Changes in secondary metabolic profiles of Microcystis aeruginosa strains in response to intraspecific interactions
Enora Briand, Myriam Bormans, Muriel Gugger, Pieter C. Dorrestein, William H. Gerwick

To cite this version:
Changes in secondary metabolic profiles of *Microcystis aeruginosa* strains in response to intraspecific interactions

Enora Briand¹²*, Myriam Bormans¹, Muriel Gugger³, Pieter C. Dorrestein⁴⁵ and William H. Gerwick²⁵

¹UMR CNRS 6553 ECOBIO, University of Rennes 1, 35042 Rennes Cedex, France, ²Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093, United States, ³Institut Pasteur, Collection of Cyanobacteria, 75724 Paris, France, ⁴Collaborative Mass Spectrometry Innovation Center, University of California San Diego, La Jolla, California 92093, United States, ⁵Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California 92093, United States.

*Corresponding author:

Enora Briand

Mailing address: UMR CNRS 6553 ECOBIO, University of Rennes 1, 35042 Rennes Cedex, France. Phone: 33 (0)2 23 23 57 13. Fax: 33 (0)2 23 23 50 26. E-mail: enora.briand@univ-rennes1.fr

Running title: Cyanopeptides production under mono and co-culture
Summary

The cyanobacteria *Microcystis* proliferate in freshwater ecosystems and produce bioactive compounds including the harmful toxins, microcystins (MC). These secondary metabolites play an important role in shaping community composition through biotic interactions although their role and mode of regulation are poorly understood. As natural cyanobacterial populations include producing and non-producing strains, we tested if the production of a range of peptides by co-existing cells could be regulated through intraspecific interactions. With an innovative co-culturing chamber together with advanced mass spectrometry (MS) techniques, we monitored the growth and compared the metabolic profiles of a MC-producing as well as two non-MC-producing *Microcystis* strains under mono- and co-culture conditions. In monocultures, these strains grew comparably; however, the non-MC-producing mutant produced higher concentrations of cyanopeptolins, aerucyclamides and aeruginosins than the wild-type. Physiological responses to co-culturing were reflected in a quantitative change in the production of the major peptides. Using a MS/MS-based molecular networking approach we identified new analogues of known classes of peptides as well as new compounds. This work provides new insights into the factors that regulate the production of MC and other secondary metabolites in cyanobacteria, and suggests interchangeable or complementary functions allowing bloom-forming cyanobacteria to efficiently colonize and dominate in fluctuating aquatic environments.

Introduction

Freshwater cyanobacteria of the genus *Microcystis* proliferate in many aquatic ecosystems, and due to expected climate and anthropogenic changes, their blooms are predicted to increase in frequency and intensity (Paerl and Paul, 2012). *Microcystis* blooms strongly impact the
functioning of aquatic ecosystems and pose a risk to animal and human healths (Codd et al., 2005; Zanchett and Oliveira-Filho, 2013) because of their ability to produce harmful toxins. Among these, the microcystins (MC), protein phosphatase 1 and 2A inhibitors (MacKintosh et al., 1990), are the most studied and widespread.

Besides the microcystins, Microcystis spp. produces a variety of other bioactive compounds valuable for the biotechnological and biomedical industries (Sivonen and Börner, 2008). They are classified into six major families depending on their structural characteristics: aeruginosins, anabaenopeptins, cyanobactins, cyanopeptolins, microginins and microviridins (Welker and von Döhren, 2006). In the last decade, efforts have been dedicated to understand the biosynthesis of these cyanopeptides. They originate from pathways involving ribosomally synthesized and post-translationally modified peptides (RiPP), non-ribosomal peptide synthetases (NRPS) or mixed polyketide synthases (PKS-NRPS) (Kehr et al., 2011). A number of bioactivities have been reported for these peptides, including cytotoxic, antiviral, antimalarial or allelopathic (Portmann et al., 2008a, b; Sivonen et al., 2010), and the biological mechanisms of action are generally through inhibition of vital eukaryotic enzymes (Ishitsuka et al., 1990; Ishida et al., 2000; Itou et al., 1999; Bister et al., 2004; Ersmark et al., 2008; Sivonen et al., 2010). Despite significant advances in our understanding of the metabolic pathways involved in their production, and of their biological activities, the natural functions and the ecological roles played by these metabolites are still not well understood.

The biosynthesis of cyanobacterial secondary metabolites consumes a great deal of metabolic energy at significant cost to the cell (Herms and Mattson, 1992). Hence, clues to the function of these compounds may be obtained by exploring the growth conditions under which they are produced. To date, most effort at understanding the function of cyanopeptides has
focused on MC. However, even for the best-studied cyanotoxins, several functions are disputed for both the intra- and extracellular forms (for a review see Kaplan et al., 2012). There have been few studies on the other cyanopeptides, and these have mainly focused on their production in several cyanobacterial strains under different growth conditions. The study performed by Rohrlack and Utkilen (2007) on the effects of nutrient and light availability on production of anabaenopeptins and microviridins by *Planktothrix* showed similar patterns of regulation to that of the microcystsins, *e.g.* higher production of the compounds under optimal growth rate conditions (Orr and Jones, 1998). Also, comparison of the production of bioactive compounds by *Anabaena* sp. 90 and its genetically engineered anabaenopeptilide-deficient mutant under various light and phosphate levels revealed that the loss of this oligopeptide resulted in a production increase of the remaining metabolites (Repka et al., 2004). Compensatory mechanisms between and within peptide classes were also observed for *Microcystis* (Tonk et al., 2005), *Planktothrix* (Tonk et al., 2009) and *Radiocystis* (Pereira et al., 2012) in response to various growth conditions. Hence a better understanding of the possible functions of these metabolites requires a holistic approach rather than focusing on a single compound in particular as natural cyanobacterial populations consist of a mixture of producing and non-producing strains for each class of bioactive peptides (Fastner et al., 2001; Welker et al., 2004). Thus, it can be speculated that the production of a range of peptide variants by co-existing cells may be regulated through intraspecific interactions.

To investigate this hypothesis, we studied *Microcystis aeruginosa* strains with the potential to produce several secondary metabolites under different culture conditions. We first compared the growth and the production of bioactive peptides in monoculture of a wild type strain PCC 7806 (WT), its MC-deficient mutant (MT) and a naturally non-MC-producing strain
PCC 9432 (NT). Secondly, we explored whether co-culturing the MC- with one of the non-MC-producing strains affected the growth or the production of cyanopeptides of each strain. For these purposes, we developed a co-culturing chamber device and evaluated variations in metabolite production using MS/MS molecular networking (Wartrous et al., 2012).

Results

_Growth of Microcystis aeruginosa strains in mono-, co-culture and MC-LR addition experiments._ The growth of the three strains (WT, MT and NT) displayed the same growth rates in monoculture (Table 1). In the WT/MT co-culture experiment, no significant difference was observed in growth of WT and MT, whereas in WT/NT co-culture experiment, the NT exhibited a higher growth rate (0.15 ± 0.005 day\(^{-1}\)) than the WT (0.10 ± 0.015 day\(^{-1}\)). In addition, purified MC-LR added to the MT monoculture did not impact its growth response (Table 1).

_Description of secondary metabolic profiles of Microcystis aeruginosa strains in monocultures._ For the three strains in monoculture, we could identify by LC-MS 12 peptides produced by the WT, the same ones without microcystins by the MT, and 12 others by the NT (Fig. 1). From these 24 metabolites (Table 2), 14 could be identified as previously described compounds: 2 anabaenopeptins (compounds 18-19, Fig. 1C), 6 cyanobactins (compounds 2, 10-12, Fig. 1A and B, compounds 16-17, Fig. 1C), 4 cyanopeptolins (compounds 5-8, Fig. 1A and B) and 2 microcystins (compounds 3-4, Fig. 1A) and these compounds were added to the Global Natural Products Social Molecular Networking (GNPS) library (see _Experimental Procedures_). A total of seven other peptides could be identified as members of a known peptide class: one Cl\(_2\)-aeruginosin (compound 1, Fig. 1A and B, Fig. S1), six cyanopeptolins (compound 9, Fig. 1A and
B and compounds 20-24, Fig. 1C), while three peptides remain uncharacterized (compounds 13-15, Fig. 1C, Fig. S2).

The cyanopeptolins were the main peptide class produced by all 3 strains, and 10 variants were identified (compounds 5-9 and 20-24 in Fig. 1). Cyanopeptolins are relatively easily identified from fragment spectra deriving from the highly conserved amino-hydroxy piperidone (Ahp) unit (Welker et al., 2006). Four were already described cyanopeptolin metabolites (Cya A, B, C and Cya 963A with a quasi-molecular ion at \(m/z\) 946 [M-H\(_2\)O]+), whereas the other six are new species with \(m/z\) 896, 915, 949, 963, 965 and 979 Da (Fig. S3 and S4). Fragment patterns obtained from several cyanopeptolin variants (Cya A, B, C, 895 and 963A) produced by the WT and MT strains showed a diagnostic series of fragments indicative of the partial structure Ahp–Leu–MePhe (\(m/z\) 134, 181, 209, 258 and 370; Fig. S3). The cyanopeptolins produced by the NT presented another partial sequence, Ahp–Phe–MePhe, as characterized by the following series of fragments \(m/z\) 134, 215, 243, 292 and 404 (Fig. S4).

Additionally, several cyanobactins were produced by the three strains, all corresponding to previously identified compounds. The WT and MT strains produced the cyclic ribosomal peptides aerucyclamide A, B, C and D (respectively, compounds 10, 11, 12 and 2 in Fig. 1A and B) and the NT produced two short linear cyanobactins: aeruginosamide B and C (compounds 16 and 17 in Fig. 1C). Also, two anabaenopeptins produced by the NT were identified as the cyclic hexapeptides ferintoic acids A and B (compounds 18 and 19 in Fig. 1C).

Changes in the production of the major cyanopeptides by Microcystis aeruginosa strains in mono- and co-culture. We studied changes in cyanopeptides production (1) by the engineered MT when compared to its WT in monoculture and (2) by the three strains in response to intraspecific strain interactions. WT and MT strains produced the same peptides besides MC,
while the NT produced completely different peptides than the other two strains. Hence for the WT in co-culture and the MT in mono- and co-culture, we normalized the concentrations against the WT in monoculture, while for the NT in co-culture we normalized against NT in monoculture.

Figure 2A shows the relative concentrations of the major intracellular compounds for the WT and MT strains under mono- and co-culture conditions. MT, unable to produce any MC, produced significantly greater concentrations of Arg 684, Cya 963A, Aer A, B and C compared to WT in monoculture condition (WT in WT/WT versus MT in MT/MT). Also, a significant production increase of the compounds produced by WT and MT, with the exception of des-MCLR and Aer D, was observed in the co-culture treatments (WT in WT/WT versus WT in WT/MT and MT in MT/MT versus MT in WT/MT). The up-regulated compounds observed under WT/MT co-culture condition were always higher in MT cells than in WT cells (WT in WT/MT versus MT in WT/MT). We further investigated the effects of adding purified MC-LR on the intracellular metabolic profile of MT in monoculture (Fig. S5). No significant change in the production of peptides between the MT and MT+MCLR in monoculture was observed (MT in MT/MT versus MT+MCLR in MT/MT). Moreover the peptides concentrations in MT+MCLR were always lower than in MT cells under co-culture conditions (MT in WT/MT versus MT+MCLR in MT/MT). Also, no significant difference was observed in the production of the compounds by the WT when co-cultured with the NT (WT in WT/WT versus WT in WT/NT). However, a significant production increase of five major compounds produced by NT (Cya 962, Cya 978, Aeg B, Fer A and P 646) under co-culture conditions was observed (NT in NT/NT versus NT in WT/NT, Fig. 2B).
The cell-free supernatant of the strains under mono- and co-culture conditions was analyzed by HPLC-MS/MS in order to determine whether the major compounds detected in the intracellular fraction are released into the media by the cells due to lysis and/or active transport. Most of the compounds detected in the intracellular fractions were also detected in the media of the three strains in mono- and co-culture experiments (Fig. 3A and B), except the compound Arg 684 that was not detected in the media in both co-culture experiments (Fig. 3A). Compounds detected at higher concentrations in MT cells compared to the WT were not necessarily found at higher concentrations in the MT media (WT in WT/WT versus MT in MT/MT; Fig. 3A), except for Arg 684 and Cya A. Likewise, only Cya 963A in WT/MT co-culture (Fig. 3A) and Aeg B and C in WT/NT co-culture (Fig. 3B) were detected at a significantly higher concentration in the media under co-culture than under monoculture. In co-culture conditions, the relative concentrations of compounds were not significantly different between the two compartments as expected from diffusion of dissolved compounds across the membrane (data not shown).

**Molecular networking as a powerful tool for identification and relative quantification of analogues and unknown compounds.** Ionizable metabolomes of the three strains under the different culture conditions were subjected to mass spectral networking and searched against annotated reference spectra in the GNPS spectral library. The resulting network of WT and MT intra- and extracellular metabolomes under mono- and co-culture conditions represented 263 total nodes (Fig. 4A). Analysis of the molecular networking data resulted in dereplication of MC-LR, Des-MCLR (Fig. 4B), Cya A, B, C, 963A and 895 (Fig. 4C and D), Aer A, B, C and D (Fig. 4E and F) and Arg 684 as a single node.

The network further confirmed that MC-LR and Des-MCLR were exclusively produced by the WT cells in both the mono- and co-culture experiments and were present in the media of
monoculture and co-culture with the WT (Fig. 4B).

The identification of analogues to known compounds via the molecular networking approach indicated a cyanopeptolin cluster (Fig. 4C and D) with the presence of two new analogues (m/z 918 and 936). Their precursor masses had distinctive fragmentation patterns characteristic of the partial structure of cyanopeptolin-type peptides, Ahp-Leu-MePhe, described above (Fig. S3). Interestingly, the m/z 975 node corresponded to the hydrated form of Cya A and was exclusively present in the media of these strains. The aerucyclamide cluster (Fig. 4E and F) had nodes representing two novel analogues with m/z 521 and m/z 643 closely related to Aer A and Aer D, respectively.

Another powerful feature of this approach was the quantitative color-coding of nodes according to their origin by using the pie-chart function. To evaluate the accuracy of the spectral count quantitation method, a correlation analysis was performed between the spectral counts and the internal standard methods. A highly significant correlation was obtained (Pearson’s R² = 0.7320, P < 0.0001 for intracellular and R² = 0.7042, P < 0.0001 for extracellular quantitation, Fig. S6). Hence, the spectral count with pie-chart display was considered a reliable proxy for the relative quantification of these metabolites. This approach was used to differentiate major producers of a given compound from minor ones according to the culture condition. The quantitative information given by this representation suggested that compounds (Cya C, cyanopeptolin new analogues m/z 896, 918, 936 and aerucyclamide new analogues m/z 521 and 643; Fig. 4C, D, E and F) were mainly produced by the MT strain. A detailed analysis of the LC-MS data revealed that these minor compounds were also produced by the WT strain but not detected through the molecular networking because of their low abundance.
Analysis of the molecular networking data of the WT and NT intra- and extracellular metabolomes under mono- and co-culture conditions contained 280 total nodes (Fig. 5A). It resulted in dereplication of MCs, Cya A, Cya 963A, Cya 895, Aer A, Aer B, Aer C and Aer D produced by WT and Aeg B, Aeg C, Fer A, Fer B and 5 cyanopeptolins produced by NT with the partial structure Ahp-Phe-MePhe (Cya 914, 948, 962, 964 and 978; Fig. S4). Three new analogues of aeruginosamide ($m/z$ 650, 660 and 749) were present in the media under WT/NT co-culture conditions (Fig. 5B). They were also observed in the media of NT strain under monoculture conditions but did not appear in the network due to their low abundance. The molecular networking of the unknown peptide (P 680), identified as a di-chlorinated compound (Fig. S2), indicated the presence of a new analogue ($m/z$ 611) present in the media under co-culture conditions (Fig. 5C).

**Discussion**

This is the first study to describe the potential of *Microcystis* strains to produce several bioactive compounds in response to intraspecific strain interactions. More precisely, we demonstrated changes in the relative concentration of a number of small peptides under mono- and co-culture conditions.

Our innovative purpose-built chamber allowed two different strains to be physically separated while sharing a common culture medium allowing the study of intraspecific interactions mediated by diffusible signals that can influence the growth of the strains and the production of targeted cyanobacterial secondary metabolites. In previous studies, the use of co-culture chambers without physical contact has facilitated the investigation of chemical

**LC-MS and molecular networking approaches revealed the high chemodiversity of *Microcystis***. To profile the chemical output from MC- and non-MC-producing *Microcystis aeruginosa* strains, two complementary approaches were used. The LC-MS approach allowed a rapid identification of major secondary metabolites while the MS/MS-based molecular networking approach helped to visualize and identify structurally related compounds as well as unknown ones. A total of 32 peptides were detected, including 8 highlighted only from molecular networking. Among them, 14 could be identified as previously described metabolites: 2 microcystins, 4 cyanopeptolins, 6 cyanobactins and 2 anabaenopeptins. A total of 14 additional peptides could be identified as members of known peptide classes (1 Cl2-aeruginosin, 8 cyanopeptolins, 2 aerucyclamides and 3 aeruginosamides), while 4 peptides remain to be characterized (P 680, P 646, P 612 and P 610). Further chemical studies are needed to firmly establish their structures.

We also gained new insight into the wide diversity of secondary metabolites produced by these freshwater cyanobacteria as the spectral networks identified a total of 546 compounds. Recent genome mining of the studied strains has revealed their potential to produce more natural products than the microcystins, cyanopeptolins, aeruginosins, cyanobactins and anabaenopeptins (Frangeul *et al.*, 2008; Humbert *et al.*, 2013; Leikoski *et al.*, 2013). The WT and NT genomes contain, respectively, 7 and 9 gene clusters involved in the biosynthesis of secondary metabolites (Humbert *et al.*, 2013). Notably, they possess the microviridin and microginin biosynthetic gene clusters, but none of these compounds were identified in our study, suggesting that they were not produced under the growth conditions selected, or alternatively, not detected because their mass
spectrometry signals were too weak. In addition, three orphan PKS gene clusters encoded unknown compounds were described in these strains (Humbert et al., 2013) including a PKSI iterative gene cluster containing halogenase genes that may encode for enzymes involved in the biosynthesis of the unidentified but structurally related non-, mono- and dichlorinated peptides produced by the NT strain in this study (P 680, P 646, P 612 and P 610). As this PKS1 iterative gene cluster was recently found in other cyanobacterial genomes not related to Microcystis (Calteau et al., 2014), additional biochemical studies are needed to compare the metabolite profiles of the corresponding strains and identify this family of compounds. The expansion of genomic mining techniques to predict structures and identify products of new pathways remains a challenge and opportunity for the future.

**Microcystis bioactive peptides may have complementary or interchangeable functions.** Despite their different metabolic profiles, the three strains grew comparably under monoculture conditions, as reported for the WT and MT under several growth conditions (Dittmann et al., 1997; Hesse et al., 2001; Briand et al., 2012). Similarly, an Anabaena mutant with a disrupted anabaenopeptilide synthetase grew similarly to the wild-type under variable growth conditions (Repka et al., 2004), suggesting that cyanobacterial bioactive compounds are not essential for the growth of the producing cells.

Changes in the intracellular concentrations of peptides between WT and MT strains in monoculture were observed. The production of several peptides by MT strain was stimulated with a significant increase in concentration up to 12-fold for Arg 684, 8-fold for Cya 963A and from 3 to 4-fold for Aer A, B and C over WT, and several new analogues of the cyanopeptolins and aerucyclamides were mainly produced by the MT. The addition of purified MC-LR to the MT did not affect the level of the other peptides in MT cells, and the intracellular concentration
of these other peptides in MT cells remained much higher than in the WT ones. This suggests that the MC-LR was not sensed as a signaling peptide regulating the production of the other peptides in the MT. Our results are in accordance with those found for an anabaenopeptilide-deficient mutant of *Anabaena* sp. 90 (Repka *et al*., 2004) in which higher concentrations of the remaining oligopeptides (anabaenopeptins and microcystins) were observed. A differential allocation of the energy used for secondary metabolism between the WT and the MT may explain the observed quantitative changes in intracellular metabolic profiles. The MT may allocate more energy in the production of the remaining peptides. Alternatively, compensatory intracellular mechanisms might cause the observed effect, as they were also observed within classes of peptides in *Microcystis aeruginosa* PCC 7806 (Tonk *et al*., 2009), in *Planktothrix agardhii* NIVA-CYA 126/3 strain (Tonk *et al*., 2005) and in *Radiocystis fernandoii* (Pereira *et al*., 2012). These studies suggest the possibility of a compensation for the absence or decreased production of one compound by increasing the production of others and that these compounds may have interchangeable or complementary functions. A further suggestion of a mutual function of these cyanobacterial metabolites would be a common distribution of a group of secondary metabolites within a species. Recently, the microcystins, aeruginosins and cyanopeptolins were reported as the most widely distributed secondary metabolites in *Microcystis* (Humbert *et al*., 2013), while anabaenopeptins, cyanopeptolins and microcystins are metabolites that occurred most frequently in *Planktothrix* (Kurmayer *et al*., 2014). Further genome sequencing efforts of cyanobacterial species are required to study the distribution of genes involved in the biosynthesis of cyanobacterial secondary metabolites.

**Co-culture enhanced the production of Microcystis bioactive peptides.** Under co-culture conditions, although no significant difference between the WT and MT growth rates was
observed, the NT strain displayed a higher growth rate than the WT strain. Previous co-culture studies involving direct cell contact, between WT and MT strains (Van de Waal et al., 2011; Briand et al., 2012) and between MC- and naturally non-MC-producing *Microcystis aeruginosa* strains (Kardinaal et al., 2007; LeBlanc Renaud et al., 2011; Van de Waal et al., 2011; Lei et al., 2015) or *Planktothrix agardhii* strains (Briand et al., 2008), showed differences in the fitness of the compared strains under different growth conditions. However, these differences cannot be easily compared due to the diversity of abiotic factors tested and the specificity and ecophysiology of the different strains studied, highlighting the difficulty to predict clear competition outcome. We suggested in a previous related study (Briand et al., 2012) that differences in the fitness between monocultures and co-cultures with physical contact were attributable to cooperation processes involving diffusive chemicals. However, in the absence of physical contact as highlighted in this study, it cannot be ruled out that WT-derived compounds may induce chemical signals facilitating the growth of the NT strain. Rather than MC-LR that was found not to be the inducible metabolite (Briand et al., 2012; this study), compounds exclusively detected in the co-culture media (yellow nodes in molecular networks) should be considered and studied in more details. However, a final answer can only be found through bioassay-guided structure elucidation of the active compounds. Taken together, these results suggest that cooperation processes may take place preferentially when the cells are physically in contact, but additional experiments need to be performed to explore this hypothesis.

This work shows that even without cell contact, physiological responses to co-culturing are reflected in the stimulation of the production of the major produced peptides. Likewise, it was previously observed that MC production was increased in WT under co-culture conditions with MT (Briand et al., 2012). Moreover, the addition of MC-producing cell extract, external
MC-LR, micropeptin or microginin induced a specific response on secondary metabolite gene expression (Schatz et al., 2007; Makower et al., 2014). Although in this study, the addition of MC-LR did not affect the synthesis of the other metabolites, it cannot be ruled out that MCs and/or other secondary metabolites may play roles in cell-to-cell communication and that the expression of these metabolites may be interrelated, as suggested in previous studies (Schatz et al., 2007; Makower et al., 2014). Likewise, the compound Arg 684, that was present in WT and MT monoculture media, was not detected in the co-culture media (WT/MT and WT/NT), indicating an obvious interaction involving released metabolites. Inhibition of excretion or transformation of this compound by the co-cultured strains might cause the observed effect. Co-culture experiments are a powerful experimental approach that have enhanced the production of constitutively present compounds and/or induced silent gene clusters in order to reveal an increased chemical diversity of microorganisms (mainly bacteria and fungi) (Bertrand et al., 2014; Marmann et al., 2014). From an ecological perspective, co-cultures mimic ecological situations as microorganisms always co-exist within complex microbial communities, and thus are useful to the study of intra- and interspecies interactions (Paul et al., 2013; Schatz et al., 2005; Briand et al., 2012).

**Role and ecological relevance of cyanobacterial bioactive peptides.** The production of a range of peptide variants with interchangeable or complementary functions may provide cyanobacteria with a competitive advantage in a variable environment through inter- and intraspecific interactions. Indeed, it should be an ecological advantage for species to co-exist with chemically diverse strains: (1) as defensive compounds against grazers (Wiegand and Pflugmacher, 2005; Agrawal and Agrawal, 2011; Penn et al., 2014; Sadler and von Elert, 2014), (2) as allelopathic metabolites against other cyanobacteria, algae and plants (Leão et al., 2009; Svercel, 2013), or
(3) to provide resistance against rapidly evolving parasites (chytrid fungi, cyanophages). It has recently been shown that microcystins, microviridins and anabaenopeptins can reduce the virulence of parasitic chytrid fungi to *Planktothrix* due to inhibition of chytrid proteases that are involved in their pathogenicity (Rohrlack *et al*., 2013). Because cyanobacterial metabolites are predominantly intracellular, a biological role of cyanopeptides as defense mechanisms against fungal parasites and most likely other pathogens which are endocellular parasites is highly plausible. From this study, a further indication of the intracellular compartmentalization of these bioactive compounds was suggested. The absence of higher concentrations of metabolites in the media in relation to higher concentrations measured in the cells suggested that the studied cyanopeptides are only modestly released. Numerous studies suggested an intracellular role of MC giving competitive advantage to the toxic strains under different abiotic conditions. These include putative roles in the tolerance of oxidative stress (Alexova *et al*., 2011; Zilliges *et al*., 2011) or in carbon metabolism (Van de Waal *et al*., 2011). Hence, we can wonder whether the expression and the production of the other peptides are modified under stress conditions. Subsequent studies will have to be undertaken to further explore the regulation of the overall secondary metabolites in response to biotic and abiotic stress conditions. Most likely, these secondary metabolites have several functions, thus allowing cyanobacteria to efficiently colonize and dominate highly variable aquatic environments. However, the lack of field studies, showing the presence of active substances in the environment and their effect on target organisms, is evident and needs to be filled.

**Experimental procedures**

*Co-culture setup*
A purpose-built (Plexiglas) growth incubator was constructed in-house (SIO), in which two cultures were physically separated by a 0.45 µm cellulose nitrate membrane filter (Whatman, Buckinghamshire, UK) that enabled the passage of fluids and dissolved substances, but not cells (Fig. 6).

The exchange of dissolved organic compounds between the two chambers was tested knowing that compounds of around 100 Da diffuse within the first few hours (Paul et al., 2013). Thus, we monitored after 12, 24, 48 and 72 h the diffusion of the MC-LR standard (995 Da, Abraxis-LLC, Warminster, PA, USA, 0.1 mg l\(^{-1}\) final concentration), one of the highest molecular weight metabolites produced by the studied strains. The concentration of MC-LR nearly reached equilibrium between the two compartments within 48 h (Fig. S7).

**Strains and culture conditions**

Three axenic *M. aeruginosa* strains with different metabolic profiles (Frangeul et al., 2008; Humbert et al., 2013) were used: the MC-producing PCC 7806 (WT), a non-MC-producing mutant of PCC 7806 (MT, Dittmann et al., 1997) and a naturally occurring non-MC-producing strain PCC 9432 (NT). The cultures were cultivated in BG1\(_{10}\) medium supplemented with 1.8 mM of NaNO\(_3\) and 10 mM of NaHCO\(_3\) (hereafter BG1\(_{10}\) modified medium, Rippka and Herdman, 1992) and 5 µg ml\(^{-1}\) of chloramphenicol was added to the MT. The cultures grown under a 12:12 h light:dark regime using daylight white fluorescent tubes (Toshiba, 15 W, FL15D) with 35 µmol m\(^{-2}\) s\(^{-1}\) illumination at a constant temperature of 23 °C. The cultures were maintained in exponential growth phase by repeated dilution in fresh culture medium, while the axenicity was regularly evaluated as in Briand et al. (2012). The strains are available from the Pasteur Culture collection of Cyanobacteria (http://cyanobacteria.web.pasteur.fr/).

**Mono- and co-culture experiments**
Monocultures of each strain (WT/WT, MT/MT and NT/NT) in duplicate were initiated by inoculation of $2.5 \times 10^6$ cells ml$^{-1}$ in each culture chamber. Inoculates of cultures were centrifuged (10 min at $4000 \times g$ and $23 \, ^{\circ}C$) and washed with sterile Milli-Q water twice before being added to the respective chamber containing 425 ml of BG110 modified medium without Cm in order to exclude any impact of the antibiotic on cellular metabolism. Co-culturing experiments (WT/MT) and (WT/NT) in triplicate were inoculated into both chambers under the same culture conditions. The devices were gently shaken manually twice a day. Samples (1 ml) were withdrawn every two days to estimate cell growth. In addition, a monoculture of MT supplemented with MC-LR (0.1 mg l$^{-1}$ final concentration) was performed to investigate the possible role of the purified toxin on growth and/or secondary metabolite profiles of MT.

**Growth monitoring**

*Microcystis* growth was estimated by converting optical density (OD) at 750 nm, measured using a DU 800 spectrophotometer (Beckman Coulter, Brea, CA, USA), into cell abundance (cells ml$^{-1}$) based on the highly significant positive correlation between these two parameters ($R^2 = 0.9547$, $N = 50$; $P < 0.0001$, data not shown). Cell abundance was estimated using a Neubauer counting chamber with an Olympus IX51 inverted microscope at 400× magnification (Olympus Optical Co, Tokyo, Japan). Growth rates were calculated during the exponential growth phase according to the following equation: $(\ln N_2 - \ln N_1) / (t_2 - t_1)$, where $N_1$ and $N_2$ are cell densities at time $t_1$ and $t_2$, respectively.

**Sampling, extraction and internal standard addition**

At the end of the experiment (day 12, corresponding to the end of the exponential growth phase), the volume of each chamber was harvested separately and centrifuged (for 10 min at $4000 \times g$ and $23 \, ^{\circ}C$). Freeze-dried biomass was extracted up to three times in 2:1 dichloromethane:
methanol (DCM:MeOH, JT Baker, Center Valley, PA, USA) and were evaporated to dryness. The supernatants were extracted on SPE-C18 cartridges (GracePure™, 5000 mg, Columbia, MD, USA). Samples were evaporated to dryness under N₂ and kept frozen until analysis.

Dry intra- and extracellular extracts were dissolved with acetonitrile (ACN, EMD Chemicals, Gibbstown, NJ, USA) and internal standard (BOC-L-protected Ornithine, 0.25 mg ml⁻¹ in ACN, Chem-Impex International, Wood Dale, IL, USA) were added before HPLC-MS/MS analysis.

**High-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS/MS) analysis**

Each sample (20 µl) was injected twice into a reverse-phase HPLC system using a Phenomenex Kinetex C18 column (5 µm, 100 mm × 4.60 mm) with a gradient of 5%-99% ACN in water with 0.1 % formic acid over 12 min, held at 99% for 5 min, and then returned to 5% at 22 min for another 3 min. The solvents were LC-MS grade (JT Baker, Center Valley, PA, USA). The flow rate was 0.7 ml min⁻¹.

The HPLC eluate was electrospray ionized (capillary temperature at 325 °C, source voltage at 5 kV and a sheath gas flow rate of 69 l min⁻¹) and analyzed in the positive mode in the mass range of m/z from 300 to 2000 using a Thermo-Finnigan LCQ Advantage ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). MS/MS spectra were obtained in a data-dependent manner using collision-induced dissociation at 35 eV.

**Identification of metabolites, molecular networking and relative quantification**

We used two complementary approaches to identify cyanobacterial secondary metabolites. In the first approach, mass spectra were scanned for molecular ion masses of known peptides and their respective adducts (e.g. sodium adducts with Δm = 22 Da compared to the singly protonated
molecular mass), and loss of water peaks ($\Delta m = 18$ Da). Further, the mass differences between and the relative intensity of isotopic peaks were analyzed to gain information on the presence of possible halogen atoms (e.g. chlorine, bromine). MS/MS data were then analyzed for indicative fragments or series of fragments by comparison to fragment spectra of known peptides according to Welker et al. (2006).

In our second approach, we applied a MS/MS-based Molecular Networking (Wartrous et al., 2012). The MS/MS data were subjected to the Molecular Networking workflow of Global Natural Products Social Molecular (GNPS at gnps.ucsd.edu, M. Wang et al., in preparation) using the Group Mapping feature. The input data were simultaneously searched against annotated reference spectra in the GNPS spectral library. Algorithms assumed a precursor mass tolerance of 2 Da and a fragment mass tolerance of 0.5 Da. Pairs of consensus spectra were aligned if both spectra fell within the top 10 alignments for each of the respective spectra, the cosine of their peak match scores was $\geq 0.7$ and the minimum matched peaks was 6. The maximum size of connected components allowed in the network was 50 and the minimum number of spectra to form a cluster was 2. The networks were visualized in the software program Cytoscape (Smoot et al., 2011), where consensus spectra are represented as nodes connected by edges to aligning nodes. If different strains in different growth conditions were found to produce the same compound, then that compound’s node was represented with a multi-colored pie chart (Winnikoff et al., 2014). To further simplify the network, the background nodes from solvent and internal standard were removed.

In order to compare relative concentrations of specific metabolites between different treatments, the peak area was determined with the software Xcalibur (Thermo) using the Peak Detection Genesis Algorithm. The quantification was based on the ratio of the peak area of
metabolites and the added internal standard, BOC-L-protected ornithine. Data were then normalized to the dry weight for intracellular metabolites and to the volume for extracellular metabolites as described in Winnikoff et al. (2014).

**Statistical analysis**

Differences in growth rates and normalized relative concentrations were evaluated using One-way ANOVA with Tukey’s post test or Two-tailed t-test. The analyses were conducted with the software Graphpad Prism 4.00 (San Diego, CA, USA). In all cases, differences were accepted as significant when $P < 0.05$. Values are given as mean ± standard deviation (SD).
Acknowledgments

EB was supported by a Marie Curie IOF Fellowship within the 7th European Community Framework Program (FP7-PEOPLE-2011-IOF, grant number 301244-CYANOMIC). Mass spectrometry and molecular networking was supported by NIH grant GM107550. We are thankful to the two anonymous reviewers for their constructive comments, which helped us to improve the manuscript. The authors declare no conflict of interest.

Conflict of Interest Statement

The authors declare no conflict of interest.
References


Lei, L., Li, C., Peng, L., and Han, B.P. (2015) Competition between toxic and non-toxic


Table and Figure legends

Table 1. Growth rates of the wild type PCC 7806 (WT), its MC-deficient mutant (MT, with or without addition of MC-LR standard) and the PCC 9432 (NT) strains in mono- and co-culture (WT/MT, WT/NT) conditions. Different letters indicate significant difference. Tukey’s test, \( P < 0.05 \) after one-way analysis of variance (ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>Monoculture</th>
<th>Co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT/MT</td>
</tr>
<tr>
<td>WT</td>
<td>0.10 ± 0.02 (a)</td>
<td>0.12 ± 0.01 (ab)</td>
</tr>
<tr>
<td>MT</td>
<td>0.09 ± 0.02 (a)</td>
<td>0.12 ± 0.02 (ab)</td>
</tr>
<tr>
<td>NT</td>
<td>0.12 ± 0.01 (a)</td>
<td>0.15 ± 0.01 (b)</td>
</tr>
<tr>
<td>MT + MC-LR</td>
<td>0.11 ± 0.01 (a)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Peptides produced by the studied strains.

<table>
<thead>
<tr>
<th>Peptide class</th>
<th>[m/z [M + H]^+]</th>
<th>Peak number (retention time in min)</th>
<th>Assignment (reference)</th>
<th>Comment</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystin (MC)</td>
<td>981</td>
<td>3 (11.1)</td>
<td>Dee-MCLR (Mayumi et al., 2008)</td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>995</td>
<td>4 (11.2)</td>
<td>Microcin 604</td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Acruginosin (Arg)</td>
<td>955</td>
<td>1 (9.3)</td>
<td>Acruginosin 955</td>
<td>Cl₂</td>
<td>WT, MT</td>
</tr>
<tr>
<td>Cyanopeptolin (Cys)</td>
<td>906</td>
<td>9 (13.5)</td>
<td>Cyanopeptolin 895</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>928</td>
<td>5 (11.4)</td>
<td>Cyanopeptolin B (Martin et al., 1993)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>943</td>
<td>6 (11.5)</td>
<td>Cyanopeptolin C (Martin et al., 1993)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>946 [M-H₂O]^+</td>
<td>8 (13.0)</td>
<td>Cyanopeptolin 963A (Elsie et al., 2004)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>957</td>
<td>7 (11.6)</td>
<td>Cyanopeptolin A (Martin et al., 1993)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>915</td>
<td>22 (12.4)</td>
<td>Cyanopeptolin 914</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>949</td>
<td>23 (12.5)</td>
<td>Cyanopeptolin 948</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>963</td>
<td>24 (12.9)</td>
<td>Cyanopeptolin 962</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>965</td>
<td>20 (12.1)</td>
<td>Cyanopeptolin 964</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>979</td>
<td>21 (12.2)</td>
<td>Cyanopeptolin 978</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>cyanobactin (Aer)</td>
<td>917</td>
<td>11 (14.9)</td>
<td>Aerocyclamide C (Portmann et al., 2008a)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>933</td>
<td>12 (14.6)</td>
<td>Aerocyclamide B (Portmann et al., 2008a)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>953</td>
<td>10 (14.0)</td>
<td>Aerocyclamide A (Portmann et al., 2008a)</td>
<td>WT, MT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>903 [M + OH]^+</td>
<td>2 (10.7)</td>
<td>Aerocyclamide D (Portmann et al., 2008b)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>anaibaenaspeptin (Fer)</td>
<td>975</td>
<td>18 (11.3)</td>
<td>Acruginosinol A (Lukaski et al., 2013)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>974</td>
<td>17 (11.6)</td>
<td>Acruginosinol C (Lukaski et al., 2013)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>987</td>
<td>13 (9.8)</td>
<td>Peptide 612</td>
<td>Cl₂</td>
<td>NT</td>
</tr>
<tr>
<td>unknown (P)</td>
<td>631</td>
<td>14 (9.7)</td>
<td>Peptide 646</td>
<td>Cl₂</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>647</td>
<td>12 (10.1)</td>
<td>Peptide 690</td>
<td>Cl₂</td>
<td>NT</td>
</tr>
</tbody>
</table>
Fig. 1. Chromatograms obtained for WT (A), MT (B) and NT (C) in monoculture. Peaks legend (full names are given in Table 2): 1- Arg 684, 2- Aer D, 3- Des-MCLR, 4- MCLR, 5- Cya B, 6- Cya C, 7- Cya A, 8- Cya 963A, 9- Cya 895, 10- Aer A, 11- Aer C, 12- Aer B, 13- P 612, 14- P 646, 15- P 680, 16- Aeg B, 17- Aeg C, 18- Fer A, 19- Fer B, 20- Cya 964, 21- Cya 978, 22- Cya 914, 23- Cya 948, 24- Cya 962, IS: Internal Standard (retention time = 12.6 min).
**Fig. 2.** Relative concentrations given as a fold change over WT (A) or NT (B) in monoculture of selected intracellular compounds for WT, MT and NT strains under mono- (WT/WT, MT/MT, NT/NT) and co-culture (WT/MT, WT/NT) conditions and statistical results. MC: Microcystin, Arg: Aeruginosin, Cya: Cyanopeptolin, Aer: Aerucyclamide, Aeg: Aeruginosamide, Fer: Ferintoic acid.
**Fig. 3.** Relative concentrations given as a fold change over WT (A) or NT (B) in monoculture of selected extracellular compounds for WT, MT and NT strains under mono- (WT/WT, MT/MT, NT/NT) and co-culture (WT/MT, WT/NT) conditions and statistical results. MC: Microcystin, Arg: Aeruginosin, Cya: Cyanopeptolin, Aer: Aerucyclamide, Aeg: Aeruginosamide, Fer: Ferintoic acid, nd: not detected.
A: WT/MT

Cells

WT in WT/WT
MT in MT/MT
WT in WT/MT
MT in WT/MT

Media

Arg 684
Fig. 4. A) WT and MT intra- and extracellular molecular network under mono- (WT/WT, MT/MT) and WT/MT co-culture conditions. Larger circles indicate matches with the GNPS library. B) Molecular network of MS/MS spectra of microcystin variants: MC-LR (m/z 995) and Des-MCLR (m/z 981). C) and D) Molecular network of MS/MS spectra of cyanopeptolin variants with the common structure Ahp-Leu-MePhe. E) and F) Molecular network of MS/MS spectra of aerucyclamide variants.
Fig. 5. A) WT and NT intra- and extracellular molecular network under mono- (WT/WT, NT/NT) and WT/NT co-culture conditions. Larger circles indicate matches with the GNPS library. B) Molecular network of MS/MS spectra of aeruginosamide variants. Compounds produced by WT are in black rectangles and those produced by NT are in grey ones. C) Molecular network of MS/MS spectra of unknown peptides.
**Fig. 6.** Co-culture device to grow strains without physical contact but ensuring the exchange of dissolved solutes including secondary metabolites. **A)** The disassembled device with all parts required for the setup, and **B)** the assembled co-culture device. A 0.45 µm membrane separates both chambers that can each be filled with up to 425 ml medium. The two chambers were held together by four screws and sealed with an O-ring. Each culturing chamber had a 20 mm opening at the top for filling and sampling purposes. The black bar represents 10 cm.


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Identification of Cl₂-aeruginosin 684. The WT and MT strains produced an aeruginosin with a mass of \( m/z \) 685Da. This compound presented an isotopic pattern corresponding to a dichlorinated peptide (detection of pseudo-molecular peaks of M+, M+2 and M+4 Da, top). Its fragmentation pattern was characteristic of the aeruginosins with two intense fragments peaks at \( m/z \) 140 and 122 Da indicative of the Choi-immonium ion and its dehydrated derivative (bottom, Welker et al., 2006).

**Fig. S2.** Identification of three new structural related compounds (P 612, 646 and 680). Partial mass spectrum of NT strain showing three molecular ion peaks (\( m/z \) 613, 647 and 681) with a 34 Da increment indicative of non-, mono- and dichlorinated variants of a peptide. This was also supported by the detection of pseudo-molecular ion peaks (M+2 Da) with increasing relative intensity, corresponding well to theoretical isotopic patterns for chlorinated peptides.

**Fig. S3.** Identification of cyanopeptolins variants produced by WT and MT strains. MS/MS data of each variants: A) Cya A, B) Cya B, C) Cya C, D) Cya 895 and E) Cya 963A. Grey bars indicate fragment series used to distinguish cyanopeptolins.

**Fig. S4.** Identification of cyanopeptolins variants produced by NT strain. MS/MS data of each variant: A) Cya 914, B) Cya 948, C) Cya 962, D) Cya 964 and E) Cya 978. Grey bars indicate fragment series used to distinguished cyanopeptolins.
**Fig. S5.** Relative concentrations given as a fold change over WT in monoculture of selected intracellular compounds for WT, MT and MT with MC-LR under mono- (WT/WT, MT/MT) and co-culture (WT/MT) conditions and statistical results. MC: Microcystin, Arg: Aeruginosin, Cya: Cyanopeptolin, Aer: Aerucyclamide.

**Fig. S6.** Correlation between the relative concentrations of major compounds produced in the WT/MT experiment determined by the addition of internal standard and by the spectral counts obtained for each compound and represented by the pie-chart. A) Intracellular compounds and B) extracellular compounds.

**Fig. S7.** Over time diffusion of MC-LR through a 0.45 µm membrane in co-culture chambers.