Review on the PhD thesis entitled

"Polyphasic analysis and secondary metabolite patterns in unbranched heterocytous cyanobacteria with different life strategies"

by Andreja Kust

submitted to University of South Bohemia, Faculty of Science, Ceské Budejovice, Czech Republic, 145 pp.

Reviewer:

Rainer Kurmayer

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Preamble

In general I enjoyed reading this compilation of five peer-reviewed publications reporting novel insights on the morphology, phylogeny and secondary metabolite synthesis pathways among various filamentous unbranched cyanobacteria (characterized by specialized cells such as heterocytes and akinetes). As within the phylum of cyanobacteria the morphological variability is most impressive, careful systematic studies on environmental factors regulating the cell size and location of specialized cells within filaments as well as possible ecophysiological adaptations are needed and one part of the thesis (chapters I+II) goes into this direction. A second part (chapters III, IV, V) is dedicated to the analysis of the distribution of secondary metabolite synthesis including toxic metabolites such as the microcystins. The synthesis pathway for another family of compounds namely the pederins is described from a free-living cyanobacterium (chapter IV). Finally, for one pathway a not so common possibility involving alternative biosynthetic starter units is described (chapter V) as a means to increase overall structural diversity of an abundant peptide family namely the lipopeptides. Overall this second part highlights the genetic basis for the impressive diversity of secondary metabolites in this group of cyanobacteria. All chapters I-V were already published in international journals subsequent to the general peer reviewing process. Consequently the scientific value of the submitted PhD thesis is high and the PhD thesis is undoubtedly acceptable..

General description

The introduction section provides an overview on (i) (unbranched) cyanobacteria classification and the potential of natural product synthesis (as inferred from genomic information), (ii) the general thio-template oriented synthesis pathways (polyketide synthases and non ribosomal peptide synthetases), (iii) cyanotoxins with special reference to bioactive lipopeptides.

One reservation is that research objectives are not presented which is unusual when compared with PhD thesis submitted at other Universities. No hypothesis have been developed which limits to some extent also the coherence of the individual chapters in the PhD study. In fact one might argue that the PhD thesis contains largely two parts, with one part oriented towards variability in ecophysiology and a second part oriented towards secondary metabolism. The efforts to link this two parts are not visible.

The PhD thesis author was fully responsible for the papers I, III and IV, constituting the first author. She further partly contributed to papers II, V. According to the information provided the PhD candidate performed data collection for papers I+II, and was responsible for genome sequencing, purification of compounds, screening of strains by PCR and LC-HRMS, bioactivity testing, and writing. Overall, I consider this list less informative in order to evaluate the scientific achievements of the PhD candidate herself. Nevertheless the scientific standard of all the individual chapters is high as has been documented by the scientific reviewing process. Taking this high scientific standard into account the thesis is considered well worthy of being defended and there is no doubt that the thesis as such is acceptable.

Specific description

In chapter I four strains of benthic Anabaena are compared with regard to their morphoplogy, phylogeny and ecophysiology under a gradient in temperature and light intensity conditions. While the differences in morphology and growth under temperature and light conditions are reported I wonder why any statistical means have been used to test the observed differences. For example in Table 3 not even error bars on Chlorophyll readings between replicates (?) have been provided which makes it difficult to interpret the reported results. The lack of appropriate statistical tools appears to be in contrast with the rather sophisticated experimental facility which has been used in this study. The latter is certainly difficult to design, as simply low temperature/highest irradiance (or vice versa) oppose the most challenging to the cyanobacteria under study.

In chapter II a larger number of planktic and nonplanktic taxa collected from two geographic regions has been compared with regard to numerous morphometric parameters. I found this part driven by interesting hypothesis leading to research questions on environmental factors regulating morphological variability. Basically it is argued that smaller cell size relates to more extreme temperature and irradiance as observed under terrestrial (benthic) vs planktic conditions.

In chapter III a larger number of benthic strains of the genus *Nostoc* sp has been analyzed phylogenetically as well as for cyanotoxin production including the toxic microcystin. Perhaps surprisingly, only one strain was found to produce microcystin, implying that the overall frequency of microcystin producers within the section IV cyanobacteria is rather low. Overall this observation fits well to the reports in the literature as only a rather few modern more narrow lineages retained the possibility to produce microcystin such as the genus *Dolichospermum*. Its evolution has been dated two billion years ago probably preceding the monophyletic groups of section IV and V. This hypothesis also can explain why still individual rare genotypes are discovered that produce microcystin such as this single *Nostoc* strain. The interesting but less understood part of the story is whether maintenance of microcystin synthesis to some extent is more directly related to speciation processes, as observed for some lineages, such as the planktonic life form or also a facultative potential symbiotic lifestyle with fungi (lichens).

In chapter IV a putative synthesis pathway for a polyketide of the pederin family is described for a planktonic cyanobacterium *Cuspidothirix issatschenkoi*. While compounds of this structural family are typically found among symbiotic bacteria, this structural variant (named Cusperin) constitutes the first example described from a free-living nonsymbiotic organism respectively a cyanobacterium. The new Cusperin compound has been purified and the chemical structure was determined by 1D and 2D NMR. Genomic sequence information was obtained from single filaments and a PKS/NRPS gene cluster was identified with high similarity to Nosperin described from a symbiotic *Nostoc* strain. As a common problem in this field any direct evidence for the genetic prediction such as through genetic manipulation is lacking. Nevertheless this study is an important contribution to the growing knowledge on secondary metabolite production potential in planktic cyanobacteria beside the well known cyanotoxins.

In chapter V synthesis pathways for another group of secondary metabolites, so called lipopeptides are proposed from five genome-sequenced strains of cyanobacteria of section IV (*Cylindrospermum* and *Anabeana*). Lipopeptides have a cycle of several amino acids linked to fatty acids and have also been shown to be bioactive through interaction with membranes of eukaryotes. The synthesis pathways for two closely related lipopetide groups are compared, namely puwainaphycin and minutissamide. While both pathways seem to share homologous evolution the structural variability is high for both groups. Clearly, the numerous NRPS modules contribute to the peptide variability while two different starter modules introduce variability for the integration of the fatty acid derived substrate of the molecule. While the use of alternative starter modules in NRPS has been described earlier, it is less frequently observed when compared with the ubiquitous genetically based variability in adenylation domains responsible for substrate activation during NRPS.

Questions (for presentation and discussion during the oral defense)

+) in general I have to state that I missed a section on objectives of the study. This is not to decrease the value of the many observations reported in this thesis but rather to support scientific understanding and the generation of scientific hypotheses. Please describe your research objectives and whether they were met.

+) another point to address would be a more detailed description of the individual contributions of the PhD thesis author to the chapters I-V. During the defense it should be possible to demonstrate the relevant scientific competence.

+) One more general idea mention in the introduction section is to compare the nonplanktic with the planktic lifeform resembling an ecostrategy. It is argued that ecological factors (light, temperature) vary more under non-planktic compare with planktic conditions and some data (eg in chapter I) are provided to support this idea.

My question would be on the (hypothethical) evolution of planktic from non-planktic (terrestrial or benthic) form(s): In the view of the PhD candidate, would a suggestive evolution have occurred from planktic or benthic or the other way round?

+) Which consequences could be foreseen if this evolutionary steps from the benthic vs planktic life styles could be better understood? Such as in relation to variability in morphological results, perhaps even in relation to other traits such as secondary metabolites?

Hint: The candidate may think about the more original life form (planktic vs benthic or terrestrial). I would say that the majority of the microfossils points to a non-planktic (benthic or terrestrial lifestyle) as the more abundant and original one (probably for billions of years). Perhaps planktic ecostrategies evolved repeatedly. If this is true and individual planktic genotypes developed from benthic genotypes wouldn't we expect a much reduced ecophysiological variation within the planktic lifestyle (when compared with terrestrial ones)?

+) In chapter III the surprisingly low number of microcystin-producing strains among (free-living) *Nostoc* isolates is reported. Overall I do not doubt that this result is close to reality, however, in the view of the PhD candidate, what could be potential confounding factors to test the hypothesis of reduced microcystin production within benthic cyanobacteria lifestyle?

Hint: The candidate may think of biogeographic factors, maybe linked to phylogeographic influences (not everything grows everywhere) as well as technical biases, such as through cultivation techniques. Were there any attempts to isolate symbiotic Nostoc genotypes?

+) Which other methods could be applied to test the observation of chapter IV more directly?

Hint: Using tandem LC-MS/MS the > 200 structural variants of microcystins can be reliably targeted even in complex matrices such as peptidic extracts from *Nostoc* biofilms in relation to biomass or dry weight. On the other hand due to the high conservation of microcystin synthesis genes potential microcystin producers can be quantified by means of quantitative PCR based methods. These measurements could be compared on a larger geographic scale for planktonic and benthic communities of section IV.

+) How do you explain the discovery of a pederin like compound in a so-called free-living cyanobacterium *Cuspidothrix isatschenkioi*?

What may be the potential functions of these compounds? In my opinion although Pederin and related compounds like Nosperin have been described from symbioses, a potential bioactivity related to chemical defense eg against eukaryotes may also be relevant for the free living lifestyle.

Mondsee, 27.2.2019

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Your ref: Our ref: Date:

18th February 2019

Review of the PhD thesis of Ms Andreja Kust

Dear Prof Vrba,

With great interest, I have read the thesis Ms Andreja Kust has submitted.

Cyanobacteria are nowadays acknowledged as a valuable source for novel secondary metabolites with intriguing bioactivities. Especially cytotoxic compounds are often isolated from these organisms. As these toxic metabolites can potentially be used as lead structures in anticancer drug development or pose a threat to other organisms in the respective habitats, studying their chemistry as well as their distribution is of great interest and value for the drug discovery and ecotoxicology communities.

Ms Kust has contributed to these efforts by characterizing filamentous cyanobacteria of the Nostocales, both on a taxonomic and a natural product chemistry level.

The thesis is a cumulative thesis, based on five manuscripts that have already been accepted and published mostly in international peer-reviewed journals, three of them with Ms Kust as lead author. Her contributions have clearly been stated and justify her position as first author.
After a short introduction into cyanobacteria in general, she gives a solid overview over the current state and recent developments of the taxonomic classification of cyanobacteria, especially of filamentous nitrogen fixing genera. She also discusses the potential of cyanobacteria in natural procuct chemistry and the main biosynthetic pathways (NRPS/PKS), focusing on cyanotoxins and lipopeptides.
She then summarized the most important outcomes of her thesis by shortly describing the main results of her published research, paper by paper. The concise literature reference section is followed by reprints of her publications.

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The thesis is very well written and follows a logical approach. Minor flaws (e.g. missing explanations of abbreviations in figure captions; it would have been nice to include the Supporting Information of the manuscripts) do not significantly compromise the work.

As already stated, Ms Kust presents five published publications, so this major part of her work has already been approved by external reviewers. While not all five manuscripts have been published in higher-ranking journals, this is still adequate for six years PhD studies. Her written thesis as a whole is a well-crafted work.

Thus I recommend to **accept** the thesis and proceed with the oral defense.

Sincerely yours,

Prof. Dr. Timo Niedermeyer