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Review of Loredana Peca's PhD theses entitled "Metal-inducible promoters of *Synechocystis* sp. 6803 and their use for whole-cell bioreporter development"

Cyanobacteria are photosynthetic prokaryotes that include uniquely suitable objects for studying diverse biological phenomena, such as photosynthesis, cell differentiation, thermal adaptation and symbiosis. The survival of their aquatic species is ensured by quick and specific reacting to changes in water chemistry and, through the simple structure and well known genomes of the common laboratory strains, these functional responses can conveniently be studied at the molecular level. Taking advantage of this, the objective of the present Ph.D. work was the identification and characterization of selective transcriptional responses that are elicited by elevated concentrations of metal or semimetal ions. The accumulation of these ions is a frequent result of environmental pollution, which endangers the safety of natural water resources. Accordingly, the topic of the dissertation is timely, quite competitive, and well integrated into the research of the Molecular Stress- and Photobiology Group of the Institute of Plant Biology, BRC HAS. It relies on a wide scale of up to date experimental methods, and successfully connects basic science with its potential practical applications.

The theses are properly edited, with well-proportioned chapters that follow each other in conventional order. A merged 'Results and discussion' chapter proved helpful in avoiding unnecessary redundancies. The text was written in very good English, with remarkably few typing mistakes left overlooked. The addition of the well selected list of abbreviations may prove very helpful for the readers. I think that the 'References' and the listing of the applicant's publications, as integral parts of the dissertation, should have preceded the English and Hungarian summaries, which are only required appendices. The illustrative material is of high quality. Occasionally, for clarity, the bases of relative values, or the units in which the data had been expressed, could have been better defined in the figure legends. Nevertheless, the informative and transparent figures and tables give strong and solid support to the text.

The novel results presented are as follows:

(1) Detailed analysis of the transcriptional responses of nine *Synechocystis* sp. PCC 6803 genes involved, or implicated in metal transport.

(2) Characterization and testing of *Synechocystis* strains with cobaltous/zinc- and nickel/cobaltous-responsive reporter constructs, and the generation of an arsenide/arsenate-responsive bioluminescent construct.

(3) Establishing the inducing conditions for the *slr5038* gene that encodes a putative chromate transporter.

(4) Determination of the ranges of metal ion concentrations that elicit specific transcriptional and oxidative stress responses in *Synechocystis*.

In relation to the results I would like to whether, in addition to studying the expressional regulation, there are specific plans from the side of the research team to pursue practical applications of the metal-inducible reporter constructs they developed.

The value of the scientific results is well reflected by their publishing in the prestigious FEMS Microbiology Letters, with the candidate's first authorship, and with her co-authorship in the Acta Biologica Hungarica (IF: 0.688). The Ph.D. work of Loredana Peca is of high standard, and it is presented in a very finely prepared dissertation. Considering these merits that fulfill the requirements of the doctoral school, I recommend that, in case of a successful defense, she be awarded the PhD degree of the University of Szeged.

Szeged, January 11, 2010

Szekeres Miklós
senior researcher

Review

of the PhD thesis entitled

"Metal inducible promoters of Synechocystis sp. PCC 6803 and their use for whole-cell bioreporter development"

written by Loredana Peca

BRC, Szeged

The thesis describes gene expression-based characterization of metal stress responses in Synechocystis and provides some example how the obtained information can be used for environmental monitoring of metal pollution. The growing concern of the environmental accumulation of toxic compounds, including metals, and the need for economic and accurate methods to assess the associated hazards give the actuality for these investigations.

The thesis is based on two publications with the total impact factor of 2.7 and the applicant is the first author of both of them. These publication parameters fulfill the basic requirements set up by the Biology PhD School of the University of Szeged.

As the formal criteria are considered, the thesis is organized in a traditional way and is written on 101 pages (including the English and Hungarian summaries). The English usage is good; I found only few spelling errors (page 1: "a…ATPases"; "[Tottey et al. 2001)"; page 3: "metal (Szpunar 2005)-dependent" - metal-dependent (Szpunar 2005); page 10: "upstream gene, (Huckle et al. 1993)."; page 12: the sentence "All group D sigma factors …" is repetaed; page 13: Hik33 and Hik 33; Fig 5 legend "bioreporter for based on"; 5.3.1 heading "strai0s"; Fig. 12. title "transcripts"; page 63 "increase ~~increase~~").

My detailed remarks and questions in an order corresponding to the organization of the thesis:

Title:

The thesis can be thematically divided into four parts as 1) the expression of genes implicated in metal ion homeostasis, 2) the functioning of whole cell metal ion biosensors, 3) the characteristics of a chromate transporter operon, and 4) the concentration dependence of specific and non-specific metal ion responses are reported in it. However, the title is covering only the first two points. Furthermore, the title misleadingly suggests that the promoters themselves were the subject of the investigations. However, the thesis reports mainly gene

expression studies. Therefore a correct title would be something like this: "Metal-induced gene expression responses and the development of whole cell bioreporters in Synechocystis"

List of abbreviations:

I found this list rather short and incomplete.

Introduction

This chapter is a summary of Synechocystis operons having role in the maintenance of metal homeostasis. In my view, it is an unnecessary repetition of the corresponding part of the "Research background" chapter. Introduction should rather put the applicant's work into a more general context explaining the reasons why the subject and the approaches were selected and considered to be actual.

Research background:

It is a well organized and well detailed summary of the knowledge on metal regulated gene expression in prokaryotes. It proves that the applicant is an expert of the field.

Few remarks:

Page 1. First the applicant mentions "all organisms possess resistance mechanisms" and than discusses specific responses "These processes are usually regulated by metalloregulatory proteins of either the MerR and ArsR/SmtB families." I do not think that it really concerns all organisms.

Page 12. Heading 2.7 "Synechocystis response to general and metal ion stress" – Not the stress but the response to the stress can be considered as "general", but myself I would rather use even for stress responses the "specific" and "non-specific " classification instead of "specific" and "general". The heading correctly could be "The response of Synechocystis to various stress factors including metal ions"

Page 24. The luminescence equation is inserted into a wrong place and it should be referred in the text.

Aims of the study:

These aims are a bit too specific for me. The general aims of the study should have also been mentioned. Moreover, as there were indications in the Research background for several preliminary results (e.g. gene expression studies, previously generated bioreporters, putative chromate transporter) the aims should have been formulated to emphasize the new aspects to be investigated and the new results to be expected.

Materials and methods:

In general it is sufficiently detailed. The range of the used methods was not very wide, but they were up to date and adequate to reach the objectives. However, I missed the information

considering the number of biological repetitions and the statistical treatment of the data in general (see also later).

Page 27. Definition for IC_{max} and IC_{min} are wrong: IC_{max} is the lowest and not the highest tested concentration where no further growth was observed and IC_{min} is the highest and not the lowest one with any growth effect.

Table 1. (primers) should have been referred in the section 4.3. describing qRT-PC.R

The construction of reporter strains described under section 4.5 is rather a research background as it was carried previously by others and not the applicant. Consequently, it was unnecessary to detail all construction steps in the frame of this thesis; the description of the final setup of the constructs should have been sufficient.

Results and Discussion:

I have several problems with the organization of the chapter and the presentation of the data as detailed below:

Figures and Tables:

- Figures and Tables are frequently not at the proper place, i.e. far from the text discussing their data.
- They are often not referred at the first time of the mention of their data (e.g. Fig. 12) and sometimes they not referred in the order of their appearance (e.g. Fig. 11 is referred before Fig. 10, Fig. 15B before Fig. 15A, Fig. 27 before Fig 23.).
- Instead of Fig. 29E, Fig12C should have been referred (page 40).
- arsC expression is shown on Fig.13A, but it is not even mentioned in the text.
- On Fig. 14. CPS is not defined, it's A and B panels are not referred in the legend.

Statistical evaluation of the data is poor in general or at least poorly presented. There are several figures where neither the number of repetitions nor the meaning of the bars are given in the legend. In many figures only single values are shown with no indication of any repetitions. On Fig. 16 and Fig 29 means of two experiments are shown with error bars: in such case it is better to show both data, but to use at least three repetions is more advisable.

Section 5.1. "Effect of metal ions on cell growth" – As it is demonstrated on Fig. 8, the three selected concentrations of the metal ions were insufficient to correctly determine the IC values (e.g. in Table 2 values as larger than X, or smaller than Y). Due to this uncertainity, in the gene expression studies concentrations were used for which no growth inhibition data were determined. In my opinion it would have been advisable to carry out a more refined, statistically supported, growth inhibition experiment based on the preliminary data of Fig. 8,

and correctly determine e.g. an IC_{50} value and to use these concentrations throughout the experiments. In this case the response to the various metal ions could have been better compared. I think the presented data do not allow such comparison although that was made by the applicant on page 35 using the inexact IC_{max} and IC_{min} values: as an example the applicant states the IC ratio is 4-fold for copper as IC_{min} was claimed as 1.25 μM and IC_{max} as 5 μM. However, due to the few data points, it can not be excluded that IC_{min} is 1.5 and IC_{max} is 3, and than the ratio is only 2.

Section 5.2.1. Page 36. Four growth stages are mentioned in the text but data are shown only for three (Fig. 9). It is claimed that RNA samples were collected from four stages of the exponential phase: is it correct? Not different growth phases should have been compared? It would have been helpful to show a growth curve any way.

Sections 5.2.2. and 5.2.2.1: the results of the experiments with one and several concentrations of metal ions are unclearly referred in the text. In section 5.2.2 one selected concentration is mentioned to be selected for qRT-PCR measurements, but subsequently section 5.2.2.1. starts with a sentence that the ziaA gene responded to several Zn concentrations which is followed by a reference to Fig. 11 that shows data in response of single concentrations while Fig. 12 with the concentration-dependent data is referred only later.

It is mentioned in the text that the obtained results in some cases are contradictory to results described in the literature considering the induction of some of the genes by metal ions (e.g. ziaA and coaT), but no possible reasons are discussed. What could be the reasons for these contradictions (method, strain, condition)?

Page 40. The 5.2.2.2 subsection is devoted to the *nrs* operon in its title but also discusses O/P *coaT* inducibility.

Section 5.3.1. It is not clear how many bioreporter strains were made previously in the laboratory and by the applicant herself: page 28 "two reporter strains were previously generated in our laboratory"; page 43 "Three reporter strains were previously constructed"; Fig-s 6 and 7 show four (*coa, nrs, ars, zia*) reporter constructs.

If the reporters were constructed previously it means that they were constructed before the gene expression studies were made? If yes, it needs some explanation.

Page 45. The bioluminescence studies were also carried out in darkness and the response was shifted to higher concentrations. How darkness affects the metal-inducibility of the corresponding and the other investigated genes? Was it investigated? If not, why?

Section. 5.4. Fig. 28 Why the artCT gene expression is less induced by As^{3+} in the absence of the artR repressor?

In Section 5.5. the applicant investigated the metal-induced expression of genes that have been shown to respond to other stresses, especially to oxidative stress. In my opinion this section could have been amalgamated with section 5.2 forming a common chapter of metal-induced gene expression studies.

My questions regarding to these experiments aiming to determine metal concentrations evoking non-specific or secondary responses such as ROS generation:

Why gene expression was used as a stress marker? Direct measurement of oxidative stress (e.g. ROS generation, enzymatic defense reactions, etc.) could not have been more straightforward? Or these measurements are not possible in Synechocystis?

The selected genes respond to oxidative stress in a wide range by 4- to 130-fold induction. Does the 2-fold induction threshold selected for all genes means the same response?

The selected genes responded differentially to the metal stress (Fig. 29): does not it mean that they are not appropriate reporters of the general, non-specific responses they were selected for? In my view, the link between metal and oxidative stress responses can not be properly established based on the reported gene expression data. Again: direct measurement of oxidative stress would have been more adequate to establish this link.

It seems from the gene expression data that genes coding for chaperons (hspA and dnaJ) gave the more uniform response to all treatments therefore protein denaturation might rather be a more general secondary response to high metal concentrations that needs further attention.

On Fig. 29 it can also be well recognized that many investigated genes were actually down regulated at high metal concentrations. What could be the reason?

Conclusions:

Tables in this section should have also been numbered and referred in the text by numbers.

Table on page 66. Non-specific response should have been used instead of general and oxidative stress response.

Why it is stated that no stress occurred below the highest tested Co concentration (32 μM) if hspA was induced more than 2-fold already at 2 μM and hspA expression was selected as a stress marker?

Acknowledgements:

Fig. 33 is referred here but there are only 29 figures in the thesis.

The English and Hungarian summaries are correct, well organized and well written.
It would have been better if they were after and not before the references which correspond only to the main text and not to the summaries.

Summaryzing:

The requirements to obtain the PhD degree are met by the applicant. The thesis is well written but the organization and presentation of the data could be better and some aspects could have been investigated in more details to better support the conclusions. Most of the scientific results presented in the thesis have already been published in international journals and therefore are rather solid. I consider the following results as the major contributions of the applicant to the progression of the research field:

The applicant,

- provided comprehensive data on metal-induced gene expression in Synechocystis
- demonstrated the potential of genetically engineered Synechocystis strains as whole cell bioreporters to monitor nickel, cobalt and zinc accumulation in the environment
- developed a cyanobacterial whole cell reporter for arsenic salts
- described the regulation of a putative chromate/sulfate transporter operon in Synechocystis

Based on these achievements, the publication parameters and the quality of the written thesis, I recommend that following a successful defense the applicant will be awarded by the PhD degree.

Prof. Attila Fehér PhD

BRC, Szeged, 31.01.2010