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Invited paper

Heterologous expression of *mlrA* in a photoautotrophic host – Engineering cyanobacteria to degrade microcystins☆

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ABSTRACT

In this report, we establish proof-of-principle demonstrating for the first time genetic engineering of a photoautotrophic microorganism for bioremediation of naturally occurring cyanotoxins. In model cyanobacterium *Synechocystis* sp. PCC 6803 we have heterologously expressed *Sphingopyxis* sp. USTB-05 microcystinase (MlrA) bearing a 23 amino acid N-terminus secretion peptide from native *Synechocystis* sp. PCC 6803 PilA (*sll1694*). The resultant whole cell biocatalyst displayed about 3 times higher activity against microcystin-LR compared to a native MlrA host (*Sphingomonas* sp. ACM 3962), normalized for optical density. In addition, MlrA activity was found to be almost entirely located in the cyanobacterial cytosolic fraction, despite the presence of the secretion tag, with crude cellular extracts showing MlrA activity comparable to extracts from MlrA expressing *E. coli*. Furthermore, despite approximately 9.4-fold higher initial MlrA activity of a whole cell *E. coli* biocatalyst, utilization of a photoautotrophic chassis resulted in prolonged stability of MlrA activity when cultured under semi-natural conditions (using lake water), with the heterologous MlrA biocatalytic activity of the *E. coli* culture disappearing after 4 days, while the cyanobacterial host displayed activity (3% of initial activity) after 9 days. In addition, the cyanobacterial cell density was maintained over the duration of this experiment while the cell density of the *E. coli* culture rapidly declined. Lastly, failure to establish a stable cyanobacterial isolate expressing native MlrA (without the N-terminus tag) via the strong cpcB560 promoter draws attention to the use of peptide tags to positively modulate expression of potentially toxic proteins.

1. Introduction

Cyanobacterial biotechnology has experienced dramatic growth in recent years owing to both a unique metabolic capacity and the increased political/ecological consideration for more sustainable industrial processes. Currently, greater than 20 commercially relevant chemicals have been produced via the metabolic engineering of photoautotrophic metabolism in a variety of cyanobacterial species (Angermayr et al., 2015; Dexter et al., 2015). Unfortunately, production of target metabolites from heterologous pathways in cyanobacteria under strict photoautotrophy is often not economically competitive when compared to petrochemical or established heterotrophic bioprocesses (Ducat et al., 2011; Gomaa et al., 2016). To address these production issues, there has been development of new genetic tools for use with cyanobacteria (Berla et al., 2013; Gordon et al., 2016; Kim et al., 2017a; Ma et al., 2014), increased clarification of their biochemical systems (Cai et al., 2016; Jordan et al., 2017; Montgomery et al., 2016), and expansion of the genetic characterization of novel cyanobacterial isolates (Leao et al., 2017; Yu et al., 2015; Zhu et al., 2017). Industrially relevant enzymes

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Abbreviations

ACN	acetonitrile
GST	glutathione S-transferase
IPTG	(isopropyl β-D-thiogalactoside)
MC	microcystins
MC-LR	microcystin-LR
MlrA	microcystinase
PCR	polymerase chain reaction
TFA	trifluoroacetic acid

have also been successfully introduced into cyanobacterial systems (Bartsch et al., 2015). Industrial enzymes present diverse potential as co-products within a photoautotrophic biomass production platform, with higher value/exclusive niche applications compared to chemical feedstocks. Furthermore, successful commercial scale deployment of new photoautotrophic biotechnologies is critically dependent on the innovative utilization and progressive valorization of the resultant biomass fractions (Chew et al., 2017; Shurin et al., 2016). Towards such integrative objectives and as an alternative technology to the extensive focus on target metabolite production via heterologous gene expression in cyanobacterial biotechnology as outlined above, we describe the generation of a cyanobacterium expressing *mlrA*-based degradation of microcystins (MC).

MC contamination of surface waters is a global public health concern (Harke et al., 2016). MC are the most observed cyanotoxins in freshwater, with increasing eutrophication due to agricultural runoff and increasing temperatures poised to amplify the problem with expanding commercial agriculture and climate change (Lürling et al., 2017). While conventional water treatment processes can be effective in removing low levels of MC, significant oxidative input is required after filtration of live MC producers (to remove the intracellular toxin load) (Jeong et al., 2017; Kim et al., 2017b) and cyanobacterial bloom conditions may overwhelm water treatment capacity (Bullerjahn et al., 2016). Biological degradation of MC has been shown to be the primary natural process for remediation of contaminated waters, with the ecological presence of *mlr* genes associated with greatly accelerated toxin degradation compared to consortia utilizing *mlr*-independent degradation pathways (Lezcano et al., 2016; Morón-López et al., 2017). Biochemical characterization of the enzymes present in the *mlr* gene cluster has been undertaken, with heterologous expression of isolated genes in *E. coli* (Bourne et al., 2001; Dziga et al., 2012, 2016; Yan et al., 2012). MlrA is a metalloprotease acting specifically at the peptide bond between Adda- and the fourth position amino acid, catalyzing the initiating reaction in the *mlr*-dependent degradation pathway, linearizing the heptapeptide ring structure, and resulting in a 2100-fold decrease in toxicity, essentially rendering a non-toxic product (Dziga et al., 2012). Expression in *E. coli* has resulted in increased whole-cell MlrA activity compared to the *Sphingomonas* sp. ACM-3962 strain, with 6800-fold MlrA activity increase comparing cellular extract to that of the natural host (Dziga et al., 2012). Immobilization of MlrA expressing *E. coli* in alginate beads for use as whole cell biocatalyst has also been documented, with a 65% decline in activity after 72 h of exposure to continuous flow of lake water (Dziga et al., 2014).

An MlrA-expressing photoautotrophic host is considered to have potential advantages to the use of heterotrophic bacteria, given the lake water culture matrix. Such advantages may include: a) increased *in situ* stability of the photoautotrophic biocatalytic biomass, b) reduced input requirements for the generation/maintenance of said biocatalytic biomass, leading to c) reduced cost of novel MC treatment methodologies, potentially allowing for d) MC treatment methods that can address MC-contamination of commercial aquaculture (Drobac et al., 2016; Hu et al., 2017) and agricultural systems (Cao et al., 2018; Jia et al., 2017; Lee et al., 2017). This may be of importance as the references noted in the previous statement indicate multiple detrimental effects of MC in these scenarios, leading to both reduced target agricultural yields and significant human dietary exposure to bioaccumulated MC. Additional advantages may also include e) modular integration of such a technology with the current suite of photoautotrophic biotechnological investigations targeting commodity production. Thus, the primary goal of our research was a) to determine if MlrA production and activity can be installed in a cyanobacterial chassis. Additional tasks were: b) attempt to utilize the *Synechocystis* sp. PCC 6803 PilA (*sll1694*) N-terminus secretion tag, as described by Sergeyenko and Los (2003), for the secretion of the MlrA to the extracellular space, c) to estimate the level of MlrA activity in comparison to both a natural gene host and heterologous MlrA-expressing *E. coli* strains, and d) to perform preliminary experiments on the stability of such an expression system in semi-natural conditions.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic acid (TFA) was from Sigma (St Louis, MO, USA), acetonitrile (ACN) and Pursuit C18 were obtained from Merck (Darmstadt, Germany). Microcystin-LR (MC-LR) was extracted and purified from a culture of *Microcystis aeruginosa* PCC 7813 strain (the Pasteur Institute, Paris; Gajdek et al., 2003); acyclic dmMC-LR was produced by linearization of cyclic MC-LR by MlrA and was purified by HPLC followed by MS analyses (Dziga et al., 2012). pUC57 vector was obtained from Genscript, (USA) whereas pTZ57R\T vector was obtained from Fermentas. Alkaline phosphatase, T4 ligase as well as restriction enzymes BamHI, NdeI and XhoI were from Thermo Scientific; Gel-Out DNA purification kit and Clean-Up DNA purification were from A&A Biotechnology. Easy-A High-Fidelity PCR Cloning Enzyme was from Agilent Technologies.

2.2. Strains and growth conditions for growth assay

Synechocystis PCC 6803 (6803) was cultured using standard BG-11 media. Filter-sterilized glucose was added to the BG-11 agar media at 5 mM final concentration, unless otherwise noted. When culturing the cyanobacterial transformants, kanamycin was supplemented at 50 $\mu g\,ml^{-1}$ (Km50) final concentration. Erlenmeyer flasks (250 ml) were used with cyanobacterial working culture volumes of 40 ml, flasks were stoppered with gauze-wrapped cotton then topped with aluminum foil to facilitate gas exchange while preventing/reducing contamination, respectively. Liquid cultures were grown in an incubating shaker (model BHWY-200, Haishu Saifu Tset Instrument Factory, Ningbo, China) modified with the addition of a single 20 W fluorescent light (model T4-JL, Jinli Illumination, China) to provide sufficient irradiance for photoautotrophic growth. This light was arranged in parallel to the (maximum of three) Erlenmeyer flasks within the incubating shaker, with a distance of approximately 7 cm from the naked bulb to the flask surface (widest point at base of flask). Cultures were grown at 28 °C, 130 rpm, 24 h continuous light regime. Unless otherwise noted, cyanobacterial growth in liquid culture was monitored via spectrophotometric absorbance at 730 nm (OD_{730}) using a spectrophotometer (model T-6, NanJing PHILES Instrument Company, China). BG-11 agar plates were incubated at 28 °C under

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