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Peer-Review Record:

Evolutionary Aspects and Regulation of Tetrapyrrole Biosynthesis in Cyanobacteria under Aerobic and Anaerobic Environments

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Reviewer 1: Anonymous
Reviewer 2: Anonymous
Editor: Robert Haselkorn and John C. Meeks (Guest editor of special issue "Cyanobacteria: Ecology, Physiology and Genetics")

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First Round of Evaluation

Round 1: Reviewer 1 Report and Author Response

This is a comprehensive and authoritative review of the chlorophyll biosynthesis pathway in cyanobacteria, with an emphasis on how the organisms adapt to varying levels of oxygen. The authors have done an excellent job of summarizing a complex literature that can be very confusing to nonspecialists. For example, many of the important genes have multiple names in different types of organisms. I think the authors have done a good job of explaining this complexity.

Response: Thank you for your constructive comments and suggestions on our manuscript.

The figures are nicely done and quite informative. However, some of the legends are too wordy and repeat some things that are in the text, for example Figure 2 and 3. I think these could be shortened and not lose any information.

Response: To shorten these legends as you suggested, we removed 7 Lines (Lines 106–108 and 113–116) in Figure 2, and 14 Lines (Lines 189–194 and 199–206) in Figure 3. Figure 3C was also removed.

The one place where I think the manuscript falls down a bit is in the discussion of the evolutionary transition from the anaerobic enzymes that are found in various types of anoxygenic phototrophic bacteria to the aerobic versions that are found in cyanobacteria. Some key literature references here are not included. These include also some reviews on radical SAM enzymes.

- Ouchane *et al.* (2004) JBC 279: 6385;
- Raymond and Blankenship (2004) Geobiology 4: 199;
- Hassani *et al.* (2010) JBC 285: 19281;
- Tang *et al.* (2011) BMC Genomics 12: 334;
- Boldareva-Nuianzina (2013) App. Env. Micro. 79: 2596;
- Broderick *et al.* (2014) Chem. Rev. 114: 4229.

Response: Thank you for your citation recommendation, which was really useful to improve our manuscript. All papers were cited and some were mentioned briefly the contents of the papers.

- *Ouchane et al. (2004), Ref number 46; Lines 229–230.*
- *Raymond and Blankenship (2004), Ref. number 122; Lines 646–653.*
- *Hassani et al. (2010), Ref number 48; Lines 230–234.*
- *Tang et al. (2011), Ref number 49; Lines 234–235.*
- *Boldareva-Nuianzina et al. (2013), Ref. number 50; Lines 235–239.*
- *Broderick et al. (2014), Ref. number 17; Line 108.*

Round 1: Reviewer 2 Report and Author Response

The review by Y. Fujita and coworkers addresses the impact of an important environmental factor on the control of tetrapyrrole biosynthesis. The increasing oxygen levels in the atmosphere has challenged microorganisms and resulted in the further development of proteins with increasing oxygen tolerance during evolution on earth. In this respect the review is written to the right time and from those, who have substantially contributed to the description of the different strategies how cyanobacteria cope with ambient and low oxygen conditions. The manuscript contains also many valuable and substantial information. The manuscript attracts the reader with a generally well-understandable synapsis of significant data. It becomes obvious that, particularly the second part, as the manuscript leaves the simple report of published data and the authors describe and develop their own ideas and hypotheses, the manuscript becomes more appealing and favorable. However, I admit I feel the paper is still in a very preliminary state, as too many pitfalls and flaws could be noticed, which tremendously require a thorough revision. More accuracy in verbalization and syntax is demanded. Generally it seems that the paper has been hastily submitted. As reviews are generally highly appreciated and acknowledged, when they present the ideas in a preferably short version, the authors should be obliged to take an additional effort to shorten the manuscript, apart from the need to correct misleading and confusing sentences.

Response: Thank you for your constructive comments and suggestions on our manuscript.
According to your suggestion, to shorten this manuscript, we removed several paragraphs; Lines 113–116, 189–194, 199–206, 336–344, 352–355, and 721–726, and Figure 3C. In addition, we rewrote two paragraphs to be more succinct; Lines 255–262 and 311–323.

Line 7: Improve the perspicuity of the title. As the manuscript describes the heme oxygenase, it is recommended to talk about tetrapyrroles instead of chlorophyll, e.g. control of tetrapyrrole biosynthesis under aerobic and anaerobic conditions. Evolutionary implications …

Response: According to your suggestion, we changed the title to "Regulation of Tetrapyrrole Biosynthesis in Cyanobacteria Under Aerobic and Anaerobic Environments: Evolutionary Implications".

Line 62: As the paper benefits from data obtained with anaerobic photosynthetically active bacteria and higher plants, the intention is arbitrary to confine the manuscript mainly on results obtained with cyanobacteria. The work on anaerobically growing photosynthetic microorganism is substantial and more references on the control of low oxygen will improve the paper.

Response: We mentioned briefly extensive works on photosynthetic bacteria in Introduction section (Lines 59–63);
"Extensive studies on the regulatory mechanisms of photosynthesis, including bacteriochlorophyll biosynthesis, have been performed in the purple bacteria, Rhodobacter capsulatus and R. sphaeroides. Several regulatory proteins, such as RegB-RegA, FNR, and CrtJ/PpsR, have been identified as the key factors for control of the metabolic changes in response to redox changes caused mainly by oxygen in these purple bacteria [6]." (Ref 6: Bauer, C.E.; Setterdahl, A.; Wu, J.; Robinson, B.R. Regulation of gene expression in response to oxygen tension. In The Purple Photosynthetic Bacteria, Hunter, C.N.; Daldal, F.; Thurnauer, M.C.; Beatty, J.T., Eds. Springer Science + Business Media B.V.: Houten, The Netherlands, 2009; pp. 707–725.)

Line 50: Revise the sentence, at least a word is missing.

Response: We revised this sentences follows:
"In contrast, at night, oxygen is quickly consumed by respiration of heterotrophic bacteria and cyanobacteria, which reduce oxygen levels to near anaerobic levels." (Lines 51–53).

Line 74 + 77: Revise the sentences, it will improve the readability and understandability (differing oxygen: …differentiated?)

Response: We revised these sentences as follows:
"Expression of genes encoding these enzymes is mainly controlled at the transcriptional level in response to cellular oxygen tension. In the following sections, we will introduce these reactions and review how two enzymes with differing oxygen sensitivity are regulated in cyanobacterial cells." (Lines 87–90)

Lines 86, 85, 98, 110, 267: The content is wrong, the sentences require revision. For example: Enzymes are not transcribed. Check the need of each single work of these sentences.

Response: We revised these sentences (Lines 97–98 and 109–110, and the sentence of the original Lines 113–114 was removed). Although we could not specify some sentences pointed out (original Lines 98, 110, and 267) because the line numbers the reviewer specified were different from those of our original copy. However, this revised manuscript was extensively revised by an English editing service, so we believe that these points must be corrected.

Line 97: Check, whether all abbreviations have been introduced, here SAM.

Response: We checked all abbreviations have been introduced including S-adenosylmethionine (SAM) at Line 108.

Line 125: It is not an alternative!

Response: We revised this sentence (original line 128) as follows:
"This property may allow utilization of endogenous oxygen produced by photosynthesis under microoxic conditions." (Lines 132–133)

Mg ProtoME cyclase: The substantial work from C. Astier and S. Ouchane has not been acknowledged. They generally contributed to adaptation to oxygen and to analysis of HEMIN. Apart from this drawback, the authors should also acknowledge other group's work on this enzyme in bacteria, cyanobacteria and higher plants. It starts already at line 170 ff, regards also line 208, 218 and 238, where several references are omitted.

Response: As you pointed out, we cited Ouchane et al. (2004) and Ouchane et al. (2007) (Reference numbers 46 and 47, respectively) and rewrote sentences to acknowledge their works (Lines 229–234):
"BchE is the sole MPE cyclase in the purple photosynthetic bacterium R. capsulatus [45] whereas another purple bacterium, R. gelatinosus, uses both ChlA (AcsF) and BchE systems [46]. In the latter species, ChlA and BchE as well as HemF and HemN are differentially expressed in response to oxygen levels by the oxygen-responding transcriptional factor FnrL [47], and terminal cytochrome oxidases contribute to lowering of the cellular oxygen tension to aid expression of bchE and hemN and their operations [48]. In the filamentous anoxygenic photosynthetic bacterium Chloroflexus aurantiacus, both chlA and bchE genes were found in the genome [49]."

Line 252 + 317: Improve the sentences.

Response: Paragraphs including both sentences (original lines 259 and 324) were rewritten (Lines 256–261), and undertaken by English editing service:
"DPOR consists of two separable components, L protein and NB protein, which are homologs of two components, Fe protein and MoFe protein, of nitrogenase, respectively [54–56]. The 3D structures of both components are very similar to that of nitrogenase [57–59]. The L protein is a BchL/ChlL dimer and has a role as an ATP-dependent electron donor for the NB protein [60,61]. The NB protein, a heterotetramer of BchN/ChlN and BchB/ChlB, provides the catalytic center for Pchlide reduction [62,63]."

Line 306: The sentence is not clear and requires replacing.
Line 308: What means "other many"?

Response: Paragraph including this sentence was rewritten (lines 304-311). In this process, the relevant sentences were removed:
"Most Chl pigments have an ethyl group at the C8 position, which is contrasted by the last common precursor Proto shared with heme in which the C8 position is a vinyl group. Thus, the C8-vinyl group must be converted to an ethyl group by an 8-vinyl reductase somewhere along the reactions from Proto to Chlide. There are three evolutionarily unrelated 8-vinyl reductases. One enzyme is NADPH-dependent 8-vinyl reductase (N-DVR, BciA), which is distributed among plants [78], some marine cyanobacteria [78], and some photosynthetic bacteria [79,80]. A second enzyme is ferredoxin-dependent 8-vinyl reductase (F-DVR, BciB), which is mainly found in fresh-water cyanobacteria [81,82] and some photosynthetic bacteria."

Line 322: Correct the sentence.

Response: Paragraph including this sentence was rewritten (lines 312-323). In this process, the relevant sentence was removed:
"N-DVR from Arabidopsis thaliana shows high substrate specificity to 3,8-divinyl Chlide, which suggests that the 8-vinyl reduction step occurs mainly at the Chlide stage in A. thaliana [83]. In contrast, cyanobacterial F-DVR (Synechocystis 6803) shows a broad substrate specificity for conversion of 3,8-divinyl Pchlide, 3,8-divinyl Chlide, and 3,8-divinyl Chl a to their ethyl derivatives, which suggests that the 8-vinyl reduction occurs potentially at all three steps: Pchlide, Chlide, and Chl a [64]. The highest activity was detected for 3,8-divinyl Chlide, which implies that F-DVR catalyzes 8-vinyl reduction at the Chlide step in cyanobacteria [83](Figure 3B). The activity for 3,8-divinyl Pchlide may become obvious in cells, such as the chlL mutant that accumulates Pchlide. In a dark-grown ΔchlL mutant of L. boryana, the accumulated Pchlide is only the 3-vinyl form (Yamamoto and Fujita, unpublished result). In contrast, mutants lacking chlAI or chlAII of Synechocystis 6803 accumulated 3,8-divinyl MPE [33]. Thus, it is suggested that F-DVR does not catalyze the 8-vinyl of MPE in vivo in spite of the broad substrate specificity in vitro."

Line 328: Rewrite the sentence.

Response: *This may point the sentence at Line 336 (original line). We removed the paragraph (original Lines 336–344) to shorten, because descriptions of F-DVR heterologous expression and characterization of BciB are not so important in this review.*

Line 334: What do the authors want to explain?

Response: This may point the sentence at Line 342 (original line). We removed this paragraph as above (original Lines 336–344).

Line 382: This is likely to be misunderstood.

Response: This may point the sentence at Lines 389–390 (original line). We confirmed the content of this sentence and a relevant paper was cited (Lines 363–365):
"In addition, the product of HO, biliverdin IXα, and its reduced derivative bilirubin, are potent antioxidants that may contribute to alleviation of oxidative stress [97]."

Line 387: Rephrase the sentence to express clearly the intention.

Response: This may point the sentence at Line 395 (original line). We rephrased it to be two sentences (Lines 369–372):
"Interestingly, these three genes, chlAII, ho2, and hemN, which code for anaerobic/microoxic-type enzymes, form a small gene cluster in the genome of Synechocystis 6803 (Figure 5A). The expression of these genes is induced under microoxic conditions whereas their transcription levels are very low under aerobic conditions [22,33,89]."

Line 398: Some words at the end of the sentence are missing.

Response: This may point the sentences at Lines 400–404 (original line). We rewrote these sentences (Lines 377–381):
"In the following sections, we describe the characterization and physiological significance of ChlR for tetrapyrrole biosynthesis under low-oxygen conditions, especially nitrogen fixation requiring anaerobic conditions [100]. The evolutionary implications related to the drastic change in environmental oxygen levels that occurred on the geological scale will also be discussed."

Line 411: A word is missing only a spelling mistake?

Response: This may point the sentence at Line 422 (original line). We revised this sentence (Lines 398–400):
"This similarity suggests that Sll1512 acts a role as a transcriptional regulator in Synechocystis 6803 and, thus, was designated "chlR" [98]."

Line 466: Here the reader is entirely lost. The best is to revise the paragraph from line 463.

Response: We revised this paragraph at lines 463-487 (original line) to be two paragraphs (Lines 429–436 and 437–455) and one new figure (Figure 6A) of sequence alignment of ChlR and SarZ. This new Figure 6 also contained the working model of ChlR (as panels B and C).

Line 469: Which protein is meant?

Response: This may point the sentence at Line 477 (original line). The rewritten paragraphs (Lines 437–455) shown above include this sentence. To clarify the description of ChlR, we divided the ChlR proteins into two types: 1Cys- and 4Cys-types, which is also shown in the sequence alignment (Figure 6A).

Line 475: Did the authors introduce ChlR-like proteins?

Response: In the revision of the paragraph (Lines 437–455), we introduced two types of ChlR protein; 1Cys- and 4Cys-types and showed them in Figure 6A (the sequence alignment of ChlR proteins of 1Cys- and 4Cys-types).

Line 505: Is it correct to refer to Synechococcus 7002 (compare Figure 5B).

Response: This may point the gene number SYNPCC7002_A2198 (original Line 516). This description was correct. We redrew Figure 5 to be recognized by readers the gene arrangements of two cyanobacteria Synechocystis 6803 (Figure 5A) and Synechococcus 7002 (Figure 5B).

Line 517: no sentence.

Response: This may point the sentence at Line 528 (original line). We rewrote these sentences (Lines 515–518):
"Except for chlAII and hemN, the transcript levels of the other 26 genes were almost the same in wild-type and ΔchlR irrespective of aerobic and microoxic conditions [108]. Thus, chlAII, ho2, hemN, and psbA1, may only be the ChlR-targets in Synechocystis 6803."

Line 584: Improve the structure of the sentence.

Response: This may point the sentence at Line 595 (original line). We rewrote this sentence (Lines 580–582):
"However, some strains lack chlR in despite the presence of the genes encoding for these anaerobic/microoxic enzymes (Cyanothece sp. ATCC 51142 and T. elongatus BP-1)."

Table 1: I suggest to leave sll1917, as nothing is really known about the function of the encoded protein. On the other hand: Why did the authors omit the CHLE/BchE gene in cyanobacteria?

Response: We removed the column of sll1917 and introduce a new column of bchE in Table 1. According to the distribution of bchE in cyanobacteria (only two strains Cyanothece strains PCC 7475 and PCC 7822 in the 37 strains in Table 1 carry the probable bchE orthologs), we added sentences to explain the cyanobacterial bchE homologs (Lines 246–248 and 574–576):
"However, by extensive blast search among cyanobacteria, probable bchE orthologs were found in a limited number of cyanobacteria, including Cyanothece spp. PCC 7425 and PCC 7822 (Table 1)."
"In addition, the anaerobic MPE cyclase, BchE, is found in only two species, Cyanothece spp. PCC 7822 and PCC 7425. The bchE gene is located just downstream of the chlAII-ho2-hemN gene cluster in Cyanothece sp. PCC 7425. In these Cyanothece strains, the bchE gene may be regulated by ChlR."

Table 2: BchE function is not clear to me. Therefore, the classification of the cyclase is quite arbitrary.

Response: Although biochemical evidence is still missing, bchE is regarded as the anaerobic MPE cyclase itself or a critical subunit as discussed in the paper (Boldareva-Nuianzina et al. (2013), reference number 50). Thus, we describe bchE as the anaerobic MPE cyclase counterpart of the aerobic MPE cyclase chlA/acsF in this review.

Line: 695ff: I understood that ChlAII is not an anaerobic enzyme?

Response: It is correct. To be precise ChlAII is a microoxic-type enzyme similar to HO2 not an anaerobic enzyme. We added a phrase in the annotation d (Lines 686–688): "d Combination of anaerobic and aerobic enzymes (anaerobic/aerobic) or microoxic and aerobic isoforms (microoxic/aerobic) in extant cyanobacteria exemplified by Synechocystis 6803"

Line 710: Bioinformatics provide additional tools to predict evolution of pathways and proteins. The assignments of proteins can be substantiated by other experimental and theoretical methods.

Response: We cited a new paper, Boldareva-Nuianzina et al. (2013) reference number 50, (Lines 235–239), in which they showed phylogenetic trees of BchE and AcsF(ChlA) from many purple bacteria, suggesting that bchE undertook complex evolutionary histories including horizontal gene transfer and loss events in contrast to the tree of acsF that was consistent with that of 16S rRNA. This bioinformatic result may supportive for our evolutionary scenario shown in Figure 7.

Line 723: Did the authors introduce HmuO?

Response: HmuO is also HO in this bacterium. We removed HmuO and just mention HO at Line 727.

Figure 7: What is the novelty of this figure compared to Figure 1? How does this figure contribute to clearance? It seems to be better explained in the text.

Response: We regard this figure is still important because it is useful to propose hypothetical biosynthetic pathways in evolution. However, we revised this figure to be combined with two panels A and B of the original Figure 6 to show the probable timing of the hypothetical pathways (Figure 7).

Check the reference list, whether all references were cited in the text.

Response: We checked all references were cited in the text after extensive revision.

Second Round of Evaluation

Round 2: Reviewer 2 Report and Author Response

The authors improved substantially the manuscript and provide a manuscript with a justifiable readability of manuscript. However, I see that the length of the manuscript has not been changed, so in the last third of the review the authors got lost in details. As announce, although the manuscript might be corrected by a text editing service the revised sentences did not seem to be considered and still show often a need for revised word order.

In general, all issues have been addressed by the authors. Apart from these introductory comments, I repeat my impression that the review is a highly informative survey of the differences in control of tetrapyrrole biosynthesis under oxygenic and microoxic conditions mainly of cyanobacteria.

Response: Thank you for your suggestions on our revised manuscript in Round 2. We revised manuscript further, as shown below.

- My suggestion for a title was not completely written out and the authors should find a solution how they embed the notion ‘evolutionary implication” into the title. For example: Evolutionary aspects/implications and regulation of tetrapyrrole.

Response: According to your suggestion, we changed the title:
“Evolutionary Aspects and Regulation of Tetrapyrrole Biosynthesis in Cyanobacteria Under Aerobic and Anaerobic Environments”.

- Line 259 ff.: Make sure that the reader has the impression that DPOR consists of three different subunits, which can be separated in the L subunit and the two subunits N and B (or bchN/bchB and ChlN/ChlB). The term “Components” should be replaced by “two complex moieties”. Is it true that the authors want to give the impression that NB is a single protein? Please, provide clarity not only at the end of the paragraph.

Our response: As you suggested, we rewrote the introductory sentences for DPOR as follows:
“DPOR consists of three subunits, BchL/ChlL, BchN/ChlN and BchB/ChlB, whose amino acid sequences show significant similarities to those of NifH, NifD and NifK of nitrogenase subunits, respectively. BchL/ChlL forms a homodimer called L protein, and BchN/ChlN and BchB/ChlB form an a2b2 heterotetramer complex called NB protein, which are homologs of Fe protein (a NifH dimer) and MoFe protein (an a2b2 heterotetramer complex of NifD and NifK), of nitrogenase, respectively [54–56]. The 3D structures of both L protein and NB protein are very similar to those of nitrogenase Fe protein

and MoFe protein, respectively [57–59]. The L protein has a role as an ATP-dependent electron donor for the NB protein [60,61]. The NB protein provides the catalytic center for Pchlide reduction [62,63]."

- Line 308: Revise the phrase ", which is contrasted by the last …

Our response: As you suggested, we rewrote the sentences as follows:
"Most Chl pigments have an ethyl group at the C8 position. However, the last precursor common to heme, Proto, has a vinyl group at the C8 position."

- Line 308: Revise the phrase ", which is contrasted by the last…
  Comments to line 382, 387 and many other lines of the first manuscript:
  Regrettably, the authors corrected other sentences then I have asked for. I addressed certain lines of my copy of the first original manuscript, which apparently contained different sentences in the authors' copy. As result, the authors corrected other sentences, but sometimes corrected additionally also the sentences and phrases, I have addressed (or as consequence of the text editors' recommendations). Apparently, we all agree that enormous efforts are still required to edit the manuscript
- In this context, the previously issued sentences of Lines 375–378 should be rephrased to provide more clarity what the authors wish to express. "This response can be interpreted … at the transcriptional level."

Our response: We rewrote the sentences as follows:
"This induction can be interpreted as one of cyanobacterial hypoxic responses in order to maintain continuous supply of Chl, heme and bilins even under oxygen-limited conditions. How is the low-oxygen induction of these genes regulated at the transcriptional level?"

- Former line 398, now line 390: Sentence was not sufficiently corrected. What is meant with "conditions to be discarded"?

Our response: We rewrote the sentences as follows:
"A graduate student in our laboratory, Aoki, happened to find a single colony on an agar plate of Δho1. The agar plate was incubated under microoxic conditions followed by exposure to aerobic conditions, and later left on a bench for disposal."

- At least from my comment line 466: The authors always address entirely different parts of the manuscript. My comment referred to line 466, original paper, and concerned the sentence: "Ludwig *et al.* pointed out that… You may suggest a revision.
- The comment to Line 469 concerned the sentences "The most probable candidates for the two Cys …(In which protein?), was not addressed.

Our response: To address above two comments, we rewrote these sentences as follows:
"To support this model four Cys residues are required for holding one [4Fe-4S] cluster. Considering that ChlR is a homodimer, two Cys residues are needed per one polypeptide. If limited to 14 cyanobacterial species, four Cys residues are conserved in the ChlR proteins (we call them "4Cys-type" ChlR) (Figure 6A). Two of the four Cys could be involved in chelating one [4Fe-4S] cluster per homodimer. The most probable candidates involved in the [4Fe-4S] cluster are N-terminal Cys18 and Cys25 in ChlR of Synechocystis 6803 (Cys7 and Cys14 in ChlR of Synechococcus 7002) because the sequence of Cys-X_{4-6}-Cys-Pro is quite similar to the well-known iron-sulfur motif

Cys-X_2-Cys-X_2-Cys-X_3-Cys-Pro found in [4Fe-4S] type ferredoxins (Figure 6A) [99]. The use of iron-sulfur clusters to sense oxygen is found in other types of transcriptional regulators, such as FNR and SoxR in E. coli [104]. Further biochemical analysis is needed to understand coordination of the iron-sulfur cluster. Furthermore, it remains unclear how ChlR homologs that have only a single conserved Cys residue operate (Figure 6A). This "1Cys-type" ChlR may use the single Cys to sense oxygen levels, or may constitute a paralog that has other physiological functions."