors involved are RegA (◯, based on gene expression studies; ⬮ based on both gene expression and DNA-binding studies), FnrL (◇, repressor; ◈, activator), AerR (⊗), CrtJ (★), HbrL (✿). *Arrows* indicate a feedback control by heme operated through CrtJ or HbrL. Genes identified from bioinformatics analysis only, with no experimental studies, are specified with a question mark

in the transcription of *hemA* under semi-aerobic and anaerobic conditions underlines a function of the RegA–RegB system, namely stimulation of the production of the general tetrapyrrole precursor δ-ALA in coordination with the synthesis of the pigment-binding proteins of the photosystem.

13.4.4.2 CrtJ

Present in the photosynthesis gene cluster is a redox-responding transcription factor called CrtJ or PpsR depending upon the species. Studies have demonstrated that CrtJ/PpsR is an aerobic repressor of heme (*hem*), Bchl (*bch*), and carotenoid (*crt*) biosynthesis genes in *R. capsulatus* (Ponnampalam and Bauer 1997; Elsen et al. 1998; Smart et al. 2004). CrtJ also aerobically represses synthesis of light-harvesting II peptides (*puc*) that bind Bchl and carotenoids in these species (Ponnampalam et al. 1995). CrtJ cooperatively binds to two copies of the palindromic sequence TGT-N_{12}-ACA that is present in many *hem* promoter regions (Ponnampalam and Bauer 1997; Elsen et al. 1998; Ponnampalam et al. 1998; Smart et al. 2004).

The characterization of a *crtJ* mutant strain has revealed its involvement in the transcriptional control of several *hem* genes, summarized in Fig. 13.2 (Smart et al. 2004). The strongest regulation is the activation of *hemH* expression, whatever the redox condition is. CrtJ is also required to induce the translation of *hemZ* under semi-aerobic conditions. In the same redox conditions, CrtJ exhibits a dual role of repressor of *hemC* and activator of *hemE* at the same time. This is puzzling as these genes share a common promoter region, containing a CrtJ-binding site (Smart

et al. 2004). Elucidating how, in constant redox conditions, the interaction with this promoter induces two opposite events will be a challenge.

13.4.4.3 FnrL

The redox regulator FnrL appeared to have only moderate effect on transcription of *hem* genes (Fig. 13.2), as either an activator or a repressor, depending on the gene and/or on redox conditions (Smart et al. 2004). This makes it hard to clearly define its mode of action and function in the regulation of tetrapyrrole synthesis. On the one hand, FnrL significantly represses *hemA* in aerobic conditions and *hemB* and *hemC* in semi-aerobic and anaerobic conditions. On the other hand, it dramatically activates the transcription of *hemE* in semi-aerobic conditions. FnrL seems to be mostly involved in the early steps of the tetrapyrrole synthesis, the universal trunk, and the first step of the porphyrin branch. It was shown in other photosynthetic bacteria, such as *R. sphaeroides* and *Rubrivivax gelatinosus*, to have a crucial role in the transcription of *hemZ*, which did not appear to occur in *R. capsulatus* (Smart et al. 2004; Ouchane et al. 2007).

13.4.4.4 AerR

The aerobic repressor AerR (Dong et al. 2002) has a limited although important role in the transcriptional regulation of the tetrapyrrole synthesis, consisting in the activation of *hemE* and *hemH* whatever the redox conditions and the repression of *hemC* in semi-aerobic conditions are (Fig. 13.2). Overall, AerR seems to back up CrtJ in its target genes, with the exception of *hemZ* (Smart et al. 2004).

13.4.4.5 HbrL

The heme-binding regulatory LysR-type (HbrL) protein was identified in 2006 from the screening of a cosmid library for suppressors defective in heme synthesis using *hemB* as a reporter (Smart and Bauer 2006). From these experiments, an 804 bp ORF was isolated and cloned and the deduced amino acid sequence showed a LysR-type transcriptional regulator type (LTTR) signature: A helix-turn-helix DNA-binding domain at the N-terminal followed by an LTTR substrate-binding motif or coinducer-binding motif. In addition to being ubiquitous in prokaryotes, the LTTRs represent the largest family of DNA-binding protein. They regulate functions as diverse as metabolism, cell division, quorum sensing, oxidative stress, virulence, nitrogen fixation, etc. (Maddocks and Oyston 2008). As shown in Fig. 13.2, HbrL appears to be involved in the heme-mediated control of the expression of *hemA*, *hemB*, and *hemN1* (*hemZ*). Whereas it acts as a strong activator of *hemA* and *hemN1* in the absence of exogenous heme, it is a moderate repressor of *hemB*. Recombinant HbrL was obtained by heterologous expression in *E. coli* and purification. It exhibited heme-binding properties either in vivo by supplementing the expression medium with δ-ALA or in vitro by adding heme to the crude lysate containing the apoprotein. Finally, recombinant HbrL was found to interact with

the promoter region of the genes it regulates and the DNA-binding properties were affected by the presence or absence of heme (Smart and Bauer 2006). Interestingly, out of seven strains from the laboratory freezer, only four displayed an intact HbrL-encoding gene (unpublished observation).

13.4.4.6 Irr

The iron response regulator (Irr) is a transcription factor related to the ferric-uptake regulator (Fur) family of metalloregulators. It has been extensively studied in *Bradyrhizobium japonicum* where it is a global regulator of iron homeostasis that acts in particular as a repressor of heme synthesis in iron-limited conditions. It is constitutively expressed and its activity mostly controlled by post-translational modification. Indeed, its degradation is triggered by binding to both redox states of heme: The ferric form binds to an N-terminal heme regulatory motif (HRM), while the ferrous form interacts with a histidine-rich domain (Small et al. 2009). In the genome of *R. capsulatus*, an ORF was annotated as a Fur transcription factor (rcc02670 in http://onco.img.cas.cz/rhodo and RRC01140 in http://www.ergo-light.com/). Analysis of the sequence revealed that it belongs to the Irr subgroup rather than to the Fur one stricto sensu, as seen from divergence in key residues involved in metal coordination in the Fur group (Rudolph et al. 2006). Compared to the well-characterized *B. japonicum* Irr, it does not carry any HRM, which indicates a presumably different mechanism of action. On the other hand, the DNA-binding helix is very well conserved. It is noteworthy that, among the α-proteobacteria subfamily, *R. capsulatus* is the only representative that carries Irr as the sole regulator involved in the iron-responsive network. The usual master regulators Fur and RirA are indeed missing (Rodionov et al. 2006). This fact indicates a potential major role of Irr in *R. capsulatus* regarding the management of iron metabolism. Preliminary studies have shown that an *irr*-deleted strain of *R. capsulatus* presents abnormal levels of heme, approximately 25% less and 25% more than the wild-type strain in aerobic and photosynthetic conditions, respectively (unpublished data).

13.5 The Porphynoid Branch

13.5.1 The Siroheme Sub-branch: The Other Fe Branch

Siroheme, the prosthetic group of various reductases, was reported to occur in *R. capsulatus* strain E1F1. A 17 kb region contains genes encoding an assimilatory nitrate reduction system, which consists of (i) putative regulatory genes *nsrR* and *nasTS*; (ii) an ABC-type nitrate transporter coded by *nasFED*; (iii) genes coding for the apo-nitrate and apo-nitrite reductases, *nasA* and *nasB*; (iv) a gene encoding a siroheme synthase, *cysG*, responsible for synthesis of the nitrite reductase cofactor (Pino et al. 2006). Although the siroheme synthase was not biochemically studied,

the nitrite reductase NasB has been heterologously expressed in *E. coli* and purified recombinant protein exhibited a siroheme spectral signature (Olmo-Mira et al. 2006). CysG (EC 2.1.1.107) was characterized in *Salmonella enterica* and in *E. coli* where it was shown to be a bimodular homodimer that catalyzes the four reactions that converts uro'gen III into siroheme (Stroupe et al. 2003).

13.5.2 The Corrin Sub-branch: From Uro'gen III to VitB$_{12}$

13.5.2.1 An Unusual Pathway

Cobalamin synthesis requires more than 30 enzymatic steps with genes that encode these enzymes taking up approximately 1% of a typical bacterial genome (Roth et al. 1993; Martens et al. 2002). There are two distinct cobalamin biosynthesis pathways that exist in different microorganisms: the well-studied aerobic pathway, represented by *Pseudomonas denitrificans*, and the partially resolved anaerobic pathway, represented by *Salmonella typhimurium, Bacillus megaterium,* and *Propionibacterium shermanii* (Scott and Roessner 2002). The two pathways diverge at precorrin-2, a compound synthesized by dimethylation of uro'gen III (Raux et al. 1999). These two pathways primarily differ in their requirement for molecular oxygen and the timing of cobalt insertion. In the aerobic pathway, molecular oxygen is incorporated before the ring contraction, where the carbon bridge (C20) between the rings A and D is excised, and cobalt chelation occurs nine steps after the synthesis of precorrin-2. In contrast, in the anaerobic pathway, cobalt is inserted directly into precorrin-2. Ring contraction is likely facilitated by the different oxidation states of the cobalt ion (Martens et al. 2002; Warren et al. 2002). The existence of distinct cobalamin synthesis pathways is also reflected at the genetic level with organisms containing genes unique to one pathway or the other. Interestingly, *R. capsulatus* carries genetic hallmarks for an aerobic pathway although cobalamin synthesis occurs under both aerobic and anaerobic conditions (McGoldrick et al. 2002). A *cobG* gene, encoding a mono-oxygenase, is missing in *R. capsulatus.* This mono-oxygenase catalyzes the contraction of the corrin ring, which is a characteristic reaction in the aerobic pathway. Instead, an isofunctional enzyme, CobZ, was identified (McGoldrick et al. 2005), which may mediate the ring contraction process under both aerobic and anaerobic conditions. Details of enzymes at additional steps of this pathway are beyond the scope of this review but can be obtained from a recent review of this complex branch in the pathway by Warren (Warren and Deery 2009).

13.5.2.2 *cob* Gene Regulation

Riboswitch: Regulation at the Post-transcriptional Level

The regulation of cobalamin biosynthesis is mostly reported to occur post-transcriptionally via an RNA structure called a riboswitch. Riboswitches are metabolite-binding regions located within the 5′-UTR of messenger RNAs that function as regulatory elements for target genes (Mandal et al. 2003). Riboswitches

are widespread in prokaryotes and interact with various target metabolites. In *B. subtilis*, seven different metabolites, including guanine, adenine, lysine, thiamine pyrophosphate, FMN, SAM, and adenosylcobalamin, regulate at least 68 genes via a riboswitch mechanism (Mandal et al. 2003). In cobalamin biosynthesis, adenosylcobalamin is known to be an effector regulating translation of several cobalamin genes (Nahvi et al. 2002; Rodionov et al. 2003). A B_{12}-binding element exists within the 5′-UTR of *btuB* mRNA in *E. coli* and within the 5′-UTR of cobalamin biosynthesis operon in *S. typhimurium* that binds adenosylcobalamin (Nahvi et al. 2004). At elevated adenosylcobalamin concentrations, the translation of cobalamin transport protein (BtuB) and *cob* operon is repressed by adenosylcobalamin binding to the B_{12} box (Nahvi et al. 2004). Such interactions induce a conformational change in the mRNA secondary structure at the B_{12} box that prevents ribosome binding (Nou and Kadner 2000). Rodionov et al. (2003) employed the combination of a comparative approach of gene regulation, positional clustering, and phylogenetic profiling for identifying cobalamin-regulated biosynthesis/transport genes. They identified a conserved RNA structure called the B_{12} element that is widely distributed in eubacteria. For example, 13 B_{12} elements are present in UTR regions of the *R. capsulatus* genome with potential target genes, including *cobW*, *cbiMNQO*, *btuFCD*, *btuD*, and *btuB*, as well as upstream of several non-cobalamin biosynthesis/transport genes (Rodionov et al. 2003).

Transcriptional Regulation

Examples of cobalamin genes regulated at the transcriptional level are not abundant. In *S. typhimurium*, the global regulators Crp/cAMP and ArcA/ArcB are responsible for indirect redox and carbon controls of cobalamin biosynthesis by controlling synthesis of the positive regulatory protein, PocR. PocR subsequently uses propanediol as an inducer of *cob* gene expression (Rondon and Escalante-Semerena 1992, 1996). Other global regulators directly regulating *cob* genes are not known at present.

Recently, we observed that the global RegA–RegB signal transduction cascade not only controls expression of the Bchl and heme branches but also controls expression of enzymes at two steps in the cobalamin branch, the *cobK* gene and the *cobWNHIJ* operon (Li 2009). Thus, expression of cobalamin enzymes appears to be regulated by feedback control via a riboswitch mechanism as well as in response to redox via the RegA–RegB signal transduction cascade.

13.6 The Bchl Branch: From Proto IX to Bchl

13.6.1 The Bchl Biosynthesis Gene Cluster

Analysis of genes involved in the Mg branch of the tetrapyrrole biosynthesis pathway was primarily advanced by the work of Marrs and coworkers who used a generalized transducing agent (GTA) from *R. capsulatus* to map the location of Bchl

biosynthesis genes (Marrs 1974; Yen and Marrs 1976). These studies established that carotenoid and Bchl biosynthesis genes were present in the *R. capsulatus* chromosome in a tight linkage order. The transduction mapping studies were followed by the isolation of an R′ plasmid that was shown by marker rescue and transposition mapping analyses to contain all of the essential genetic information needed to synthesize Bchl (Marrs 1981; Biel and Marrs 1983; Taylor et al. 1983; Zsebo and Hearst 1984). Structural and functional information regarding these loci was refined by complete sequence analysis of the R′ by Hearst and coworkers (Alberti et al. 1995) and by construction of defined interposon mutations in each of the sequenced open reading frames (Giuliano et al. 1988; Young et al. 1989; Yang and Bauer 1990; Bollivar et al. 1994a, b). Similar clustering of Bchl biosynthesis genes occurs in other purple bacterial species (Igarashi et al. 2001; Jaubert et al. 2004).

Several of the enzymes encoded by one or more of the Bchl biosynthesis genes have been expressed in *E. coli* and shown to exhibit enzymatic activity at specific steps of the Mg branch. The first committed step of the Mg branch involves insertion of Mg into proto IX by the enzyme Mg chelatase (EC 6.6.1.1) to form Mg–proto IX. Gibson et al. (1995) were the first to successfully heterologously express subunits of Mg chelatase encoded by the *bchH*, *bchI*, and *bchD* genes in *E. coli* to definitively establish the subunit composition of this enzyme. Activity is dependent on Mg, ATP, and proto IX. The multimeric Mg chelatase is a typical class 1 metal chelatase (Schubert et al. 2002) where BchH binds proto IX (Gibson et al. 1995) while the structures of BchI and BchD show that they both have ATPase domains of the AA+ class (Fodje et al. 2001).

The next step of the pathway involves SAM-dependent methylation of the carboxyl group of Mg–proto IX by the enzyme SAM Mg–proto IX-*O*-methyltransferase (EC 2.1.1.11) to form Mg–proto IX monomethyl ester (MPE). Confirmation that the *R. capsulatus bchM* encodes this enzyme occurred when this gene was heterologously expressed in *E. coli* with cell lysate extracts exhibiting activity (Bollivar et al. 1994a, b).

Oxidative cyclization to form the fifth ring of Bchl is catalyzed by the enzyme MPE oxidative cyclase (EC 1.14.13.81) that is coded by the *bchE* gene to form the product protochlorophyllide (PChlide). This enzyme has not been well characterized biochemically owing to the difficulty in observing activity in vitro. However, analysis of the *R. capsulatus* BchE peptide sequence indicates that it may be a SAM enzyme that also uses vitB_{12} as a cofactor (Gough et al. 2000). Indeed depletion of vitB_{12} in vivo leads to accumulation of MPE in this branch of the pathway (Gough et al. 2000).

Reduction of the D ring of PChlide to form chlorophyllide (Chlide) occurs by the enzyme NADPH–PChlide oxidoreductase (EC 1.3.1.33). In nature there are two unrelated enzymes that can catalyze this reduction. One is a light-dependent version that is present in cyanobacteria, alga, gymnosperms, and angiosperms, while the other is light-independent and present in anoxygenic bacteria, cyanobacteria, alga, and gymnosperms (Fujita and Bauer 2003; Heyes and Hunter 2005). The dark operative form is present in *R. capsulatus*, encoded by *bchL*, *bchN,* and *bchB*. Interestingly, it has a high degree of primary sequence similarity to nitrogenase

(Fujita and Bauer 2003). Biochemical characterization of the *R. capsulatus* enzyme indicates that it contains iron sulfur centers and requires ATP, ferredoxin, and a reducing agent for catalysis (Fujita and Bauer 2003; Nomata et al. 2005). Reduction of ring B of Chlide by the enzyme Chlide a reductase (EC 1.3.1.75), encoded by *bchX*, *bchY*, and *bchZ*, also uses an enzyme that is very similar to dark PChlide reductase (Nomata et al. 2006).

There are several latter steps of the pathway that have not been biochemically characterized. The one exception is the esterification of the propionate on ring IV by the enzyme Bchl *a* synthase, encoded by *bchG*. This enzyme is membrane bound and difficult to isolate. However, it was heterologously expressed in *E. coli* with membrane fractions shown to exhibit this activity (Oster et al. 1997).

13.6.2 Regulation of Bchl Biosynthesis Gene Expression

13.6.2.1 CrtJ/PpsR

Studies have demonstrated that CrtJ/PpsR is an aerobic repressor of Bchl genes, *bch*, in *R. capsulatus* (Ponnampalam and Bauer 1997; Elsen et al. 1998). As discussed below, anaerobic induction of Bchl, carotenoid, and light-harvesting genes also requires phosphorylated RegA. So together, CrtJ and RegA regulate synthesis of Bchl biosynthesis genes by coordinating aerobic repression and anaerobic activation, respectively (Fig. 13.2).

CrtJ cooperatively binds to two copies of the palindromic sequence TGT-N_{12}-ACA that is present in all of the characterized *bch* promoters (Alberti et al. 1995; Ponnampalam and Bauer 1997; Elsen et al. 1998; Ponnampalam et al. 1998). The palindromic sequence is found either 8 bp apart, or at sites that are distantly separated. For example, the *R. capsulatus bchC* promoter region has a CrtJ recognition palindrome that spans the –35 promoter region and a second CrtJ palindrome located 8 bp away that spans the –10 promoter region (Ponnampalam et al. 1998). Binding to these two palindromes is cooperative so if the 8 bp space between the two palindromes in the *bchC* promoter region is altered by the addition or deletion of just a few base pairs, then CrtJ is unable to bind to either palindrome effectively (Ponnampalam et al. 1998).

CrtJ also cooperatively binds to two palindromes at other promoters but these palindromes are separated by more than 100–150 bp (Elsen et al. 1998). An example of this type of binding occurs in the intergenic region between *crtA* and *crtI* that contains two promoters, one that is responsible for driving expression of the *crtA–bchI–bchD* operon and a second divergent promoter >100 bp away that is responsible for expression of the *crtI–crtB* operon (Elsen et al. 1998). The promoter for the *crtA–bchI—bchD* transcript has a single CrtJ-binding site that spans the –10 promoter sequences while the *crtI–crtB* promoter also has a single CrtJ recognition sequence that spans the –35 recognition sequence. Cooperative binding of CrtJ to these two palindromes coordinately represses expression of both the *crtA–bchI–bchD* and *crtI–crtB* operons (Elsen et al. 1998). This affects synthesis of both Bchl

and carotenoids since BchI and BchD are subunits of Mg chelatase (Bollivar et al. 1994a, b) and *crtI* and *crtB* code for phytoene dehydrogenase and phytoene synthase that are enzymes for the first two committed steps of carotenoid biosynthesis, respectively (Armstrong et al. 1990). Presumably, binding to distant sites involves looping of the DNA so that tetrameric CrtJ can bind cooperatively to both of the recognition palindromes (Elsen et al. 1998).

13.6.2.2 RegA–RegB

The Mg branch of the tetrapyrrole biosynthetic pathway is also anaerobically activated by the global two-component system RegA–RegB (Willett et al. 2007). Specifically, expression of the large *bchEJG-orf428-bchP-idi* operon that encodes numerous enzymes in Bchl biosynthesis, as well as an enzyme involved in carotenoid biosynthesis, requires phosphorylated RegA for maximal expression (Bollivar et al. 1994a, b; Alberti et al. 1995; Hahn et al. 1996; Suzuki et al. 1997; Bollivar 2006; Willett et al. 2007). In addition, phosphorylated RegA is also required for expression of the *crtA–bchIDO* operon that codes for early enzymes involved in the Bchl branch as well as early enzymes in the carotenoid biosynthesis pathway (Alberti et al. 1995).

13.7 Co-dependent Syntheses of Tetrapyrrole End Products

The trifurcated tetrapyrrole biosynthetic pathways not only share common early intermediates but also form an intricate network where the end products cobalamin and heme are involved in each others and Bchl biosyntheses. For example, in the Bchl branch, Gough et al. (2000) identified a cyclase encoded by *bchE* that catalyzes the conversion of MPE to PChlide. The sequence similarity between this MPE-cyclase and a cobalamin-dependent P-methylase from *Streptomyces hygroscopicus* indicates an involvement of cobalamin in MPE-cyclase activity. Although no in vitro cyclase assays have been reported, MPE-cyclase activity was demonstrated to be cobalamin dependent in vivo as cobalamin depletion or a *bchE* knockout results in an accumulation of MPE.

In addition to the dependence on cobalamin for Bchl biosynthesis, the synthesis of cobalamin is dependent on the presence of heme. Specifically, one of the most complicated steps in the *R. capsulatus* cobalamin branch is the corrin ring contraction that is catalyzed by the cofactor-rich enzyme, CobZ. CobZ contains heme as a cofactor as well as flavin and two Fe–S centers (McGoldrick et al. 2005). The dependence on heme as a cofactor of CobZ thus makes cobalamin synthesis heme dependent.

Finally, synthesis of heme is also cobalamin dependent as heme synthesis requires *S*-adenosylmethionine as a methyl group donor and its synthesis involves a cobalamin-dependent enzyme (Drennan et al. 1994; Layer et al. 2003; McGoldrick et al. 2005). The interdependent relationship among all three tetrapyrrole compounds as discussed in McGoldrick et al. (2005) suggests that codependence of

these different tetrapyrrole branches on different tetrapyrrole end products is a form of regulation used to coordinate the levels of these different tetrapyrrole derivatives.

13.8 Conclusion

The tetrapyrrole biosynthetic pathway of *R. capsulatus* is quite complex involving several branches that are responsible for synthesis of heme, vitB_{12}, and Bchl *a*. The flow of tetrapyrroles into each branch in the trifurcated pathway is highly regulated with an intricate network of transcriptional and post-transcriptional events controlling the synthesis of these various end products. This includes the control of enzyme activity by interaction with tetrapyrrole end products and transcriptional control either in response to redox poise or in response to an interaction with a tetrapyrrole. There is also a riboswitch control of translation via interaction of mRNA with a tetrapyrrole. Many players in this complex regulatory web have been identified, although it will not be surprising to find that there are additional players involved in controlling this complex pathway.

Among many challenges going forward will be to determine how multiple transcription factors coordinately regulate the synthesis of individual enzymes in the pathway. For example, the *hemC* promoter appears to be controlled by at least four different activators/repressors. Where these factors bind to the *hemC* promoter and how binding of individual transcription factors affects binding of other transcription factors remain to be determined. The study of this complex pathway has been an ongoing for over five decades and, owing to its complexity, will likely remain an area of focus for some time.

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