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Title: Heat shock and iron limitation modulates the metabolic profiles of the cyanobacterium *Microcystis aeruginosa* NIES-88.

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Short running title: OSMAC applied to *Microcystis aeruginosa*.

Abstract

The freshwater cyanobacterium *Microcystis aeruginosa* NIES-88, which can produce microcystins, micropeptins, and argicyclamides was subjected to a one strain many compounds (OSMAC) analysis. We report its response to two environmental stressors, temperature and iron limitation, by means of untargeted and targeted metabolomics. The results demonstrated a slower specific growth rate of 0.20/day and 0.16/day in adverse conditions of 37 °C and iron limitation, respectively. The metabolic signature of *M. aeruginosa* was highly dependent on incubation temperatures. Production of microcystins LR and RR were severely downregulated while that of argicyclamide B was significantly upregulated with a highest 10-fold increase on day 14 in heat shock treatment. *M. aeruginosa* NIES-88 was found to produce a new compound, argicyclamide D (**1**) in iron limited medium which shares the same macrocyclic structure as the previously reported analogs. Hence, it is proposed that acclimation of *M. aeruginosa* to environmental stressors might be mediated by a change in the metabolic pathways as well as modulation of their expressed metabolites.

Keywords: argicyclamide, microcystin, OSMAC, metabolomics

Introduction

Cyanobacteria are an ancient group of photosynthetic gram-negative prokaryotes that have colonized diverse habitats. They have been successfully mined for natural product derived drug candidates (Demay et al. 2019). One such drug currently on the market and used in cancer treatments is Brentuximab vedotin which was developed from dolastatin 10 isolated from the cyanobacterium *Symploca hydroides* (Van de Donk and Dhimoletta 2012). In the past decades, the issue of redundancy in natural product research has become a concern with the repeated isolation and characterization of natural products that have already been reported in the literature (Hussain et al. 2017). Moreover, with the advent of genome sequencing, a huge inconsistency has been found between the number of secondary metabolites biosynthetic gene clusters (BCG) encoded in a particular strain and the number of metabolites produced by that strain (Romano et al. 2018). This indicates that in typical laboratory conditions, a significant number of gene clusters are not transcribed, suggesting a considerable amount of untapped chemical potential (Pan et al. 2019). One reason for this is that the optimal conditions for the production of secondary metabolites are not essentially identical to those for growth. In their natural environment, microorganisms thrive in ecosystems together with other species and ever-changing physical parameters. Hence, the need to protect itself against the changing conditions, predation and competition for the same resources giving rise to signaling and cross-feeding between organisms that will induce the biosynthesis of novel metabolites (Romano et al. 2018).

New strategies are needed to identify and characterize the chemical diversity of these microorganisms. One such strategy is the OSMAC (One Strain Many Compounds) approach, which comes from the theory that a single strain is able to produce many compounds in different growth conditions to access the cryptic metabolic pathways that are otherwise silent (Pinedo-Rivilla et al. 2022). Culture conditions can significantly impact the production of natural products resulting in either upregulation or downregulation of certain compounds or even the production of new metabolites through the activation of cryptic biosynthetic pathways (Da Silva et al. 2023). Many studies have reported the impact of cyanobacterial culture conditions with various objectives: to increase biomass, lipid content, improve production of bioactive compounds or to investigate the ecology and physiology of strains related to harmful algal blooms (Crnkovic et al. 2018). In a previous study, increased levels of capsular/bound polysaccharide was observed when fermentation of *Leptolyngbya* sp. was carried out in biofilm culture in conico-cylindrical flask compared to the culture in planktonic

mode in Erlenmeyer flask (Veerabadhran et al. 2018). Metabolomics can also be used to analyze the behavior of cyanobacterial strains subjected to stress conditions. As metabolites are considered as the end products of cellular metabolism, their levels can be regarded as the direct readout of biochemical activity in response to genetic or environmental changes (Sindelar and Patti 2020). Using such an approach, it was found that the strains *Nostoc* sp. UIC 10110 and *Scytonema* sp. UIC 10036 showed an upregulation of the primary metabolites heterocyst glycolipids when cultivated in low nitrate medium. Low phosphate levels displayed a selective increase in the production of the secondary metabolite merocyclophane C in *Nostoc* sp. UIC 10110. *Scytonema* sp. UIC 10036 also showed an upregulation of tolytoxin in high phosphate conditions and the induction of a new peptide when grown in Z45₀ medium (Crnkovic et al. 2018).

We have selected *M. aeruginosa* NIES-88 for this study as it is a well-studied cyanobacterial strain and prolific producer of many bioactive natural products such as microcystins, micropeptins, aeruginosins, microginins, anabaenopeptins and microviridins (Okino et al. 1993, Murakami et al. 1997, Kisugi and Okino 2009). Also, we have recently reported the isolation and structure elucidation of the bis-prenylated antibacterial cyanobactin argicyclamide A (2) which has made a breakthrough to access prenylation on basic/hydrophilic side chain amino acid residues by the prenyltransferase AgcF found in *M. aeruginosa* NIES-88 (Phan et al. 2021) and demonstrated the natural products biosynthetic potentials of this strain. Factors such as temperature, nutrient availability, pH, and light intensity have been extensively studied in this genus with a major focus on microcystins (Alexova et al. 2011, Giannuzzi et al. 2016, Wang et al. 2016, 2018) while other secondary metabolites produced by *Microcystis* species have received little attention. With this in mind, to improve the chemical diversity of *M. aeruginosa* NIES-88, we aimed to evaluate the production of metabolites (particularly argicyclamides and microcystins) in *M. aeruginosa* NIES-88 in response to various physico-chemical parameters such as media composition (addition of trace metals, nutrient depletion, nutrient limitation), pH and temperature. In the present study, we focused on the effects two environmental stressors, temperature and nutrient limitation through untargeted and targeted metabolomics using high-performance liquid chromatography (HPLC) coupled with high-resolution accurate mass spectrometry (TOF-MS).

Material and methods

General experimental procedures

¹H NMR spectra were recorded with a JEOL NMR spectrometer at 500 MHz. Column chromatography was performed using ODS silica gel ODS (YMC-Gel, 150 µm). High-performance liquid chromatography (HPLC) experiments were performed with a JASCO HPLC system equipped with a PU-980 pump and UV-970 UV/vis detector. HR-ESIMS experiments were performed with an Agilent 1100 Series HPLC system coupled with a micrOTOF-HS mass spectrometer (Bruker Daltonics). ESI-MS/MS data was obtained using SolariX XR Bruker mass spectrometer. All chemicals and solvents were of analytical or chromatographic grade.

Organism and culture conditions

The unialgal culture of *Microcystis aeruginosa* NIES-88 (thereafter, *M. aeruginosa*) was obtained from the NIES collection and was maintained in 500 mL BG-11 medium (Waterbury and Stanier 1981) at 25 °C under illumination of 80 µE/m²s on a 12L:12D cycle.

OSMAC analyses

Using the OSMAC (One Strain Many Compounds), we conducted a series of preliminary screenings of the production of metabolites by *M. aeruginosa* cultured in different conditions. One parameter at a time was changed while keeping the other parameters identical to the control (BG-11 medium as described above). To investigate the effect of heavy metals, 50 mM of Cd(NO₃)₂·4H₂O (Sigma-Aldrich) and 50 mM of PbCl₂ (Sigma-Aldrich) served as a source of metals. For heat shock experiments, the flasks were placed in incubators at 25 °C, 30 °C and 37 °C. To evaluate the effect of pH, modified BG-11 mediums were prepared with pH of 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0. Finally, for nutrient limitation experiments, BG-11 mediums with different concentrations of N, P, Zn, Cu and Fe were used. From a 7-day old pre-culture of the strain, 1.0-1.2 × 10⁵ cells/mL were transferred to 500 mL of medium in the appropriate condition in triplicates. Growth of the cyanobacterial strains was monitored every 2 days by counting the cells using a hemocytometer. After 7-10 days, the culture was centrifuged at 10,000 rpm and the supernatant discarded. The cells were homogenized and extracted with MeOH (10 mL × 3). Chemical profiling was conducted by LC-MS using a Cadenza CD-C18 column (2 × 150 mm, 3 µm, flow rate 0.2 mL/min at 25 °C). The mobile phase conditions were: 0–30 min 20–80% (v/v) acetonitrile with 0.1% (v/v) formic acid in Milli-Q water; 30–45 min, isocratic 80% (v/v) MeCN in Milli-Q water with 0.1% (v/v) formic acid. Injection volume was 5 µL. MS analysis was in the range of 50 – 3,000 *m/z*.

Based on the chemical profile, two conditions were chosen for 10 L scale-up: heat shock of 37 °C and iron limitation.

Heat shock treatment

1.0 – 1.2 x 10⁵ cells/mL from 500 mL culture was inoculated in 10L glass bottles. The control conditions were BG-11 medium supplemented with continuous aeration with filtered air (0.3 L/min) at 25 °C under illumination of 80 µE/m²s on a 12L:12D photoperiod and 6 mg/mL (NH₄)₅[Fe(C₆H₄O₇)₂]. The heat shock treatment consisted of *M. aeruginosa* grown in BG-11 medium aerated with filtered air at 0.3 L/min at 37 °C under illumination of 80 µE/m²s on a 12L:12D cycle and 6 mg/mL (NH₄)₅[Fe(C₆H₄O₇)₂]. All experiments were conducted in triplicates and lasted for 38 days. Cells were counted every 3-7 days using a hemocytometer. The specific growth rate at each condition was calculated based on the cell concentration data during exponential growth at day 21, using the equation (You et al. 2018),

$$k(T) = \frac{\ln(\frac{N}{N_0})}{t - t_0}$$

where N is the cell concentration (cells/mL) at incubation time t (day), N₀ is the initial cell concentration at t₀, and T (°C) is the temperature at which the cells were incubated.

Every 3-7 days, 40 mL of the cyanobacterial culture was collected aseptically and centrifuged at 10,000 rpm at 4°C. The supernatant was discarded, and the pellet stored at -30°C until further analysis. Metabolites were extracted with methanol by sonication for 20 min. The extract was filtered with a 0.45 µm syringe filter then was subjected to solid phase extraction and eluted with 100 % MeOH which was evaporated to dryness. The dried extract was then reconstituted with 1 mL of 50% ethanol (v/v) in milli-Q water prior to LC-MS analysis as described above.

Iron limitation experimental design

For the iron limited medium, *M. aeruginosa* was first transferred to 500 mL of (NH₄)₅[Fe(C₆H₄O₇)₂] free BG-11 medium for 3 days and then inoculated at a concentration of 1.0 – 1.2 x 10⁵ cells/mL to 400 L (40 × 10 L) of BG-11 medium containing only 0.6 mg/mL (NH₄)₅[Fe(C₆H₄O₇)₂] supplemented with aeration with filtered air (0.3 L/min) at 25 °C under illumination of 80 µE/m²s on a 12L:12D cycle. Cells were counted every 3-7 days using a hemocytometer to monitor growth rate. After 38 days, the cells were separated from the medium by centrifugation at 10,000 rpm. *M. aeruginosa* freeze-dried cells (15.5 g)

from 400 L of culture was homogenized at 10,000 rpm for 15 min and extracted with 80 % MeOH (250 mL × 3). The extract was then dried in vacuo and partitioned between EtOAc and H₂O. The H₂O fraction (1.5 g) was subjected to open column chromatography with a stepwise gradient elution starting with aqueous 20% MeOH to 100% MeOH. The 100% MeOH (104 mg) fraction was further purified by reverse phase HPLC (Wakosil II 5C18 AR, 20 x 250 mm, UV detection 215 nm, flow rate 4.0 mL/min) and running condition of 60% MeCN with 0.1% trifluoroacetic acid to yield **1** (0.9 mg, *t_R*=22.5 min).

Advanced Marfey's Analysis

Compound **1** was hydrolyzed (0.1 mg) using 6 M HCl at 110 °C for 16 h. The hydrolysate was evaporated to dryness and dissolved in 50 µL of 1 N NaHCO₃. FDLA (*N*^α-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide, 200 µL of 10 mg/mL solution) in acetone was added and reaction mixture was incubated at 40°C for 1 h. The reaction was quenched by 50 µL of 2 M HCl and 200 µL of MeCN was added. The reaction mixture was directly analyzed by LC-MS using a Zorbax C3 column (2.0 x 150 mm) with a gradient system starting from 25 % to 60 % MeCN supplemented with 0.1% FA over 30 min. L-*allo*-Ile and L-Ile was analyzed with a gradient system starting from 30% to 38% MeCN with 0.1% formic acid over 50 min using the Zorbax C3 (2.0 x 150 mm) column. The separation of L-Leu and L-Ile was performed with an ODS-SR-3 (Develosil, 2.0 x 150 mm) with an isocratic mode of 33% MeCN with 0.1% formic acid over 95 min.

Anti-bacterial assay

Anti-bacterial activity was determined using the microtiter broth dilution method (Wiegand et al. 2008) against *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 12600), and methicillin resistant *Staphylococcus aureus* (ATCC 43300). Briefly, serial two-fold dilutions were carried out on 96 well microplates in Mueller Hinton broth using pure compounds with final concentration 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µM. Synergistic/antagonistic measurements were carried out using 50:50 of argicyclamide A and **1** with final concentrations of each compound being 50, 25, 12.5, 6.25, 3.12 and 1.56 µM. The 96 well microplates were incubated for 24 h at 35 °C. OD measurements were taken with Multiskan JX microplate reader (Thermo Labsystems) at 630 nm. The absorbance values and no visual growth of bacteria were recorded as the minimum inhibition concentration (MIC).

Siderophore CAS assay

Siderophore detection was carried out using the chrome azurol S (CAS) colorimetric assay previously described by Schwyn and Neilands (1987). Briefly, 45 μ L of Fe-CAS-HDTMA (hexadecyltrimethylammonium bromide) complex was added to 5 μ L of 100 μ M of compound **1** and argicyclamides in 96 well plates in triplicates. Deferoxamine (10 μ M) was used as positive control. Reading was taken at 630 nm after 6 h using Multiskan JX microplate reader.

Data analysis and statistics

The raw LC-HRMS data were converted to mzXML format using the opensource ProteoWizard software. The converted data were then uploaded to the web-based metabolomics platform MetaboAnalyst 5.0 (www.metaboanalyst.ca) for peak detection, extraction and alignment (Pang et al. 2022). The data were mean centered before univariate and multivariate analyses. Data were then analyzed by mummichog 1.0.5 on the same platform to search for chemical connections based on the m/z values of metabolite ions for pathway enrichment.

Following the untargeted metabolomics analysis, microcystins and argicyclamides were quantified against the standard calibration curves of their purified compounds obtained from *M. aeruginosa* culture. The significance of each metabolite was further evaluated using Student's t tests at 95% confidence level.

Results

Growth of *M. aeruginosa* NIES-88 at different temperatures and nutrient limited medium

Microcystis aeruginosa cell density was measured every 3-7 days for a time period of 38 days (Figure 1) until the death phase was reached. Cell concentrations of *M. aeruginosa* were too low to be measured accurately during the lag phase (day 1-9), therefore measurements started from day 10 to day 38. A slightly slower growth rate was observed at 37 °C as indicated by the lower specific growth rate of 0.20/day as opposed to a specific growth rate of 0.22/day at 25 °C. At higher temperatures, cells took less time (24 days) to reach the stationary phase than at lower temperatures (35 days). The lowest specific growth rate of 0.16/day was recorded for iron limited medium and it took longer for the cells in the iron limited medium (17 days) to reach the exponential phase mainly due to a longer growth cycle. No growth was seen in BG-11 medium without Zn, Cu and Fe.

Effect of temperature on the metabolic profiles of *M. aeruginosa*

LC–HRMS analysis was performed to evaluate the global metabolomic effect of temperature variation on *M. aeruginosa*. To determine the discriminatory metabolic phenotype between cells grown at 25°C and 37°C, we identified the effects of temperature on some specific metabolites produced by *M. aeruginosa* namely microcystin LR, microcystin RR and argicyclamides A-C (2-4).

At higher temperatures, production of both microcystins LR and RR declined by at least 6-fold (Figure 2). The lowest concentration of microcystins were found at the end the of the stationary phase. On the other hand, the production of argicyclamide C seems to be significantly favored at 37°C. Production of argicyclamide C during the exponential phase of *M. aeruginosa* seems to be the highest. The highest production showed a 10-fold increase on day 14. Concentration of argicyclamides A and B seems to vary throughout the experiment with argicyclamide B showing the least change. Day 14 and 30 seems to favor the production of argicyclamide A at 37°C while the other days there is a slight reduction in their production at 37°C. This provided the first indication that the metabolic signature of *M. aeruginosa* was highly dependent on incubation temperatures.

To gain further insights into response of metabolic pathways when subjected to different temperatures, Mummichog together with results from KEGG databases was used to annotate significant metabolite features and pathway analysis in MetaboAnalyst 5.0 online programming. Several affected pathways were identified (see Appendix Table 1 in Supporting Information) and the list of top pathways ($p < 0.1$) and metabolites affected are given in Table 1. Fisher Exact's test was used to evaluate the statistical significance of the most enriched pathways to verify whether a level of association between the significant Hits and Hits Total were more than random chance. It is interesting to note that the Mummichog pathway analysis among each group showed that porphyrin and chlorophyll metabolism was the topmost enriched metabolic pathways (see Appendix Table 1 in Supporting Information) despite not significantly enriched below the 5% cut-off level. The most significantly enriched pathways based on Fisher Exact's test were the glycine, serine, and threonine metabolism, one carbon pool by folate, aminoacyl-tRNA biosynthesis, methane metabolism and starch and sucrose metabolism.

Effect of iron limitation on the metabolic profiles of *M. aeruginosa*

Compound **1** was found to be induced in iron limited medium (see Appendix S1 in Supporting Information). It had molecular ion $[M + H]^+$ at m/z 1074.7094 (calculated 1074.7079) and its molecular formula was established as $C_{57}H_{91}N_{11}O_9$ with 17 degrees of unsaturation. Compound **1** differed by 16 atomic mass units (1 oxygen unit) with previously reported argicyclamide A (Phan et al. 2021) and 1H NMR of **1** displayed similarities with the latter (see Appendix S2 and S3 in Supporting Information). 1H NMR spectra showed signals for the H_α methines of α -amino acids at δ_H 4.47, 4.40, 4.18, 4.09, 4.05 and six signals for amide NH protons at δ_H 8.71, 8.62, 8.60, 7.95, 7.42, 7.37, 7.23, 7.21. Owing to limited quantity of **1**, we were unable to get a clear carbon and 2D NMR data. Tandem MS^2 was performed, and the fragmentation pattern suggested that **1** is related to argicyclamides with the presence of proline, valine, leucine or isoleucine and phenylalanine (Figure 3). Marfey's analysis of **1** (see Appendix S4 in Supporting Information) further confirmed the presence of L-phenylalanine, L-valine, L-proline, L-leucine, and L-isoleucine. An additional OH signal at δ_H 6.66 was observed on 1H NMR of **1**. The possibility of a hydroxylated prenyl group was excluded due to splitting pattern observed in MS^2 (Figure 3). The $N^\delta H$ of arginine and the amide NH of Leu in argicyclamide A gave signals at δ_H 7.37 and 7.39 respectively. Only one signal was found at δ_H 7.37 on 1H NMR corresponding to only one proton. Therefore, structure of argicyclamide D (**1**) (Figure 4) is proposed to be similar to the octapeptides argicyclamides with macrocyclic structure composed of Val-Phe-Ile-Val-Leu-Pro-Pro- N^δ hydroxyArg with two prenyl groups on the guanidine moiety. The $N^\delta H$ is proposed to be hydroxylated as shown in structure **1**, a similar feature of N^δ -hydroxyarginine from a cyclic peptide can be seen in pentaminomycins A-E isolated from *Streptomyces*, thus supported the proposed structure of **1** (Hwang et al. 2020).

Discussion

In this study, we report the metabolomic profiles of the freshwater cyanobacterium *M. aeruginosa* cultivated at two temperatures and in iron limited medium using the OSMAC strategy. This approach has allowed us to first discover argicyclamide A-C which was previously published (Phan et al. 2021) as the latter was clearly upregulated at higher temperatures. Further analysis of different culture conditions through OSMAC led us to discover a new analog reported in this study.

Lower specific growth rate was found at 37°C which agrees with many similar laboratory based experiments (Chu et al. 2007, You et al. 2018, Zheng et al. 2020). Multiple biological

processes are affected by extreme temperatures in cyanobacteria. Through non-target metabolomics analysis, an overall view of the differential features in *M. aeruginosa* grown at different temperatures in BG-11 medium suggested that the growth inhibited cells responded via multiple pathways and mechanisms. The slower growth can be attributed to the reduced photosynthesis due to the decreased RuBisCO's affinity for carbon dioxide with increasing temperatures (González-Fernández and Ballesteros 2012). High temperatures can cause oxidative stress due to the disruption of the electron transport system, damage DNA and induce cell death (Giannuzzi et al. 2016). Zheng et al., (2020) found an increased level of ROS 64.6% at 30 °C and 87.8% at 35 °C in *M. aeruginosa* FACHB-912 cells. Microcystins are known to protect cells from reactive oxygen species by binding to cysteine residues on redox sensitive proteins (Zilliges et al. 2011). Previous studies have shown that microcystin production increases with temperature until the optimum temperature is reached. Thereof, the production decreases with increasing temperature (Mowe et al. 2015, Crettaz et al. 2017). Our results indicate a huge decrease in microcystins-LR and RR production at 37°C as well as an up-regulation of the production of argicyclamide C at high temperatures.

According to mummichog pathway analysis, the starch and sucrose metabolism was significantly enriched ($p < 0.05$) on day 17 during the early exponential phase. This is in accordance to previous studies where the cyanobacterium *Synechoscystis* sp. shifted its natural accumulation of glucosyl-glycerol toward sucrose production when grown in high temperature of 35 °C (Warr et al. 1985). Another more recent study on *Synechococcus* sp. WH8102 also showed an accumulation of carbohydrates at high temperatures. The authors concluded the enhanced carbohydrate accumulation was beneficial to relieve the cells from osmotic stress caused by the high incubation temperature (Li et al. 2019). At the beginning of the decline phase, glycine, serine, and threonine metabolism, one carbon pool by folate, aminoacyl-tRNA biosynthesis and methane metabolism were the most enriched. One carbon metabolism mediated by folate consists of many interlinking metabolic pathways such as amino acid homeostasis involving methionine, glycine and serine, biosynthesis of nucleotides, epigenetic maintenance, and redox defense (Clare et al. 2019). A study about the redox metabolism in *Synechocystis* sp. PCC 6803 demonstrated that an auxiliary pathway, comprised of one-carbon, serine, and glycine metabolism, is crucial for cellular redox homeostasis (Kugler and Stensjö 2022). Hence, an enriched glycine, serine and one carbon pool metabolism might be a result of a redox defense induced by the high temperature.

M. aeruginosa displayed the lowest specific growth rate in iron limited medium which is consistent with the literature. Iron is one of the absolute requirements for almost all microorganisms. In cyanobacteria, iron is crucial for vital processes such as photosynthetic pigment biosynthesis, respiration, nitrate and nitrite assimilation, nitrogen fixation and DNA synthesis (Alexova et al. 2011). Therefore, a decrease in biomass yield was expected. Previous studies have highlighted the negative impact of iron starvation and/or limitation on growth and photopigments (Nagai et al. 2007, Xing et al. 2007, Li et al. 2009) and increase oxidative stress as observed in *M. aeruginosa* FACHB-905 by an increase in superoxide dismutase and catalase levels (Wang et al. 2016, 2018, Yeung et al. 2016).

Most studies about iron stress in cyanobacteria have shown that microcystin production is inversely proportional to the concentration of available iron in the immediate environment (Sevilla et al. 2008, Alexova et al. 2011, Wang et al. 2016, Yeung et al. 2016). According to a study by Ceballos-Laita *et al.*, 2017, microcystin LR can bind to iron and therefore increase its bioavailability to the cells. There is also some evidence that microcystins can act as informational chemicals for intraspecies communication (Schatz et al. 2007, Zilliges et al. 2011). The authors also proposed that microcystins together with other secondary metabolites released in the surrounding medium after cell lysis have the ability to signal the remaining cells of stress conditions and serve as a signal of the extent of cell death within the remaining population.

Cyanobacteria are known to produce siderophores when exposed to iron stress (Galica et al. 2021). It was first hypothesized **1** as a siderophore since its production was only found in iron limiting medium. However, CAS assay showed negative result. Next, we tested **1** against bacteria because argicyclamide A (**2**) showed potent antimicrobial activity (Phan et al. 2021). However, the result showed **1** negligible activity. No synergistic or antagonistic effect with argicyclamide A was found. Hence, it is proposed that argicyclamides might have some cell signaling or protective functions which may explain the production of **1** in iron limitation and the up-regulation of argicyclamide B in stress conditions like high temperature where microcystins are down-regulated.

This study has demonstrated the natural products biosynthetic potential of *M. aeruginosa* and its capability of adapting mechanisms when exposed to environmental stressors. Growth was significantly inhibited when faced with high temperatures and iron limitation and metabolic pathways could be changed in response to the stress conditions. There is evidence that carbohydrate metabolism and one carbon pool metabolism were enriched to deal with

osmotic stress and redox changes to the cells caused by the high temperature. Microcystins production decreased at high temperatures while argicyclamide C levels significantly increased. When faced with iron limitation, *M. aeruginosa* produced a new analog (**1**) of the previously reported argicyclamides. Hence, it suggested that the survival of *M. aeruginosa* to high temperature and iron stress was unlikely to result from one metabolite, as multiple metabolites and signaling pathways appeared to be involved. Our study successfully reports the discovery of new compounds using the OSMAC approach and provides new targets to research on the interaction and mechanism of adaptation of cyanobacteria to adverse environmental conditions.

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Supporting Information

Figure S1: Chromatogram of crude extracts of *M. aeruginosa* NIES-88 when grown in (a) BG-11 medium, 25°C, with 0.6 mg/L of $(\text{NH}_4)_5[\text{Fe}(\text{C}_6\text{H}_4\text{O}_7)_2]$ ($1/10^{\text{th}}$ of normal concentration) (b) BG-11 medium, 25°C and 6 mg/L of $(\text{NH}_4)_5[\text{Fe}(\text{C}_6\text{H}_4\text{O}_7)_2]$ (normal concentration).

Figure S2: ^1H NMR (600 MHz) spectrum of argicyclamide A (**2**) in $\text{DMSO}-d_6$.

Figure S3: ^1H NMR (500 MHz) spectrum of argicyclamide D (**1**) in $\text{DMSO}-d_6$.

Figure S4: LC-MS chromatogram of Marfey's analysis of (a) hydrolysate **1**, (b) standards of authentic amino acids.

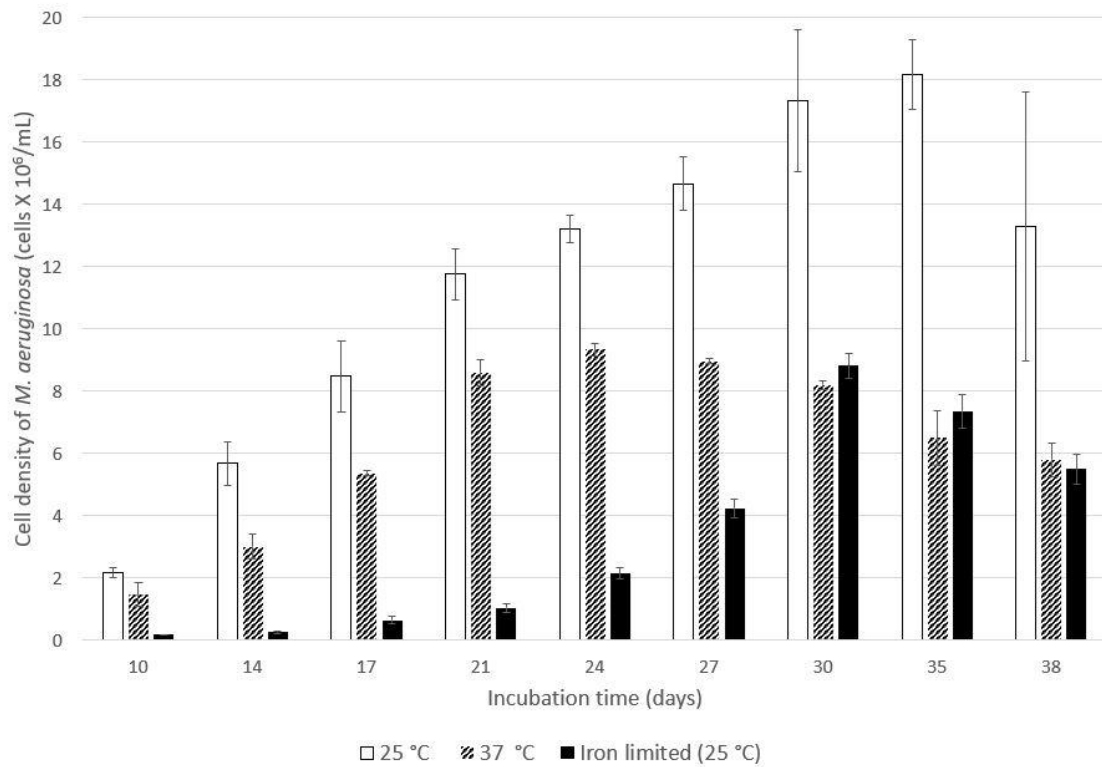


Figure 1: Growth of *Microcystis aeruginosa* at 25°C and 37°C. Data are means $\pm$ SD error bars (n = 3).

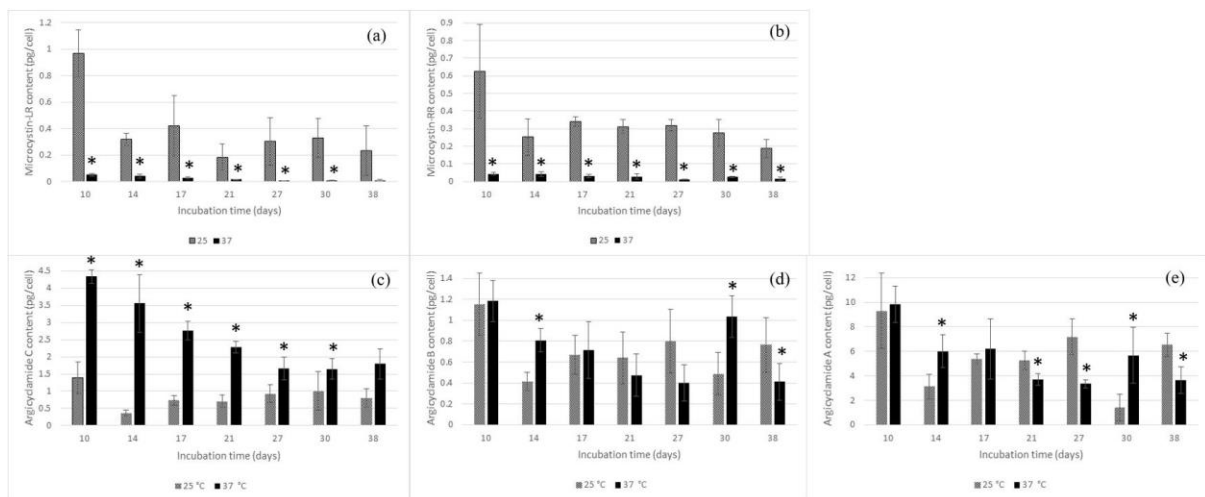


Figure 2: Production of (a) microcystin-LR, (b) microcystin-RR, (c) argicyclamide C, (d) argicyclamide B and (e) argicyclamide A by *M. aeruginosa* at 25°C and 37°C. Data are means $\pm$ SD error bars (n = 3). Asterisk on each plot denotes a significant difference between groups ($p < 0.05$).

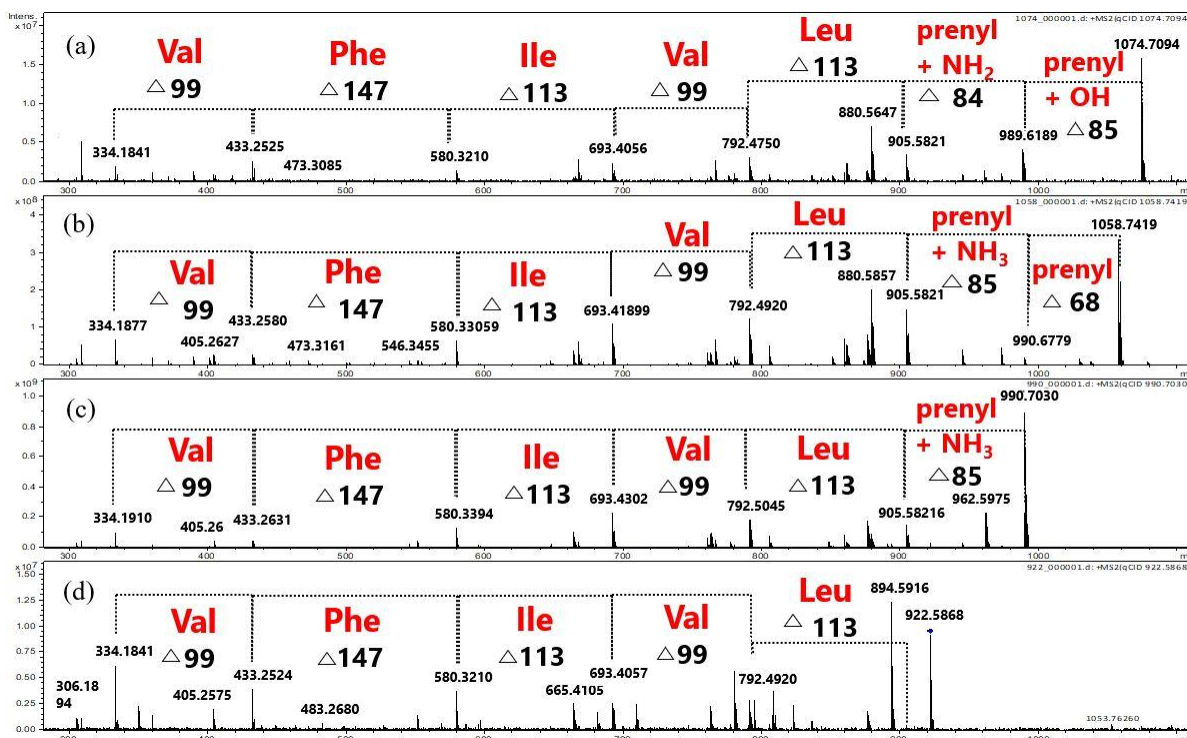
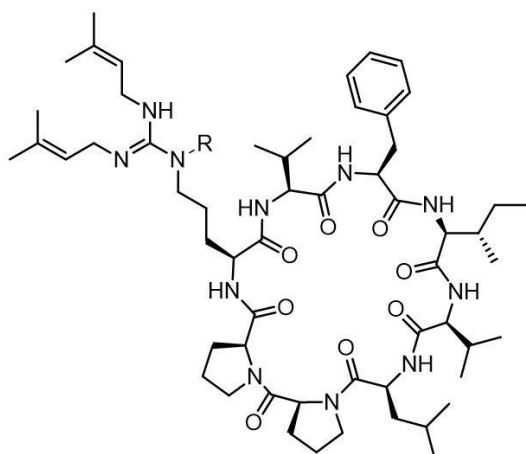
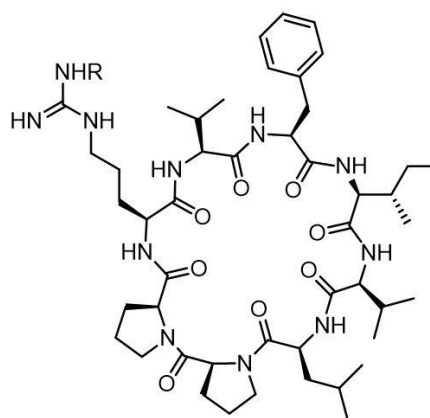


Figure 3: Tandem MS² fragmentation pattern of (a) **1**, (b) argicyclamide A, (c) argicyclamide B and (d) argicyclamide C.



Argicyclamide D (**1**) R = OH
Argicyclamide A (**2**) R = H



Argicyclamide B (**3**) R = dimethylallyl
Argicyclamide C (**4**) R = H

Figure 4: Proposed structure of **1** and structures of argicyclamide A-C (**2-4**).