MC Yeast: Stress-based detection and enzymatic degradation of the cyanobacterial toxin microcystin

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Abstract

Cyanobacteria, also known as blue-green algae, are an annual problem in many water systems worldwide. During the summer, the cyanobacteria release hepatotoxins called microcystins which pose health risks to humans and animals. Our goal was to build a two-part system to detect and then degrade these toxins.

We based our detection system on the natural oxidative stress response of the yeast Saccharomyces cerevisiae. Exposure to microcystins is linked to higher levels of oxidative stress, and we coupled the oxidative stress response to the expression of yellow fluorescent protein. Thus, fluorescence levels are expected to indicate the amount of microcystins present in a sample. To understand and validate our toxin detection mechanism, we also created mathematical and molecular models. To degrade the detected toxins, we expressed the enzyme microcystinase, which is naturally found in some gram-negative bacteria. The enzyme renders microcystins 160 times less toxic by linearizing their structure.

Our results indicated that coupling promoter regions of genes involved in oxidative stress to reporters molecules could be used to measure the amount of stress factors in yeast cells. We were able to show that the promoters we chose are a part of the yeast's rapid stress response, which means they were a good choice for experiments such as this. In addition, we demonstrated that active microcystinase can be produced in Saccharomyces cerevisiae.

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Competing Interests

The authors have declared that no competing interests exist.

Ethics Statement

N/A

Data Availability

(http://www.protocols.io/groups/aaltohelsinki-2016)

All data are fully available without restriction.
More detailed descriptions of experiments, results and conclusions can be found at our iGEM wiki page: http://2016.igem.org/Team:Aalto-Helsinki (http://2016.igem.org/Team:Aalto-Helsinki).
Protocols are available at www.protocols.io/groups/aaltohelsinki-2016 (http://www.protocols.io/groups/aaltohelsinki-2016)

Introduction

Cyanobacteria, also known as blue green algae, produce toxins that pose serious health risks to humans and animals worldwide. The most common class of cyanotoxins is the microcystins (MCs), which are potentially lethal for liver cells. Microcystins are cyclic peptides that consist of seven amino acids. They contain a unique β-amino acid Adda, and two of the seven amino acids vary between different variants of microcystin. Due to the variation in these two amino acids, more than a hundred different variants of microcystin are known. The most common variant is microcystin-LR (MC-LR), where the variable amino acids are leucine and arginine. It is also the most potent of all microcystins [1].

Comments

PLOSPatrick: Are cyanobacteria very prevalent in the environment. How would a human or animal be most likely to come across them?
snehadri: They are abundant in lake waters, especially if there has been eutrophication. Humans and animals can be exposed to them if they are using lake water for any purpose - be it for drinking or for recreational purposes.
Laura_Dress: Very interesting! I didn't know that. Would be cool to see some details like that added to the manuscript...if not already below...still making my way through :)
snehadri: Here is a link to a video on our project, which shows how cyanobacteria pose a problem in Finland: https://www.youtube.com/watch?v=1jT9AFI26oE&t=7s
PLOSPatrick: That's a brilliant video! It's very accessible to a non-scientific audience but also gives good information about the project. I'd definitely recommend including a link to this when you revise your work for submission.
teague: Do different cyanobacteria produce different microcystins? Do some species pose more of a problem than others?
snehadri: Yes, there are more than a hundred known variants of microcystin. The variant that has been the focus of our study, microcystin-LR, is most commonly found one in lake waters.

Currently, there are no inexpensive or convenient methods available to detect cyanotoxins in waters, and furthermore, no methods at all for getting rid of these toxins. This project explored the possibility of developing a system capable of carrying out both functions in an efficient and affordable manner. The yeast *Saccharomyces cerevisiae* was engineered to be able to detect and degrade the most common microcystin, MC-LR.

Comments

teague: Some more information would be nice to support your assertion that "there are no inexpensive or convenient methods...". What is the state of the art? What are the current methods, and what are their drawbacks? That can help make your proposed advance more concrete.
snehadri: Thanks for bringing this to our attention, we'll elaborate on this.
snehadri: A month or so after developing our idea, we heard about a paper-based device developed by the VTT Technical Research Centre of Finland and University of Turku which could detect cyanotoxins in 15 minutes. But we did not feel completely dejected because we also had the degradation part. Moreover, their product is yet to be commercialized so the value can only be estimated.
snehadri: More info here: http://www.vttresearch.com/media/news/biodegradable-quick-test-reveals-blue-green-algae-toxins-in-swimming-water

When MC enters mammalian liver cells, it inhibits protein phosphatases 1 and 2a (PP1 and PP2a). This results in the hyperphosphorylation of target proteins, which in turn leads to the formation of reactive oxygen species and finally to an oxidative stress response [2].

Comments

teague: What kind of organism-wide results are there? What disease or symptoms or illness do MCs cause in people?
snehadri: I suppose by this point it's clear that we need to include more context in the article, as is provided here: https://www.youtube.com/watch?v=1jT9AFI26oE
teague: Yes, in general some more context would be useful. It helps the readers recognize the impact of your work! (-:
snehadri: MC-LR mainly targets the liver, but studies have also shown it to affect the gut microflora and cause cancer of the testes in rodents.

Two important transcription factors activated by oxidative stress are Skn7p and Yap1p. They have specific binding sites in the promoter areas of genes that respond to oxidative stress. Skn7p binds to OSRE (oxidative stress response element) and Yap1p binds to YRE (Yap1p response element). Some of the best characterized promoter regions that these transcription factors bind to are the promoters for the genes *TSA1* (thioredoxin peroxidase) and *CCP1* (mitochondrial cytochrome-c peroxidase) [3]. To construct a sensor for oxidative stress caused by microcystin, we fused these promoter regions with the protein coding sequence of the yellow fluorescent protein Venus.

Comments

devang: It is unclear whether you're still discussing the mammalian oxidative stress response in this paragraph, or whether Skn7p and Yap1p are yeast TFs. Or if they are present across eukaryotes.
teague: +1 for @devang (/user/devang)'s comment. I recognize them as yeast because I'm a yeast person. Also, please consider citing previous work on synthetic Skn7-responsive promoters: http://www.nature.com/nbt/journal/v23/n12/full/nbt1162.html
snehadri: Thanks for the reference. We'll have a look at it and see if it can be incorporated in our report.
teague: (full disclosure: I work in the lab that published that manuscript.)
snehadri: Yes, like @teague (/user/teague) said those are of yeast. Thank for pointing it out, we have made the distinction in our final version.

An important prerequisite for the function of these stress promoters is that the toxin must be imported into the cell. MC-LR is unable to pass the cell membrane without the help of specific transporter proteins. In human liver cells, the membrane bound protein OATP (organic anion transport peptide) has been identified to import MCs into the cell. A previous study found that the QDR2 transporter from *S. cerevisiae* strain VL3 had the most similarity to OATP out of all *S. cerevisiae* proteins, and confirmed that strain VL3 was thus

capable of microcystin import [4]. To enable the induction of the oxidative stress promoters with microcystin, we replaced the original QDR2 transporter of our working *S. cerevisiae* strain (SS328-leu) with the VL3 variant of the QDR2 transporter. This was presumed to allow toxin import in the new strain, and thus enable the function of our yeast cells as a sensor.

Microcystinase (MlrA) belongs to a set of enzymes that degrade microcystins and was originally found in some Gram-negative bacteria. MlrA hydrolyzes the peptide bond between the Adda and arginine amino acids in microcystin-LR (MC-LR), thus linearizing the toxin. [5] This linearization renders MC-LR 160 times less toxic [6]. A previous study has indicated that MlrA might be a membrane protein [7]. In addition, we created a homology model of MlrA that further supported this theory [8]. However, MlrA has previously been produced in an active, soluble form in *Escherichia coli* [9]. We produced MlrA in *S. cerevisiae* to further investigate the potential of heterologously produced microcystinase to be used for degrading microcystin. Additionally, our goal was to find further information on whether MlrA truly was a membrane protein.

Comments

devang: I suggest concluding the Introduction by describing how your solution might work in practice. Are you envisioning releasing your biosensor cells into entire lakes? How would you obtain efficient depletion of microcystinase in such environments when the cyanobacteria that produce them are still able to survive and continue to release them.
snehadri: Thanks for your comment. Considering the ethical implications, our idea was for the enzyme to be used in a bucket of water and not for release into the open lake. As for the cyanobacteria, much of their presence is caused by eutrophication and that has to be addressed by other means. By not releasing the enzyme into the lakes we also delay any possible evolutionary changes in the microcystin structure to bypass the action of the enzyme.
devang: Thanks for replying. It is still unclear to me what utility degrading microcystins in a bucket might have. Are current best-practice water purification systems unable to decontaminate microcystins?
snehadri: At the time of the conception of our project, there were no solutions for the degradation and as for the detection, there were some solutions but quite expensive. Our "enzyme capsule" would be accessible to the end-user and also be cost-effective, so to speak, since we are just using genetically-engineered yeast cells. The context of our project has been explained in this video: https://www.youtube.com/watch?v=1jT9AFI26oE (Perhaps including some more context in the introduction will help global readers).
devang: Thanks, the video does clarify things.
teague: +1. I am a fan of being very concrete about the way your work is intended to be used. I'm a little unclear (even with the video) -- are you proposing that the capsule contain bioengineered yeast? Or some purified form of the MlrA enzyme produced with your yeast?
teague: Also, I'm intrigued by your statement about evolutionary pressure (in the comment above.) What evolutionary role does microcystein have? Is that known?
snehadri: Sorry for the confusion. The capsule would contain the purified enzyme, since that is the essential factor in the degradation. The part about the evolutionary pressure was just a thought I had, based on some basic research. This needs further research into several questions, such as does microcystin affect bacteria and if not, why do some bacteria even produce the microcystinase enzyme? I think that could give some insight about evolutionary processes at hand, if any. I was thinking of this might parallel antibiotic resistance but not sure if it's a fair analogy.
teague: Okay. I think a discussion of the evolutionary role is interesting, but not necessary.

Materials and Methods

Basic Methodology

Detailed protocols for experimental work can be found at [10]. A summary of the main experimental methods can be found below. Unless otherwise mentioned, *S. cerevisiae* was grown at 30 °C, and *E. coli* at 37 °C, with orbital shaking between 220-250 rpm. *S. cerevisiae* strains containing plasmids were grown in SD medium containing all amino acids except leucine, which was used for auxotrophic selection. *S. cerevisiae* strains with no plasmids were grown in YPD medium. *E. coli* with plasmids was grown in antibiotic-supplemented LB medium.

Plasmids and Strains

Escherichia coli strain TOP10 was used for cloning work, and strain BL21(DE3) for protein expression in *E. coli*. For work with *Saccharomyces cerevisiae*, strain SS328-leu was used. Protein expressed in *E. coli* was expressed in the pET28a plasmid; for *S. cerevisiae,* pRS415 was used for plasmid expression. pUG6 was used as a shuttle vector for integrating genes into the *S. cerevisiae* genome. MlrA enzyme genes were synthesized and cloned into target vectors by GeneArt; all other genes were synthesized by IDT and cloned into target vectors either with Gibson assembly, yeast recombination cloning, or restriction enzyme cloning.

Comments

devang: Please rephrase: "for S. cerevisiae, pRS415 was used for plasmid expression". Please cite BioBricks produced and/or sequence accessions for each gene and promoter element used.
snehadri: Ok, we will put that information in our final version. Another place to mention our BioBricks :)

Fluorescence Assays

Fluorescence expression measurements were performed using a microplate reader or flow cytometer.

For measurements with the microplate reader, the experiment was conducted as follows: overnight liquid cultures of tested strains were diluted to OD600 0.2 and the diluted cultures were grown to OD600 0.5. From these cultures, cell culture was added to wells of a 96-well plate along with inducing hydrogen peroxide so that a final volume of 100 uL and desired hydrogen peroxide concentration was reached. These cultures were grown on the 96-well plate, sealed with an optically clear adhesive film, with linear shaking. The microplate reader recorded OD600 and fluorescence at five minute intervals, with the excitation wavelength set at 502 nm and emission wavelength set at 528 nm. The growth medium was used as the blank for fluorescence and OD600 values. Blanked fluorescence was plotted either as a function of time or as a function of blanked OD600.

Comments

devang: "desired hydrogen peroxide concentration"-please be more specific.
lauralaiho: We will clarify these.

Fluorescence measurements with flow cytometry were performed as follows: overnight cultures of tested strains were diluted to OD600 of 0.2 and then grown to OD600 of 0.5. Induction samples were prepared by combining culture with hydrogen peroxide or microcystin so that the desired concentration of either hydrogen peroxide or microcystin and a final volume of 1.5 mL/2.2 mL was reached. These cultures were grown under normal conditions for two hours, after which final 1 mL samples for flow cytometry were prepared

into PBS. For hydrogen peroxide samples, this was done by diluting the culture directly into PBS to reach an approximate final OD600 of 0.1, corresponding to a dilution of 1:5 - 1:10. For microcystin samples, cells were spun down and re-suspended in PBS, likewise to a final approximate OD600 of 0.1. The following flow cytometer settings were used for the measurement; FSC 43, SSC 311, FITC 392, 10000 events per measurement.

Comments

teague: Please indicate the manufacturer and model of the flow cytometer; and the bandpass filter used in the FITC channel. Also, I assume the "settings" you refer to are PMT voltages?
Pihla: Yes, that's correct, clarifying this!

Catalase Activity Assay

Relative catalase activity levels of cells were obtained as follows: overnight cultures of the tested strains were re-suspended into 2X PBS and diluted to a defined OD600 greater than 1.5. The cell solution was combined with an equal volume of microcystin solution to achieve a final volume of 1 mL, with different microcystin dilutions prepared into water. These mixtures were incubated for one or four hours. After the incubation, 0.5 mL of 30 % H_2O_2 and 0.2 mL of 1% Triton X-100 were added to the mixtures, resulting in the formation of a foam layer. The foam layer was measured after five minutes. As catalase is responsible for degrading the hydrogen peroxide into oxygen and water, the foam layer can be assumed to be relative to the catalase activity level of the cell. Thus, the relative foam thickness (height divided by width) was used as a measure of relative catalase activity.

Comments

teague: So the previous assays (fluorescence, etc) are pretty standard. This one is much more novel. If you developed it yourselves, how did you validate it? If you found it elsewhere in the literature, please cite?
snehadri: We based our catalase assay on this paper: Iwase T, Tajima A, Sugimoto S, Okuda KI, Hironaka I, Kamata Y, et al. A simple assay for measuring catalase activity: a visual approach. Sci. Rep. 2013;3:3081.

Molecular and Mathematical Models

To validate the concept behind our detection mechanism and to improve the understanding thereof, two simplified molecular models representing the activation processes of the transcription factors Yap1p/Skn7p and Msn2/4p were created based on a review of the available literature.

The Yap1p/Skn7p model was then simulated using the COPASI software. In lieu of using experimental data, various ranges of reasonable parameter values were analyzed.

Protein Localization Analysis

To determine the localization of the expressed MlrA, the following analysis was conducted:

MlrA was expressed under the GAL1 promoter with galactose induction, and the cells were harvested and collected. The growth medium was concentrated and a sample was taken from it. This sample was expected to contain any secreted enzymes.

Comments

devang: Insufficient detail here. Amounts of galactose used, growth parameters, lysing protocols etc. are all important details essential for reproducibility. Alternatively, you could cite the papers from which the protocols were adapted.
snehadri: Thanks for the feedback, I agree that more detail is required here even if the link to the full protocols is included in the report.

The cells were lysed and centrifuged. The supernatant fraction contained the soluble proteins as well as the inner membranes of the yeast cells, and the pellet contained inclusion bodies and cell debris. The inner membranes were purified and a sample was taken from this, as well as from the rest of the supernatant fraction, which was expected to contain only the soluble proteins.

To assess whether the protein would be in the cell wall or plasma membranes, the pellet fraction after cell lysis was renatured to recover possible inclusion bodies in a soluble form. Samples from the renatured proteins and the rest of the pellet were taken.

All the samples were analysed with Western blot to visualize the fraction where the enzyme localized.

Comments

devang: Please cite antibody used etc.
snehadri: Thanks for pointing it out. We should have of course mentioned it here. It was an anti-6xHis antibody - more details in the final version!
VeroniqueKiermer: Indeed, good idea to list all antibodies -- and when doing so please list vendor, catalog number and lot number -- also indicate what dilutions were used for the WB. There is so much variation between antibodies that it is critical for others to reproduce
snehadri: You're of course right. We've now tried to provide as much detail as possible now in the final version :)

Enzyme Activity Assay

To measure the activity of the expressed MlrA, reaction mixtures containing the enzyme diluted to 1:10, 1:100 or 1:1000 with PBS. Each mixture contained 0.1% BSA as blocking agent and 1 µg/L microcystin-LR. The amount of MC-LR corresponds to the safety limit set by World Health Organization [11], and has been used in previous experiments [9,12].

The reaction mixtures were incubated at 37 °C with shaking at 180 rpm, and samples of 100 µl were taken at certain time points. The enzyme was immediately deactivated with the addition of 100 µl methanol after sampling. The samples were analysed with LC-MS to monitor changes in the relative amounts of the toxin over time. The relative abundance of MC-LR was plotted against time to visualize the gradual degradation of the toxin.

Comments

teague: I understand that the detailed protocols are elsewhere, but you're missing a few key details here necessary to interpret your results: what kind of column are you using? (C18? Amino? Something else?) What kind of mobile phase? (Acetonitrile? Methanol?) What MS instrument, and in what mode?
snehadri: These details have been added on the final version. Thanks for stressing on this point.

The amount of MC-LR in the prepared extract could only be estimated, so no quantitative analysis of the reaction could be done. Additionally, because of its high affinity to plastic, MC-LR had to be handled with glassware, such as Pasteur pipettes, which do not give accurate measurements. All the same, as the goal was to confirm whether the enzyme was active, working with exact amounts was not crucial.

Results and Discussion

Fluorescence induction with hydrogen peroxide

Yeast strains were transformed with vectors containing the Venus YFP gene under CCP1 and TSA1 promoters. An additional strain utilizing the constitutive GPD1 promoter to express YFP was created as a control. To test the expression of fluorescence under oxidative stress, cultures of the different strains were grown with different concentrations of hydrogen peroxide. The produced fluorescence was measured over time with a microplate reader (Figure 1). As seen in Figure 1A, CCP1 seems to produce greater fluorescent responses over time with higher induction concentrations. As it was noted that cells grew significantly slower in higher hydrogen peroxide concentrations [13], fluorescence had to be considered also relative to cell density. The general trend seen in 1A can be observed when normalizing fluorescence to cell density (Figure 1B), but the progressive increase in fluorescence with increasing induction concentrations is made even clearer. Meanwhile, the TSA1 promoter doesn't seem to respond to hydrogen peroxide with a clear fluorescent signal (Figure 1C), but plotting fluorescence against cell density reveals that when the effect of hydrogen peroxide on cell density is considered, the promoters seem to express slightly more fluorescence in the presence of hydrogen peroxide, although different concentrations don't have observable differences. The base level of expression for TSA1 is high, however.

Comments

teague: Please start each "result" in the results section with a statement motivating the experiment. Something like "in order to test our synthetic promoters' response to oxidative stress, we...."

snehadri: Will do!

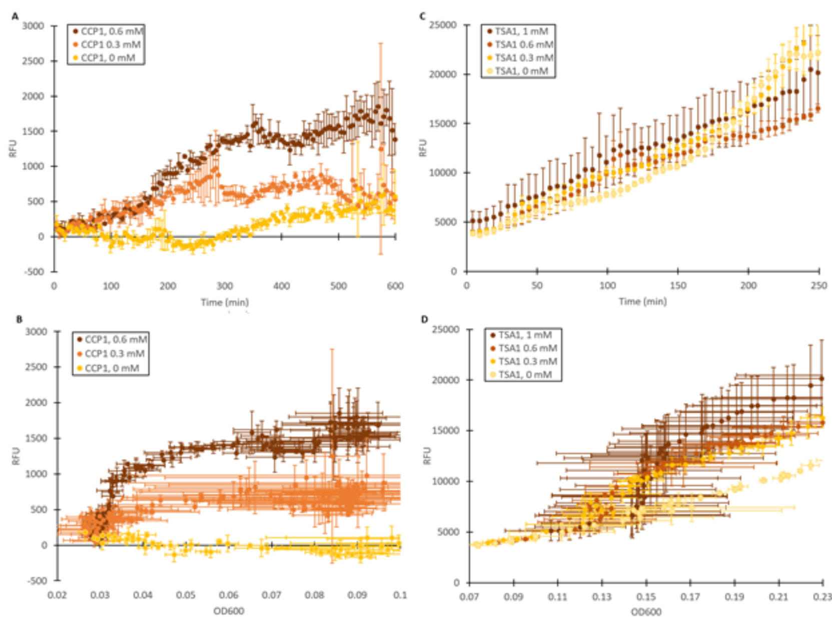

Figure 1. Fluorescence measurements with a microplate reader. (A) Fluorescence produced under the CCP1 promoter as a function of time, n=3. (B) Fluorescence produced under the CCP1 promoter as a function of OD600, n=3. (C) Fluorescence produced under the TSA1 promoter as a function of time, n=3. (D) Fluorescence produced under the TSA1 promoter as a function of OD600, n=3.

Comments

teague: Please indicate how you converted 3 measurements (n=3) to one point on the plot, and what the error bars indicate. (mean and SEM? something else?)

teague: PS - the data in 1B is very pretty. (-:

Pihla: Averages of 3 measurements were used, with error bars being standard deviation, thank you for asking for specification!

Additional verification of stress induction was obtained by measuring fluorescence with a flow cytometer (Figure 2). As seen in figure 2A, absolute fluorescence values produced under GPD1 and TSA1 promoters are significantly higher than those produced under the CCP1 promoter. The CCP1 promoter seems to have a very low base level of expression. Notably, the fluorescence seems to increase with hydrogen peroxide induction also in the control (GPD1 promoter). An explanation for this could be that since hydrogen peroxide slows culture growth, a relatively larger portion of the cells in the culture are older, meaning that they have accumulated more fluorescence. Because a defined number of cells are measured to obtain the fluorescence distribution, slower culture growth can show up as such a bias.

To account for this, fluorescence values were divided by the respective uninduced values of each promoter (Figure 2B). This shows that the relative increase in GPD1 fluorescence in higher hydrogen peroxide concentrations is significantly lower than for the stress promoters. Using this as a control for the stress promoters, it seems that both stress promoters increase the expression of fluorescence in response to hydrogen peroxide, although for the CCP1 promoter, a control promoter with a lower, comparable expression level would be more accurate. Such a control would also help account for the fact that the relative fluorescences are more easily affected by small biases when the fluorescence levels are lower. Although additional repeats would be needed to further validate these results and better characterize the promoters' responses to different induction concentrations, the fold change between uninduced and induced promoters (2B) gives strong indication that the tested stress promoters are working in principle. An important additional note is that although our tested CCP1 promoter clearly worked, its sequence did contain a single base mutation. Whether this difference has a positive or negative effect on the fluorescence would have to be confirmed by obtaining the correct promoter sequence.

Comments

teague: I'm having difficulty wrapping my head around this. You are right that, since your GPD1 fluorescence also increases due to oxidative stress, you need to normalize your measurements to something -- but is the uninduced culture the right choice? That culture is growing faster and doesn't represent, in my mind, a particularly good biological normalization.
teague: I would be interested to see what happens when you normalize the TSA1 and CCP1 results to GPD1. Then, you're dividing by a measurement that you don't expect to be directly affected by stress (and Skn7, etc), thus normalizing for stress effects on cell size, age, etc.
teague: The other thing you might consider is that stress affects *a lot* of the yeast genome -- something like 30% of genes are differentially regulated by the generalized stress response. Have a look at Audrey Gasch's work for more details. It is at least possible that the GPD1 promoter *is* responding to stress after all!
Pihla: Thank you for the insightful comments! It's true that the differences in growth speed don't make the uninduced culture an ideal normalization. We also discussed briefly the kind of bias this might cause. However, we thought that this normalization is still valid on the level of individual cells, if you acknowledge the possible sources of bias. Normalizing the TSA1 and CCP1 results to GPD1 is a good idea, but trying that out, the results in fact looked essentially the same as figure 2A – the difference being that the increase in fluorescence with increasing concentrations is slightly smaller. Thus, it doesn't give much information not already contained in fig2A and fig2B - fig2B and normalization to uninduced shows a similar result as normalization to GPD1 (that TSA1 and CCP1 react considerably more strongly to H_2O_2 concentration than GPD1). However, normalization to uninduced gives an idea of the fold change in fluorescence, unlike normalization to GPD1. As for GPD1 possibly responding to stress, that is a good insight – we didn't find information of it being regulated by oxidative stress specifically, but it is affected by osmotic stress. It might of course still be affected by other types of stress too. A very good point to acknowledge, thank you once again!

Figure 2. Fluorescence measurements with flow cytometry after 2 h hydrogen peroxide induction.
(A) Median fluorescence produced under different promoters as a function of hydrogen peroxide induction concentration, n=1. (B) Median fluorescence produced under different promoters normalized to uninduced median fluorescence values, as a function of hydrogen peroxide induction concentration, n=1.

Catalase activity measurement to verify microcystin import

The microcystin-importing QDR2 transporter from *S. cerevisiae* strain VL3 was integrated into the genome of *S. cerevisiae* strain SS328-leu. Successful integration was confirmed with antibiotic selection and PCR. Transporter functionality and microcystin import was assessed by measuring the relative catalase activity levels of different strains (Figure 3). The catalase activity response to the different microcystin concentrations in the microcystin-importing strain VL3 is observed to be similar to that reported previously by [4]. For the unmodified *S. cerevisiae* strain SS328-leu, observed catalase activities are low and show no response to microcystin. Implementation of the VL3 QDR2 transporter in SS328-leu was expected to create a catalase activity response corresponding to that observed in VL3. However, although a clear phenotype change is observed as increased catalase activity base levels, the expected response to microcystin did not occur. Because more replicates would be needed to gain further verification and more accuracy on the different strains' response to microcystin, the possibility of the phenotype change indicating microcystin transporter functionality was not ruled out.

Figure 3. Relative catalase activity of yeast strains in response to different microcystin concentrations, n=1.

Fluorescence induction with microcystin

Vectors containing Venus YFP constructs were transformed into the *S. cerevisiae* SS328-leu strain with the integrated QDR2 transporter. These strains were incubated with different concentrations of microcystin. Strains with the unmodified transporter were used as controls. Two strains containing the GPD1 promoter with and without the microcystin transporter served as a control for changes between transporter variants for a promoter unaffected by microcystin. The produced fluorescence was measured with flow cytometry (Figure 4).

Figure 4. Fluorescence measurements with flow cytometry after 2h microcystin induction.
(A) Median fluorescence produced under different promoters as a function of microcystin induction concentration, n=1. (B) Median fluorescence produced under different promoters normalized to uninduced median fluorescence values, as a function of microcystin induction concentration, n=1.

Comments

teague: Please plot these using a log-scaled X axis! (-:
Pihla: Good idea, thank you for the suggestion!

As seen in Figure 4A, none of the strains seems to show a significant fluorescent response to microcystin. Normalizing the fluorescence values to uninduced concentrations further confirms that none of the produced fluorescence changes are significantly different from the controls. There are two possible explanations for this; either microcystin doesn't induce the promoters as hydrogen peroxide does, or microcystin is not being imported into the cell. Faulty transporter functionality seems the more plausible explanation overall. Despite the phenotype change after integrating the microcystin transporter, the previous results showed no indication of microcystin producing the expected response inside the cell, thus indicating that microcystin is not imported.

Comments

teague: I have a knee-jerk reaction to the word "significant" -- I'm expecting a statistical test and a p value. You may consider using a different word, or I may be alone in my response to it.
snehadri: I'll agree that we could do better with the word choice

It is possible that as a strep-tag was incorporated on the C-terminus of the transporter, the tag interferes either with the correct expression and localization of the transporter, or with its function. Additionally, the obtained amino acid sequence for QDR2 contained one unclear amino acid, which was either a leucine or isoleucine. For the tested construct, isoleucine was chosen for this position, but it is possible that the original transporter contains a leucine instead. Furthermore, the observed phenotype change might not require the functionality of the new transporter, as long as the original transporter is removed. As the function of the VL3 QDR2 transporter is largely unknown, it is possible that it does not have the hydrogen peroxide antiporter functionality that the normal yeast QDR2 transporter has (SGD ID: S000001383). As a result, removal of the original transporter would allow hydrogen peroxide accumulation in the cell, leading to an increased catalase activity base level, regardless of the functionality of the integrated VL3 transporter. This would explain the phenotype change in absence of a functional microcystin importer.

Detection System Simulations

Molecular models were created for the activation processes of Yap1p/Skn7p and Msn2/4p. A graphical representation of these is presented in Figures 12 and 13.

Comments

teague: What *kind* of models are these? Flux-balance analysis? Something else?
Nidia_Obscura: These were kinetic models and the diagrams we showed below were the regulatory networks of Yap1p/Skn7p and Msn2p/Msn4p

Figure 5 presents the activation process of Yap1p/Skn7p. During stress, Gpx3p oxidizes Yap1p, which is therefore localized into the nucleus [14]. In the nucleus, the oxidized Yap1p forms a weak bond with Skn7p, which is turned into a strong bond by an unknown kinase [15]. The Yap1p/Skn7p complex then binds to the promoter regions of genes involved in the cell's oxidative stress response.

Figure 6 shows the process of activation for Msn2/4p. Hydrogen peroxide formed during oxidative stress causes thioredoxin to oxidize [16]. The oxidized thioredoxin then inhibits the function of a kinase that normally prevents the nuclear import of Msn2/4p, which causes Msn2/4p to localize into the nucleus [14]. The nuclear Msn2/4p then binds to the promoter regions of OSR genes.

Simulation results from our COPASI simulation indicate that Yap1p and Skn7p are involved in the cell's immediate response to stress, and are therefore suitable for use in a detection mechanism. In Figure 7 below, we show the theoretical concentration of active YFP-coding genes in the cell as a function of time. In addition, our model supported the mechanism suggested by [17] where YAP1P degradation is driven by the activity of NOT4 inside the nucleus. More detailed information regarding the modelling results can be found at [18].

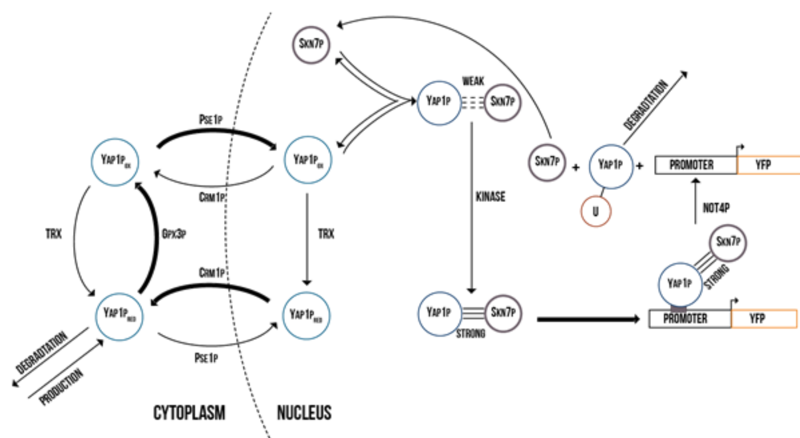

Figure 5. The molecular model of the activation of Yap1p and Skn7p during oxidative stress.
Gpx3p = thiol peroxidase, Crm1p = karyopherin for Yap1p export from nucleus, Pse1p = karyopherin for Yap1p import into nucleus, Trx = thioredoxin, YFP = yellow fluorescent protein.

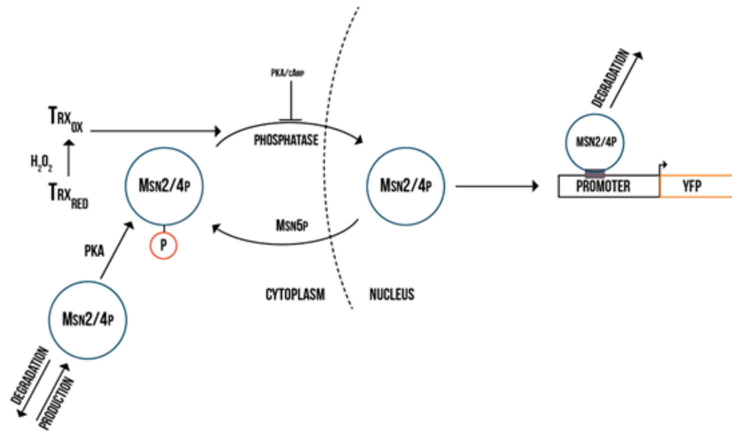

Figure 6. The molecular model of Msn2/4p activation during the oxidative stress. PKA = protein kinase A, P = phosphate, Msn5p = karyopherin involved in exporting Msn2/4p from nucleus, TRX = thioredoxin, cAMP = cyclic AMP.

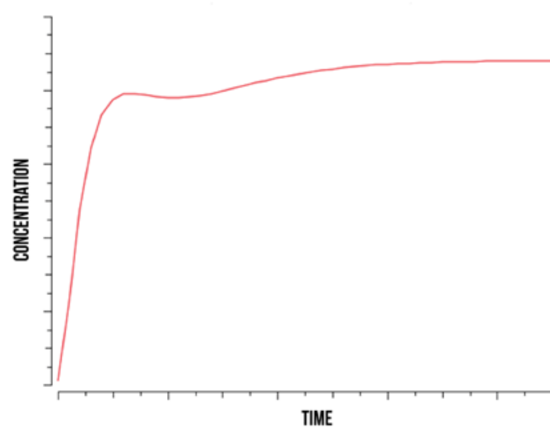

Figure 7. At higher kinase activity rates, the active gene quantity experiences a faster initial slope and subsequent immediate decrease, followed by an increase and stabilization.

Expression of microcystinase in *S. cerevisiae*

We produced and analyzed two variants of microcystinase (MlrA) to find the best way to express it in *S. cerevisiae*. The coding sequence for MlrA from *Sphingomonas* sp. ACM-3962 (GenBank accession number AF411068.2) was codon optimized for *S. cerevisiae* by GeneArt. Our first variant had the coding sequence for MlrA with a 6xHis-tag fused to its C-terminus. The second variant had a mating factor α tag fused to its N-terminus in addition to the C-terminal 6xHis-tag to assess its effect on the expression and localization of the enzyme.

After expressing the enzymes, the cells were lysed and the lysate was centrifuged. Samples were taken from all points of processing and analyzed with western blot. The results in Figure 8 shows that both variants were expressed. The calculated size of MlrA with a His-tag is 37 kDa. However, our results show a band corresponding to a protein of about 28 kDa. This is the same size we obtained when producing MlrA in *E. coli* [13]. The smaller than expected size was also established by Dziga et al., who discussed that the smaller size might be due to protease activity specific to *E. coli*, as the expected MlrA size was observed when producing MlrA heterologously in *Staphylococcus aureus* [9]. The results from Dziga et al. also indicated that any cleavage of the enzyme would happen on the N-terminus, as the C-terminally fused His-tag was not cleaved. Our results support this, as our constructs had the His-tag on the C-terminus, similar as in [9]. This indicates that there might be similar proteolytic activity in *S. cerevisiae* as there is in *E. coli* with regard to this enzyme.

Expression of MlrA with the mating factor α tag resulted in a protein of about 38 kDa, which corresponds to the calculated size of MlrA. It might be that the secretion tag in front of the enzyme protects it from the presumed proteolytic cleavage, although the secretion tag itself is apparently cleaved off during processing as expected; the calculated mass of the construct containing the signal sequence is about 47 kDa.

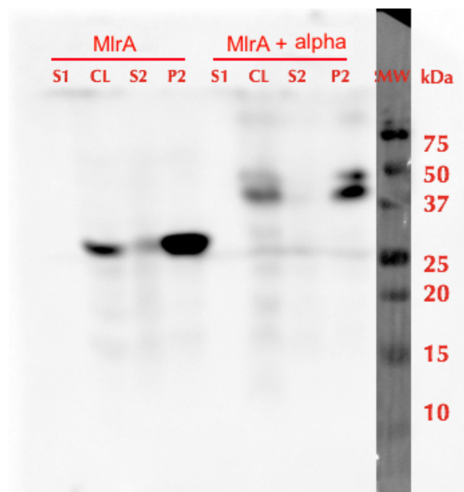

Figure 8. Western blot of different yeast MlrA constructs. S1 - the growth medium, CL - cell lysate, S2 - supernatant from CL and P2 - pellet from CL.

Comments

teague: This is one of the prettiest western blots I've ever seen an iGEM team produce. (-: Do look closely at the PLoS guidelines, though, to make sure that you're allowed to do the image manipulation that you did to the molecular weight lane.

snehadri: The MW lane comes from the digitized image of the blot and has been superimposed on the chemiluminescent image. It only serves to indicate the molecular weight ladder. I do wonder if it is necessary or if just the number lane be shown. For now we have adjusted the MW lane to merge with the rest of the blot so perhaps it looks better now.

Microcystinase localization in *S. cerevisiae*

Further analysis of the different fractions of the cell indicated that both constructs localize in the fraction comprising of inclusion bodies, plasma membrane and cell wall (Figure 9, P2 fraction). MlrA without a tag (MlrAY in Figure 9) was also localized in the fraction containing only the cell wall and plasma membrane (P3), indicating that the protein is localized in the plasma membrane. The absence of MlrA with the tag (MlrAYa) in the fractions after the renaturing step (Figure 9, S3 and P3) is most likely due to technical errors in the experiment, and based on this we are unable to conclude whether the protein is in the plasma membrane or in the inclusion bodies.

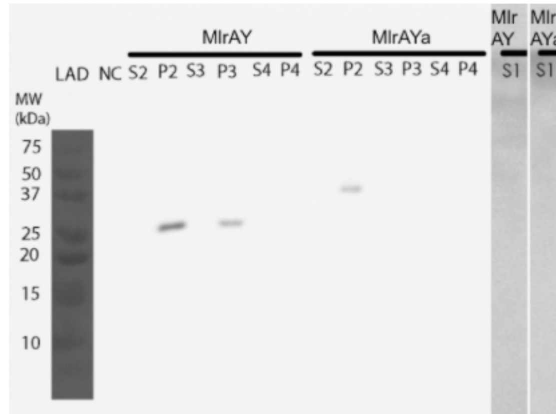

Figure 9. Localization of MlrA (MlrAY) and MlrA with mating factor α (MlrAYa). LAD - molecular weight standard, NC - S. cerevisiae SS328-leu without MlrA plasmid as negative control, S1 - secreted protein, S2 - soluble protein and inner membranes, P2 - inclusion bodies, plasma membrane and cell wall, S3 - refolded protein, P3 - plasma membrane and cell wall, S4 - soluble protein, P4 - inner membranes.

Microcystin degradation

Enzyme activity assays were performed for the cellular fractions containing the inclusion bodies, cell walls and plasma membranes (Figure 9, P2). *S. cerevisiae* not containing a microcystinase construct was used as a negative control.

The results for the degradation assay, plotted in Figure 10, show that the amount of microcystin-LR is decreasing over time, suggesting that the enzyme is active and is degrading the toxin. However, since the exact amounts of MC-LR and MlrA in the samples were not known, no quantitative analysis of the reaction could be done, e.g. to calculate reaction kinetics.

The results of the activity assay provide more verification on localization results. Based on the localization we can say that MlrA resides in the plasma membrane, but we were unable to definitively conclude the localization of MlrA with the mating factor α beyond that it localized into the fraction containing the plasma membrane, cell wall, and inclusion bodies. However, because both enzyme variants exhibited activity degrading microcystin, localization in inclusion bodies seems unlikely. Based on this, we can conclude that both variants most likely reside in the plasma membrane.

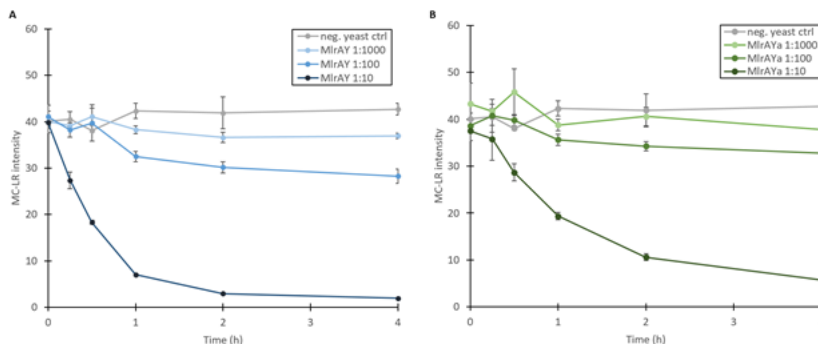

Figure 10. Enzyme activity measured by microcystin degradation. The graphs show the degradation of MC-LR with three different dilutions, n=3. (A) MlrA without mating factor α - MlrAY, and (B) MlrA with mating factor α - MlrAYa.

Conclusions

We verified the functionality of two stress promoter devices (TSA1 and CCP1) by testing their expression with hydrogen peroxide induction. The base level of TSA1 fluorescence was rather high, and relative changes were rather small. Still, it clearly seemed to be upregulated by oxidative stress. For the CCP1 promoter, YFP expression base level was near zero, absolute values of induced fluorescence were much smaller, but relative values were significantly greater. Thus, amplifying the fluorescent signal could make the CCP1

construct a viable oxidative stress sensor. Our molecular models support the viability of using these promoters in sensor purposes. According to our simulations, mechanisms based on the response to oxidative stress have good potential for detection mechanisms, as they suggest a fast response of the cell based on the transcription factors our promoters rely on.

Comments

teague: "basal" level?
snehadri: We were not sure which one it would be but perhaps your suggestion is the right one

We have a good proof of concept showing that both promoters are induced by oxidative stress using hydrogen peroxide, but more experimental work would have to be done to confirm their viability in detecting oxidative stress caused by microcystin. In order to validate this, continued work on the VL3 strain QDR2 microcystin transporter would be essential. Different variants of the transporter would have to be used in further experiments. Additionally, analysis of the expression of the transporter could produce information on the localization of the transporter. This would aid in confirming potential transporter inactivity and the reason behind it. Integrating the transporter into a different locus in the genome or expressing it in a plasmid while leaving the original transporter intact could also help pinpoint the source of unexpected transporter function.

In the case that our promoters were not functional with microcystin induction, they could have uses in other purposes. As the concept of our promoter devices is precisely to respond to oxidative stress, they could be utilized for different applications centred around this idea. We hypothesized that in fresh waters, microcystin is one of the strongest factors causing oxidative stress, and thus, our promoters would give a signal relative to its concentration. However, there are many other agents that cause oxidative stress, and thus our device could be used for studying their combinatory effect and monitor stress levels in the form of a fluorescence signal.

Comments

teague: Other agents like what?
snehadri: There are many oxidative agents found in nature like radiation, xenobiotics and air pollution but the extent of oxidative stress imposed by them would have to compared with that of microcystin.

We demonstrated that active microcystinase can be produced in *S. cerevisiae*. Combining our enzyme activity and localization analyses, the most likely localization of microcystinase in *S. cerevisiae* seemed to be the plasma membrane. This supports previous accounts of the enzyme being a putative membrane protein. Further research would have to be conducted in order to get quantifiable data about the enzyme. More detailed comparison of microcystinase production and activity levels between *S. cerevisiae* and *E. coli* would outline potential benefits of choosing a specific production host for the enzyme.

To further develop the concept of degrade microcystin from contaminated water enzymatically, a few points are worth acknowledging. Even though the microcystins are the most common cyanobacterial toxins, there are several others produced by the bacteria which this enzyme cannot degrade. In addition, to make the degradation complete, other enzymes from the microcystinase enzyme set would have to be utilized, as MlrA only linearizes the toxin. For most purposes, however, linearization would likely bring toxin concentrations to safe levels.

Comments

PLOSPatrick: Does the team have any ideas about how the enzyme could be deployed in a way that's environmentally safe and practical (i.e., recoverable, not harmful to aquatic life, not too visible, etc.)?
snehadri: What we had in mind was to use the enzyme in a bucket of water (as shown in the video linked in one the comments above). This would limit the enzyme's reach to just the bucket and it would denature upon encountering the hot temperatures of the sauna. Practically speaking then, the degradation would have to be carried out in room temperature prior to the water being used.
PLOSPatrick: Ah, that makes sense. It might be nice for readers if you could include a little about the sauna idea, perhaps expanding on this as a potential use in the discussion section. I know that when reading papers, I sometimes have difficulty imagining exactly how something would be deployed in the real world, so an example like this is really helpful.
snehadri: Thanks for the suggestion. It does give a bit of a story to our project.

Acknowledgements

LC-MS analysis was carried out by Matti Wahlsten from the Cyanobacteria group at the University of Helsinki.

We want to thank our supervisors and everyone else who has helped and supported us during our iGEM journey. In particular, special thanks go to David Fewer from the University of Helsinki, Alexander Frey, Jorg de Ruijter, Pezhman Mohammadi and Georg Schmidt from Aalto University, and Merja Penttilä, Jussi Jäntti and Dominik Mojzita from the VTT Technical Research Centre of Finland.

References

1. Welgamage Don DON, A. C. D. An investigation into the biodegradation of peptide cyanotoxins (microcystins and nodularin) by novel gram-positive bacteria. 2012; Available from OpenAIR@RGU. [online]. Available from: http://openair.rgu.ac.uk (http://openair.rgu.ac.uk)
2. Campos A, Vasconcelos V. Molecular mechanisms of microcystin toxicity in animal cells. Int J Mol Sci. 2010;11(1):268–287.
3. He XJ, Fassler JS. Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of Saccharomyces cerevisiae. Molecular microbiology. 2005;58(5):1454-1467.
4. Valério E, Vilares A, Campos A, Pereira P, Vasconcelos V. Effects of microcystin-LR on Saccharomyces cerevisiae growth, oxidative stress and apoptosis. Toxicon. 2014;90:191-198.
5. Bourne DG, Riddles P, Jones GJ, Smith W, Blakeley RL. Characterisation of a gene cluster involved in bacterial degradation of the cyanobacterial toxin microcystin LR. Environ Toxicol. 2001;16(6):523-34.
6. Bourne DG, Jones GJ, Blakeley RL, Jones A, Negri AP, Riddles P. Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR. Appl. Environ. Microbiol. 1996;62(11):4086-4094.
7. Pei J, Mitchell DA, Dixon JE, Grishin NV. Expansion of type IICAAX proteases reveals evolutionary origin of g-secretase subunit APH-1. J. Mol. Biol. 2011;410:18-26.
8. iGEM Aalto-Helsinki 2016. Aalto-Helsinki 2016 Description. In: Aalto-Helsinki 2016 [Internet]. 2016 [cited 16 Jan 2017]. Available: http://2016.igem.org/Team:Aalto-Helsinki/Description (http://2016.igem.org/Team:Aalto-Helsinki/Description)
9. Dziga D, Wladyka B, Zielińska G, Meriluoto J, Wasylewski M. Heterologous expression and characterisation of microcystinase. Toxicon, 2012;59(5):578-586.
10. Aalto-Helsinki 2016 – research group on protocols.io. Life Sciences Protocol Repository. Protocols.io. 2017 [cited 19 Jan 2017]. Available: https://www.protocols.io/groups/aaltohelsinki-2016 (https://www.protocols.io/groups/aaltohelsinki-2016)

11. WHO, 2003. Cyanobacterial toxins: Microcystin-LR in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality. Geneva, World Health Organization (WHO/SDE/WSH/03.04/57).
12. Edwards C, Graham D, Fowler N, Lawton LA. Biodegradation of microcystins and nodularin in freshwaters. Chemosphere. 2008;73(8):1315-1321.
13. iGEM Aalto-Helsinki 2016. Aalto-Helsinki 2016 Laboratory. In: Aalto-Helsinki 2016 [Internet]. 2016 [cited 16 Jan 2017]. Available: http://2016.igem.org/Team:Aalto-Helsinki/Laboratory (http://2016.igem.org/Team:Aalto-Helsinki/Laboratory)
14. Lushchak VI. Oxidative stress in yeast. Biochemistry. 2010;75(3):281-296.
15. He XJ, Mulford KE, Fassler JS. Oxidative stress function of the Saccharomyces cerevisiae Skn7 receiver domain. Eukaryot Cell. 2009;8:768–778.
16. Boisnard S, Lagniel G, Garmendia-Torres C, Molin M, Boy-Marcotte E, Jacquet M, et al. H2O2 Activates the Nuclear Localization of Msn2 and Maf1 through Thioredoxins in Saccharomyces cerevisiae. Eukaryot Cell. 2009:8(9):1429–1438.
17. Gulshan K, Thommandru B, Moye-Rowley WS. Proteolytic degradation of the Yap1 transcription factor is regulated by subcellular localization and the E3 ubiquitin ligase Not4. J Biol Chem. 2012;287:26796–26805.
18. 18. iGEM Aalto-Helsinki 2016. Aalto-Helsinki 2016 Modelling. In: Aalto-Helsinki 2016 [Internet]. 2016 [cited 16 Jan 2017]. Available: http://2016.igem.org/Team:Aalto-Helsinki/Modelling (http://2016.igem.org/Team:Aalto-Helsinki/Modelling)

Comments

PLOSFeedback: Hi @snehadri (/user/snehadri) Thanks for your submission to the PLOS iGEM Project. Our editorial team have reviewed your article and have the following feedback: This is a solid report on the work completed. The aim of this work was to build a two part system to detect and degrade toxins made by cyanobacteria. The authors have made synthetic stress responsive constructs, and they have also designed the other construct with the enzyme that would degrade the toxin. However, as the authors have not tested them with the actual toxins and the work as it currently stands it may be a little too preliminary for submission to PLOS ONE. However, the authors would be very welcome to submit their article as an iGEM Report for posting on PLOS Collections (http://blogs.plos.org/collections/). Regardless of where the article is finally submitted, it will be included in the PLOS iGEM 2016 Collection (http://collections.plos.org/igem2016).

snehadri: Hi @PLOSFeedback (/user/PLOSFeedback), thank you for your feedback! It's not clear to me what you mean by "... the authors have not tested them with the actual toxins...", because we have shown in the results that our enyzme was functional against microcystin-LR, which is the most common variant of the toxin in lake waters. It's true that we do not have additional data about the degradation, for example the kinetics, but nevertheless the enzyme has been tested on an actual toxin. I'd appreciate it if you could get back to us on this matter. We agree that the work done may not enough to be considered for submission to PLOS ONE. When writing this article, our intention was to submit this as an iGEM Report, so we will be happy to submit it as such for PLOS Collections.

> **PLOSPatrick:** Hi @snehadri (/user/snehadri) Thanks for your response! I think that the point that @PLOSFeedback (/user/PLOSFeedback) was raising was that the synthetic stress responsive constructs (mentioned in the first sub-section of your Results and Discussion) were not tested with the actual toxins. Would that be correct to say? This was a very interesting read on the whole, and looks like an interesting and innovative approach to a real-world issue, so well done to you and your team!
>
> **snehadri:** Hi @PLOSPatrick (/user/PLOSPatrick), thanks for the clarification and for your encouraging words! Coming back to the topic of toxin testing, we did test the engineered stress-responsive yeast cells for their response to microcystin, but according to the fluorescence data there was no significant effect of the toxin on the cells' fluorescence levels.

devang: I have to agree with @PLOSFeedback (/user/PLOSFeedback). I think a crucial step to generate publishable work would be to demonstrate the efficacy of the system with samples of cyanobacteria contaminated pond water. As the authors note, oxidative stress can arise from many environmental stimuli and the assumption that stress can be linked to levels of microcystins needs to be tested. Overall, the promoter characterisation work and the development of both arms of the project in the iGEM time-frame is admirable!

snehadri: Thanks to all of you for your constructive feedback. This has been a very good lesson in scientific writing for us!

teague: Overall, nicely done - as @PLOSFeedback (/user/PLOSFeedback) said, a solid report on what you accomplished. I am of mixed feelings about its publishability in a more "traditional" format -- the key result for me would have been an increase in catalase or fluorescence with the addition of MC-LR. The MlrA expression, though, and the assay you used, I think are very interesting -- especially if you expand on the validation of the assay itself.

> **snehadri:** Thanks. We agree that the detection part needs more validation but that is all the data we have for now.