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Sunscreens of Cyanobacteria: Unravelling the Genetic, Molecular, and Structural Diversity of Mycosporine-like Amino Acids

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ACADEMIC DISSERTATION

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***

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***

2 List of original publications

This thesis based on the research published in following publications:

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II. “A refactored biosynthetic pathway for the production of glycosylated microbial sunscreens” - Sıla Arsın, Endrews Delbaje, Maija Pollari, Jouni Jokela, Matti Wahlsten, Perttu Permi, David Fewer., 20 Aug 2024, In: **RSC Chemical Biology**. DOI: 10.1039/D4CB00128A

III. “Direct evidence for production of microbial sunscreens by scum-forming cyanobacteria from the Baltic Sea” - Inkeri Vuori, Greta Gaiani, Endrews Delbaje, Sıla Arsın, Jouni Jokela, Matti Wahlsten, Perttu Permi, David Fewer., 16 Jan 2025, In: **Environmental Microbiology Reports** DOI: 10.1111/1758-2229.70056

Author Contributions:

I. Sıla Arsın was involved in the design and planning of the study. She supervised and performed experiments, interpreted the results and wrote and edited the manuscript for publication.

II. Sıla Arsın was involved in the design and planning of the study. She performed most of the experiments, interpreted the results and wrote and edited the manuscript for publication.

III. Sıla Arsın was involved in conceptualisation of the study. She mentored and supported research activities, helped interpret the results and was involved in the writing & editing of the manuscript for publication.

3 Abbreviations

Ala – Alanine
Gly – Glycine
Lys – Lysine
Orn – Ornithine
Pro – Proline
Ser – Serine
Tau – Taurine
Thr – Threonine
Da – Dalton
BGC – Biosynthetic gene cluster
DAHP – 3-deoxy-arabino heptanoic acid-7-phosphate
GOE – Great Oxidation Event
HR-LCMS – High resolution liquid chromatography mass spectrometry
m/z – Mass to charge ratio
MAAs - Mycosporine-like amino acids
MGA ligase – Mycosporine-glycine-amine ligase
NMR – Nuclear magnetic resonance spectroscopy
NPRS – Non-ribosomal peptide synthetase
ROS – Reactive oxygen species
S7P – Sedoheptulose-7-phosphate
UPLC/HPLC – Ultra/High – performance liquid chromatography
UV-A – Ultraviolet A radiation
UV-B – Ultraviolet B radiation
UV-C – Ultraviolet C radiation
UV-R - Ultraviolet radiation
λ_{max} – Absorption maxima

4 Abstract

Mycosporine-like amino acids (MAAs) are small, colourless molecules with high extinction coefficients. They are highly stable and can act as effective sunscreens for the many producing organisms alongside having antioxidant and osmolyte capabilities. MAAs' unique mechanism of allows for the dissipation of the UV radiation absorbed as heat without producing radical oxygen species. Currently, over 70 different MAA structural variants have been reported with varying degrees of additional properties, such as wound healing. Their effectivity and non-toxic nature garnered three decades long interest for research and biotechnological applications. However, the bottleneck of naturally low production levels and limited understanding of their biosynthetic pathways, these compounds are yet to be utilized commercially.

Cyanobacteria are one of the most prolific MAA-producing organisms. Taking a multidisciplinary approach, this thesis attempted to broaden our understanding of the genetic and molecular diversity of MAA biosynthetic pathways in cyanobacteria as it relates to the structural diversity. Studies in this thesis focused on the cyanobacterial cultures maintained at the University of Helsinki Culture Collection (UHCC) but also extended analyses to investigate the distribution of different MAA biosynthetic pathways across other publicly available cyanobacterial genomes as well as field samples to provide a better understanding of their ecological roles and evolutionary path. Employing high-resolution liquid chromatography mass spectrometry (HR-LCMS) and nuclear magnetic resonance spectroscopy (NMR) techniques, we identified and characterized novel MAA structural variants. Namely, the novel Aplysiapalythine E and previously reported Tricore B produced by the lichen-symbiont *Nostoc* sp. UHCC 0926 and the novel diglycosylated MAA structural variants from the benthic *Nostoc* sp. UHCC 0302. We obtained complete genomes for both *Nostoc* sp. UHCC 0926 and *Nostoc* sp. UHCC 0302 to identify the corresponding MAA biosynthetic gene clusters for the structural variants detected. With a thorough genome mining strategy, we identified much more complex pathway organisations than previously reported, where the biosynthetic enzymes were encoded distantly in incomplete clusters across the genome. We described these as discontiguous biosynthetic gene clusters and noted their presence in other cyanobacterial genomes too.

Based on the structural and bioinformatic analyses, we proposed a novel biosynthetic scheme for the distinct variants synthesized in *Nostoc* sp. UHCC 0926 and applied a similar approach to investigate the biosynthesis of diglycosylated MAAs in *Nostoc* sp. UHCC 0302. The genome of *Nostoc* sp. UHCC 0302 also encoded a unique discontiguous MAA biosynthetic gene cluster. Using heterologous expression experiments in *Escherichia coli (E. coli)*, we refactored the MAA biosynthetic pathway and successfully demonstrated the collaborative activities of the enzymes encoded distantly. This work also led to identification and showcased potential activities of glycosyltransferase enzymes involved in MAA synthesis, which had long eluded researchers.

Additionally, our research showed the prevalence of MAAs in bloom-forming cyanobacteria sampled from Finnish lakes and the Baltic Sea. This work revealed high MAA producers from the *Anabaena/Dolichospermum/Aphanizomenon* (ADA) species complex. The ADA-2 and ADA-4 clades were particularly prominent in MAA biosynthetic pathways. These results suggest that MAAs may play an ecological role in the formation and maintenance of surface blooms and highlight the biotechnological potential of bloom-forming cyanobacteria.

Overall, this doctoral dissertation advances our understanding of MAA biosynthesis in cyanobacteria by exploring the genetic complexity of biosynthetic pathways and their dynamic evolution. It presents novel MAA structural variants and corresponding biosynthetic pathways, offering valuable insights for future research and potential large-scale commercial applications.

5 Introduction

5.1 “Back when the world was new…”

Our solar system and the earth are approximately 4.6 billion years old based on the dating of calcium-aluminum-rich fossils from meteorite inclusions (Bouvier and Wadhwa, 2010). This first eon was named Hadean, after *hell* and its ruler; *Hades*, as our earth was a seething cauldron, bombarded by asteroids and comets (Korenaga, 2021; Nisbet and Sleep, 2001). The atmosphere was thick with carbon dioxide and methane, basking in intense solar as ultraviolet B (UV-B) (280 nm – 315 nm) and ultraviolet C (UV-C) (100 nm – 280 nm) rays, which reached the surface unhindered by an ozone layer that had yet to form (Cockell, 1998).

The first signs of living organisms did not appear until a bit into the Archean eon that started 4 billion years ago, and the Earth’s temperature slowly started to cool down. First evidence of life dating back 3.7 billion years have been discovered in the deep oceans of the Nuvvuagittuq supracrustal belt in Northern Quebec, with haematite filament formations hinting at the presence of iron-oxidizing bacteria (Dodd *et al.*, 2017). This theory raised questions, as oxygen was thought to be scarce; however, the exact extent of this scarcity remains debated. Following studies presented hypotheses that suggest the presence of intermittent ‘whiffs’ of oxygen before the Great Oxidation Event (GOE) (Slotznick *et al.*, 2022; Anbar *et al.*, 2007). The likelihood of “whiffs” is linked to local and restricted availability of oxygen as a result of cyanobacteria initially inhabiting a few certain corners of the early Earth (Anbar *et al.*, 2007). Cyanobacteria are the most valuable players of the GOE and are dated around 3.5 billion years old based on various fossil evidence, marking a significant milestone in Earth's history (Noffke *et al.*, 2013; Chikaraishi *et al.*, 1987; Garcia-Pichel *et al.*, 2019). Inhabiting all corners of the earth, cyanobacteria played a major role shaping the planet's atmosphere by releasing oxygen through photosynthesis, gradually paving the way for the evolution of complex life forms (Shih, 2019).

5.2 The necessary evil: solar radiation

The high-energy, short-wave UV radiation rays emitted by the sun are known to cause direct damage to DNA, leading to mutations and replication errors that can result in cell death (Rastogi *et al.*, 2010). High amounts of UV-B (280-315 nm) and UV-C (100-280 nm) reaching the early earth’s surface has likely indeed posed a major challenge for the life forms as we know them, but it is also thought that without UV, life would not have emerged at all (Pressman *et al.*, 2015; Mulkidjanian *et al.*, 2003; Munñoz Caro *et al.*, 2002; Kufner *et al.*, 2025). One of many theories on the origins of life, “RNA World” which states that the life began from a nucleotide soup leading to self-assembling and replicating RNA molecules, is a widely accepted one (Gilbert, 1986). Intricacies involving the complex reactions and conditions forming the basis for this hypothesis are still under study however, effect of UV radiation might have been crucial in activating hydrogen cyanide and hydrogen sulfide to create precursors for some amino acids and lipids (Pressman *et al.*, 2015; Mulkidjanian *et al.*, 2003; Munñoz Caro *et al.*, 2002; Kufner *et al.*, 2025).

Despite potentially giving rise to RNA then leading to evolution of DNA and complex life forms, the negative effects of UV radiation exposure have been extensively documented (Biniek *et al.*, 2012; Kladwang *et al.*, 2012; Tang *et al.*, 2024; Amaro-Ortiz *et al.*, 2014; Andrady *et al.*, 2023). UV-A radiation damage tends to be linked with high oxygen levels, (which were negligible at this point) creating radical oxygen species (ROS) upon absorbtion by biomolecules (Gao and Garcia-Pichel, 2011b). UV-B on the other hand can directly damage DNA as, upon excitation the pyrimidine bases such as thymine can form dimers such as cyclobutadipyrimidines (CPD), pyrimidine - pyrimidone (6-4) photoproducts which can be further excited to isomerize into other photoproducts (Cadet *et al.*, 2005; Rastogi *et al.*, 2010). Modifications on purine bases can also occur creating unstable photoproducts and adenine dimers (Cadet *et al.*, 2005; Rastogi *et al.*, 2010). Additionally, the radiation damage can lead to double strand breaks, mispairing of bases during replication, depurination, depyrimidation causing mutations, and in oxygen rich atmosphere further oxidizing and free-radical-induced damage may also occur, potentially compromising cellular integrity (Rastogi *et al.*, 2010; Cadet *et al.*, 2005). In addition to general DNA replication and cell-cycle checkpoint, cells have also evolved specific UV damage repair mechanisms, such as photolyase enzymes that reverse dimerization of nucleobases, UV-damage endonuclease enzymes that incision of photoproducts and nucleotide mismatches and nucleotide excision repair mechanisms that can remove stretches of nucleotides around the mutation (Rastogi *et al.*, 2010; Goosen and Moolenaar, 2008). Photosynthesizing organisms that are constantly exposed to UV radiation, evolved to contain additional protective pigments such as caretenoids, scytonemin and mycosporine-like amino acids (MAAs), to mitigate the detrimental impacts of regular radiation exposure (Garcia-Pichel and Castenholz, 1991; Cogdell and Frank, 1987).

It presents a fascinating dichotomy: while solar radiation may have initiated life on Earth, it is also the most ancient and persistent threat to life, driving evolution further as life forms are forced to adapt in numerous ways to survive. This is particularly striking in the case of photoautotrophic bacteria, which, despite being obligated to expose themselves to the dangers of solar radiation, created the conditions necessary for the development of large, complex life forms of today (Rastogi *et al.*, 2014; Sabater, 2018; Schirrmeister *et al.*, 2015).

5.3 Cyanobacteria: A success story

Cyanobacteria are photoautotrophic, gram-negative bacteria that possess thicker peptidoglycan layers (10–700 nm) along with complex exopolysaccharide (EPS) layers and sheaths around their cells (Hoiczyk and Hansel, 2000; Kehr and Dittmann, 2015). Cyanobacteria are the first to evolve oxygenic photosynthesis which played a key role in the GOE (Schirrmeister *et al.*, 2015). A group of ancient endosymbiotic cyanobacteria lost most of their DNA, evolving from free-living cells into chloroplasts, organelles of photosynthesis in plants (Sabater, 2018). Owing to their billions of years of evolutionary success, cyanobacteria are morphologically diverse as they can be unicellular, filamentous, or colonial and evolved wide range of adaptations to thrive in all sorts of environments (Allaf and Peerhossaini, 2022; Komárek *et al.*, 2014). They have dominated oceans as primary

producers as well as all other corners of the earth ranging from icy Antarctic, deserts, hypersaline environments, soda lakes, hot springs, and all sorts of terrestrial surfaces (Olsson-Francis *et al.*, 2010; Yadav *et al.*, 2022; Maugeri *et al.*, 2013; Oren, 2015; Mehda *et al.*, 2021; Velichko *et al.*, 2021). Cyanobacteria can form biofilms that are also known as microbial mats or phototrophic mats which provide nutrients and physical safety to the microorganisms from harsh environmental conditions (Bozan *et al.*, 2022). Biofilms are multilayered agglomeration of bacteria which adhere to a surface and produce a complex polysaccharide medium wherein they are enclosed, and the size of this layer can range from millimetres to several centimetres. These biofilms can sustain themselves for a long time as cyanobacteria can utilize atmospheric CO_2 and N_2 and light to maintain growth (Potnis *et al.*, 2021; Bozan *et al.*, 2022; Rossi and De Philippis, 2015). Many cyanobacteria have specialized set of genes for fixing atmospheric nitrogen, N_2, and certain group evolved a specialized type of cells called heterocysts where nitrogen fixation takes place (Herrero *et al.*, 2001; Kumar *et al.*, 2010). Certain cyanobacteria are also able to form spore-like cells called akinetes that have a thick cell wall and provide protection during stressful conditions (Kumar *et al.*, 2010; Allaf and Peerhossaini, 2022). Successful immobile microbial mat formations aside, cyanobacteria also possess a range of means for motility. Some simply utilize gliding motility via type IV pili formation such as *Synechocystis* sp. or via the use of filaments like in *Oscillatoria* and *Phormidium* sp. as a form of phototaxis. Species living in marine environments can move and adjust their position in the water column using gas vesicles in their cells (Walsby *et al.*, 1995; Hoiczyk and Baumeister, 1995; Bhaya *et al.*, 2000). Overall, cyanobacteria have evolved to maximize chance of survival via numerous metabolic and physical adaptations and became crucial players for CO_2 and N_2 cycling on earth (Chen *et al.*, 2021b).

5.4 Cyanobacterial Blooms

Globally, cyanobacteria are prominent in marine and freshwater environments. In waters enriched with organic matter and nutrients such as nitrogen (N) and phosphorus (P), cyanobacterial species can thrive and dominate, forming cyanobacterial blooms (Huisman *et al.*, 2018; Zhang *et al.*, 2022b). In Finland, cyanobacteria acquired a bad reputation, as these blooms overtake many lakes and the Baltic Sea region during the summer months (Löptien and Dietze, 2022; Finni *et al.*, 2001). Cyanobacterial blooms can be dense and detrimental to the water ecosystem and lowering the safety and quality for recreational activities, fisheries, and consumption by humans and animals (Zhang *et al.*, 2022b). The World Health Organization (WHO) has an extensive review on cyanobacterial blooms including their assessment and management, providing guidance for water safety measures to ensure public health (Chorus and Welker, 2021).

Common bloom-forming genera include *Aphanizomenon*, *Cylindrospermopsis*, *Dolichospermum*, *Microcystis*, *Nodularia*, *Planktothrix*, and *Trichodesmium*. These blooms can smother aquatic vegetation by covering the surface with a thick layer, referred to as “scum”, leading to oxygen depletion and the eventual death of other aquatic organisms (Zhang *et al.*, 2022b; Rastogi *et al.*, 2015a). Cyanobacteria also produce various cyanotoxins, such as microcystins, nodularins, cylindrospermopsin, anatoxin, and saxitoxin, which can

cause digestive issues, liver damage, and neurological damage in both animals and humans (Dittmann et al. 2013; Rastogi et al. 2015a). As bloom-forming cyanobacteria grow and die, they consume nutrients and oxygen in the water and upon decomposition, they release toxins into environment. Additionally, cyanobacteria can produce odorous compounds such as geosmin and 2-methylisoborneol (MIB), which can mix with drinking water, causing unpleasant musty or chemical/medicinal odours and tastes (Graham et al. 2010; O'Neil et al. 2012; Zhang et al. 2022; Manganelli et al. 2023).

Understanding the conditions and adaptations that enable cyanobacteria to form toxic blooms is crucial for implementing preventative measures (Jalili et al. 2022; Manganelli et al. 2023). This is particularly important as warming temperatures due to climate change are expected to increase the likelihood and occurrence of these blooms globally (Visser et al. 2016).

5.5 Natural products of cyanobacteria

The evolutionary success and prominence of cyanobacteria can be attributed to their ability to synthesize a diverse array of secondary metabolites (Burja et al. 2001; Kultschar et al. 2018). Unlike primary metabolic processes, secondary metabolism and its products are not immediately essential for an organism's survival but can provide significant advantages under stressful conditions (Carmichael 1992). These compounds can function as toxins, antimicrobials, anticancer agents, antioxidants, enzyme inhibitors, surfactants, fragrant ingredients, pigments and sunscreens making them attractive to numerous industries. The curated cyanobacterial secondary metabolite database, CyanoMetDB, contains over 2000 different cyanobacterial natural products that have been reported and characterized between 1967 and 2020 (Jones *et al.*, 2021). The increasing availability of genomic data and predictive bioinformatic tools, it is becoming easier to match biosynthetic pathways and enzymes to specific compounds of interest (Welker and Von Döhren, 2006; Calteau *et al.*, 2014; Baunach *et al.*, 2024; Dittmann *et al.*, 2015).

The genera *Lyngbya*, *Microcystis*, *Nostoc*, and *Hapalosiphon* have been extensively studied and are noted for their rich production of natural products. Microbial natural product biosynthetic pathways usually involve genes encoded alongside each other in the genome, forming a metabolically efficient, co-regulated *"cluster",* often referred to as biosynthetic gene clusters (BGCs) (Medema *et al.*, 2015; Scott and Piel, 2019; Cimermancic *et al.*, 2014). Some cyanobacteria possess over 20 BGCs per genome; however, many cryptic and orphan BGCs exist whose end-products have yet to be identified (Baunach *et al.*, 2024; Popin *et al.*, 2021; Dittmann *et al.*, 2015).

The natural products belong to various structural classes, including peptides, polyketides, alkaloids, lipids, and terpenes (Burja *et al.*, 2001; Dittmann *et al.*, 2015). Apart from ribosomal peptides, which are limited to proteinogenic amino acids, many peptide-structured natural product compounds are produced by multi-modular enzyme systems such as polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) enzymes (Fischbach and Walsh, 2006). PKS can possess between 7-64 catalytic domains, and NRPS enzymes can incorporate both non-proteinogenic and proteinogenic amino acids,

contributing to the diversification and complexity of natural products (Fischbach and Walsh 2006). Additionally, cyanobacteria can produce long-chain hydrocarbons, which hold potential as a sustainable biofuel source. However, in native producers, the accumulation of these hydrocarbons is typically around 0.12 % of dried biomass (Coates *et al.*, 2014). While large-scale, sustainable production of such natural products using CO_2 and sunlight is a major attraction of cyanobacteria in biotechnology, low production levels are the main bottleneck (Coates *et al.*, 2014). Nonetheless, cyanobacteria are heavily studied to understand the underlying biosynthetic processes of these natural products, which can be harnessed by genetic engineering and synthetic biology for robust production via heterologous microbial cell factories (Kallio *et al.*, 2014, 2021; Kim *et al.*, 2015; Vavitsas *et al.*, 2021; Verma *et al.*, 2022).

6 Mycosporine-like amino acids (MAAs)

6.1 Discovery & taxonomic distribution of MAAs

The discovery of MAAs dates back to 1965 when Charles M. Leach isolated a water-soluble compound from fungal mycelium and named it P310 due to its absorption maxima (λ_{max}) of 310 nm (Leach, 1965). Initially, this UV absorbing compound was thought be a pigment involved in photo-sporogenesis in fungi. However, similar compounds were soon reported from coral and cyanobacteria species studied in the Great Barrier Reef (Leach 1965; Shibata 1969). Shibata had suggested that these compounds might either be acting directly as UV filters or are precursor to such UV absorbing pigments that provide protection to highly exposed marine organisms (Shibata 1969).

In 1976, Favre-Bonvin and colleagues had provided further structural characterization of these UV-absorbing compounds, proposed a synthesis scheme, and coined the term "mycosporine" for their discovery in fungi (Favre-Bonvin et al. 1976). By the 1980s, various reports confirmed that different marine organisms, including hermatypic corals, symbiotic zooxanthellae, dinoflagellates, starfish, mussels, cod eggs, and red algae, harboured different structural variants of MAAs, all acting as UV filters (Dunlap and Chalker, 1986; Nakamura *et al.*, 1982, 1981; Takano *et al.*, 1978; Carreto *et al.*, 1990; Jokiel and York, Jr., 1982). Many marine animals obtain MAAs via their diet and bioaccumulate them in various tissue parts such as eyes, skin and eggs to mitigate UV radiation damage (Carroll and Shick, 1996; Isla Naveira *et al.*, 2024; Bonin *et al.*, 2024). Later, surprisingly gadusols, which share structural similarity to MAA chromophores (Figure 1) and also referred to as precursors of MAAs (Geraldes and Pinto, 2021), were shown to be synthesized in zebrafish and the *mysAB*-like gene cluster encoding for 2-epi-5-epivaliolone synthase (EEVS) phosphate-cyclase and MT-Ox (methylation and oxidation) enzymes were shown to be involved in biosynthesis, and later also identified in many other vertebrates (Osborn et al. 2015). The authors suggest that vertebrates likely obtained the gadusol biosynthetic pathway via horizontal gene transfer events prior to evolution of bony fish over 400 million years ago, and while several modern animals retained these genes, they have been lost in mammals (Osborn et al. 2015).

6.2 Function and regulation of MAAs in cyanobacteria

MAAs are particularly abundant in cyanobacteria and are often found in species of the orders Synechococcales, Chroococcales, Oscillatoriales, and Nostocales, being most enriched order (Jain et al. 2017) Most cyanobacteria produces approximately 0.16 to 3 % of their dry weight as MAAs, and they seem to either accumulate in the cytoplasm or extracellular matrix, often alongside other protective compounds, such as the pigment scytonemin, which is also produced by some cyanobacteria (Garcia-Pichel and Castenholz, 1993; Hu *et al.*, 2015; Wright *et al.*, 2005; Oren and Gunde-Cimerman, 2007; Ferroni *et al.*, 2010). In general, the main function of cyanobacterial MAAs is believed to be that of a microbial sunscreen, protecting cells from harmful UV-R (Oren and Gunde-Cimerman,

2007). UV induction of MAAs have been successfully shown in cyanobacteria isolated from various environments such as including soil, desert rocks, mountain walls, pavements, the Baltic Sea, hot springs, saline cyanobacterial mats, and biofilms (Garcia-Pichel and Castenholz, 1993; Rastogi *et al.*, 2015b; Wulff *et al.*, 2007). Specifically, the UV-B radiation (280-315 nm), highest at midday, has been shown to be the most effective inducer of MAA production in cyanobacteria (Ehling-Schulz *et al.*, 1997; Sinha *et al.*, 2003; Shang *et al.*, 2018; Singh *et al.*, 2008; Rastogi *et al.*, 2015b, 2023). While UV-B radiation can induce MAA synthesis in some cyanobacteria, it's worth noting that MAA production can also occur constitutively as well (Garcia-Pichel and Castenholz, 1993; Hu *et al.*, 2015 Singh *et al.*, 2008a; Rajeshwar P. Sinha *et al.*, 2001). Light-dependent circadian regulation could be the main regulatory element for constitutive MAA production, where the synthesis is induced during the day with peak UV levels and maintained throughout during the night (Sinha *et al.*, 2001). Interestingly, far-red light response has also been noted in *Chlorogloeopsis fritschii* PCC 6912 where the MAA biosynthetic gene cluster was upregulated upon exposure, suggesting involvement in photon dissipation and thermodynamic optimisation (Llewellyn *et al.*, 2020).

Increased salinity and desiccation stress has also been shown to enhance MAA production, suggesting a potential osmolyte function for MAAs. Halotolerant cyanobacteria with high intracellular MAA concentrations, coupled with their polar, water-soluble, and uncharged structures, have led to speculation regarding their involvement in osmoregulation (Waditee-Sirisattha *et al.*, 2014; Volkmann *et al.*, 2006; Shang *et al.*, 2018; Singh *et al.*, 2008, 2020; Oren, 1997). Studies have demonstrated a synergistic effect of UV-B radiation and hyper-salinity induced by increased NaCl or NH_4Cl concentrations on MAA production (Singh *et al.*, 2020, 2008). Additionally, several studies have also reported links between synergistic upregulation of MAA biosynthesis and increased exopolysaccharide (EPS) synthesis upon the activation of salt and desiccation stress regulators via UV-B exposure (Ehling-Schulz *et al.*, 1997; Wright *et al.*, 2005; Shang *et al.*, 2018). These studies show that cyanobacterial MAAs might play additional protective roles and are a part of the complex UV-B induced response regulation pathway to mitigate various forms of damage that can arise alongside increased UV radiation (Singh *et al.*, 2010a; Shang *et al.*, 2018; Wright *et al.*, 2005).

6.3 Structural diversity in cyanobacterial MAAs

All MAAs are water-soluble and colourless molecules characterized by cyclohexenone or cyclohexenimine chromophores. The structural diversity in MAAs mainly comes from the conjugation of various amino acids onto the first and/or third carbon (C1 or 3) of the central 4-deoxygadusol chromophore (Figure 1.) along with additional modifications on the amino acid residues or the chromophore (Rastogi *et al.*, 2016; Wada *et al.*, 2015; Shick and Dunlap, 2002). In early 2000s, advances in liquid chromatography/mass spectrometry (LCMS) techniques led to establishment of effective methodologies for MAA characterization which then led to increasing numbers of different MAA structural variants being reported from various environments (Volkmann and Gorbushina, 2006; Whitehead and Hedges, 2003,

2002; Peng *et al.*, 2023; Geraldes *et al.*, 2020b). Currently there are 78 structural MAA variants reported from various organisms (Peng *et al.*, 2023) and 33 of these are present in cyanobacteria (Table 1).

Mycosporine-glycine is the most common cyclohexenone MAA variant and an intermediate, wherein there is a Gly residue attached on the 3rd carbon (C3) of the 4-deoxygadusol core (Figure 1.) (Balskus and Walsh, 2010; Wada *et al.*, 2015). In cyanobacteria, Gly can sometimes be substituted with other amino acids like Ser, Tau, Orn or Lys or amino alcohols to create different cyclohexenone MAA variants (Table 1). For many cyanobacteria cyclohexenimine MAA variants dominate, these can have various amino acid disubstitutions on the 1st carbon (C1) of mycosporine-glycine, most common ones are mycosporine-2-glycine, shinorine, porphyra-334 and palythine-Thr/Ser (Peng *et al.*, 2023; Gao and Garcia-Pichel, 2011b; Wada *et al.*, 2015)

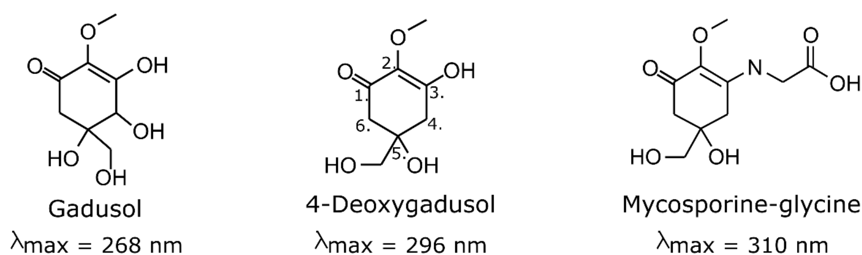

Figure 1. Chemical structures and λmax of gadusol, 4-deoxygadusol chromophore and mycosporine-glycine.

Common types of structural variation in MAAs can be in the form of further modifications of the amino acid residues and/or attachment of sugar moieties either onto the amino acid residues or the chromophore (Sakamoto *et al.*, 2019; Ishihara *et al.*, 2017; D'Agostino *et al.*, 2016; Nazifi *et al.*, 2015; Böhm *et al.*, 1995; Werner *et al.*, 2021). For example, glycosylated shinorine, porphyra-334 and palythine derivatives with hexose or pentose moieties as well as larger variants with multiple chains of sugar moieties have been reported (Table 1). The largest MAA structural variant characterized to date is the 1050 Da and is composed of three chromophores with a disaccharide moiety, exhibiting dual absorption maxima at 312 nm and 340 nm (Matsui *et al.*, 2011). While all MAA derivatives effectively absorb UVA/B and possess high photostability, the structural differences mean that there can be slight shifts in the UV absorption maxima (Whitehead and Hedges, 2005).

Table 1 MAA structural variants reported from cyanobacteria so far.

MAA Structural Variant	Chemical Structure (SMILES)	Molecular Formula	Molecular weight (g·mol^{-1})	λ_{max} (nm)	Strain of Isolation	Reference
13-O-(β-galactosyl)-porphyra-334	OC(C/1)(CO)CC(NCC(O)=O)=C(OC)C1=N\C(C(O)=O)C(OC2OC(CO)C(O)C(O)C2O)C	$C_{20}H_{33}N_2O_{13}$	509.20	334	*N. sphaericum*	(Ishihara *et al.*, 2017)
2xHexose-palythine-threonine	OC(CC(NC(C(O)=O)C(C)OC1OC(CO)C(O)C(O)C1O)=C2OC)(COC3OC(CO)C(O)C(O)C3O)CC2=N	$C_{24}H_{40}N_2O_{16}$	613.25	322	*Nostoc commune*	(Nazifi *et al.*, 2013)
586 Da MAA	OC(C/1)(CO)CC(NCCCC(C(O)=O)NC2=C(OC)C(CC(CO)(O)C2)=O)=C(OC)C1=N\CCCC(N)C(O)=O	$C_{26}H_{42}N_4O_{11}$	587.30	332	*Nostoc commune*	(Sakamoto *et al.*, 2019) *
880 Da MAA	OC(C/1)(COC2OC(COC3OC(CO)C(O)C3O)C(O)C(O)C2O)CC(NCCCC(C(O)=O)NC4=C(OC)C(CC(CO)(O)C4)=O)=C(OC)C1=N\CCCC(N)C(O)=O	$C_{37}H_{60}N_4O_{20}$	881.4	331	*Nostoc commune*	(Nazifi *et al.*, 2015)
Aplysiapalythine-D	N=C1CC(CO)(O)CC(N(C)CC(O)=O)=C1OC	$C_{11}H_{18}N_2O_5$	539.23	334	Benthic cyanobacteria isolated from Alpine Lake	(Werner *et al.*, 2021)
Asterina-330	COC1=C(CC(CC1=NCC(=O)O)(CO)O)NCCO	$C_{12}H_{20}N_2O_6$	288.30	330	*Trichodesmium* spp.	(Dupouy *et al.*, 2008)
Dehydroxyl-usujirene	/	$C_{13}H_{19}N_2O_4$	409.20	356	*Synechocystis* sp. PCC 6803	(Zhang et al. 2007)
Euhalothece-362	CC(C(=O)O)N=C1CC(CC(=C1OC)NC=C(CO)O)(CO)O	$C_{14}H_{22}N_2O_7$	330.33	362	*Euhalothece* sp.	(Volkmann and Gorbushina, 2006)
Hexose-palythine-serine	COC1=C(CC(CC1=NC(CO)C(=O)O)(COC2C(OC(C(C2O)O)O)CO)O)N	$C_{17}H_{28}N_2O_{11}$	437.18	320	*Scytonema cf. crispum*	(D'Agostino et al., 2016b)
Hexose-palythine-threonine	CC(C(C(=O)O)N=C1CC(CC(=C1OC)N)(COC2C(C(C(C(O2)CO)O)O)O)O)O	$C_{18}H_{30}N_2O_{11}$	451.19	322	*Nostoc commune*	(Nazifi *et al.*, 2013)

Hexose-porphyra-334	O[C@@](CC(NC(C(O)=O)C(C)O)=C/1OC)(COC2OC(CO)C(O)C(O)C2O)CC1=N/CC(O)=O	$C_{20}H_{32}N_2O_{13}$	509.00	334	*Nostoc commune*	(Nazifi *et al.*, 2013)
Hexose-shinorine	OC(C/1)(CO)CC(NCC(OC2C(CO)OC(O)C(O)C2O)=O)=C(OC)C1=N\C(C(O)=O)CO	$C_{19}H_{30}N_2O_{13}$	495.2	334	*N. sphaericum*	(Ishihara *et al.*, 2017)
M-343	/	/	/	343	*Synechocystis* sp. PCC 6803	(Zhang et al. 2007)
Mycosporine-2-glycine	COC1=C(CC(CC1=NCC(=O)O)(CO)O)NCC(=O)O	$C_{12}H_{18}N_2O_7$	302.28	332	*Euhalothece* sp.	(Kedar *et al.*, 2002)
Mycosporine-alanine	COC1=C(CC(O)(CO)CC1=O)NC(C)C(O)=O	$C_{11}H_{17}NO_6$	259.26	317	*Hassallia byssoidea*	(Saha *et al.*, 2021)
Mycosporine-GABA	O=C1CC(CO)(O)CC(NCCCC(O)=O)=C1OC	$C_{12}H_{20}NO_6$	274.13	310	*Nostoc commune*	(Nazifi et al., 2015a)
Mycosporine-glutamicol	COC1=C(CC(CC1=O)(CO)O)NC(CCC(=O)O)CO	$C_{13}H_{21}NO_7$	303.31	310	*Degelia plumbea*	(Roullier *et al.*, 2011)
Mycosporine-glycine	COC1=C(CC(CC1=O)(CO)O)NCC(=O)O	$C_{10}H_{15}NO_6$	245.23	310	*Chlorogloeopsis* PCC 6912	(Portwich and Garcia-Pichel, 1999)
Mycosporine-lysine	COC1=C(CC(CC1=O)(CO)O)NCCCCC(C(=O)O)N	$C_{14}H_{24}N_2O_6$	316.35	310	*Gloeocapsa sp.*, *Calothrix sp*	(Garcia-Pichel and Castenholz, 1993; Garcia-Pichel *et al.*, 1993)
Mycosporine-ornithine	COC1=C(CC(CC1=O)(CO)O)NCCCC(C(=O)O)N	$C_{13}H_{22}N_2O_6$	302.32	310	*Cylindrospermum stagnale strain PCC 7417*	(Katoch *et al.*, 2016)*
Mycosporine-serinol	COC1=C(CC(CC1=O)(CO)O)NC(CO)CO	$C_{11}H_{19}NO_6$	261.27	365	*Lichina sp.*, *Peltigera sp.*	(Roullier *et al.*, 2011)
Mycosporine-taurine	OC1=C(CC(CC1=O)(CO)O)NCCS(=O)(=O)O	$C_{10}H_{17}NO_7S$	295.31	309	*Synechocystis* sp. PCC 6803	(Zhang et al. 2007)

N-Methylpalythine/ Aplysiapalythine-C	CN=C1CC(CC(=C1OC)NCC(=O))(CO)O	$C_{11}H_{18}N_2O_5$	258.27	330	*Calothrix sp., Scytonema sp., Brasilonema octagenarum, Leptolyngbya sp., Tolypothrix sp.*	(Geraldes *et al.*, 2020b)
Palythene	C/C=C/NC1=C(C(=NCC(=O)O)CC(C1)(CO)O)OC	$C_{13}H_{20}N_2O_5$	284.31	360	*Oscillatoria spongelidae*	(Bandaranayake et al. 1996)
Palythine	COC1=C(CC(CC1=NCC(=O)O)(CO)O)N	$C_{10}H_{16}N_2O_5$	244.24	320	*Lyngbya* sp. CU2555	(Rastogi and Incharoensakdi 2014)
Palythine-serine	COC1=C(CC(CC1=NC(CO)C(=O)O)(CO)O)N	$C_{11}H_{18}N_2O_6$	274.27	320	*Anabaena variabilis* PCC 7937	(Singh et al. 2010)
Palythinol	CC(CO)NC1=C(C(=NCC(=O)O)CC(C1)(CO)O)OC	$C_{13}H_{22}N_2O_6$	302.32	332	*Gloeocapsopsis* sp.	(Rastogi et al. 2015b)
Pentose-porphyra-334	OC(C/1)(COC2OCC(O)C(O)C2O)CC(NC(C(O)=O)C(C)O)=C(OC)C1=N\CC(O)=O	$C_{19}H_{30}N_2O_{12}$	479.00	335	*Nostoc commune*	(Matsui *et al.*, 2011)
Porphyra-334	CC(C(C(=O)O)NC1=C(C(=NCC(=O)O)CC(C1)(CO)O)OC)O	$C_{14}H_{22}N_2O_8$	346.33	334	*Trichodesmium* spp.	(Subramaniam et al. 1999)
Shinorine	COC1=C(CC(CC1=NCC(=O)O)(CO)O)NC(CO)C(=O)O	$C_{13}H_{20}N_2O_8$	332.31	333	*Microcoleus* sp.	(Karsten and Garcia-Pichel 1996)
Tricore A	OC(C/1)(COC2OC(COC3OC(CO)C(O)C3O)C(O)C(O)C2O)CC(NCCCC(C(O)=O)NC4=C(OC)C(CC(CO)(O)C4)=O)=C(OC)C1=N\CCCC(NC5=C(OC)C(CC(CO)(O)C5)=O)C(O)=O	$C_{45}H_{70}N_4O_{24}$	1051.00	334	*Nostoc commune*	(Matsui et al., 2011)
Tricore B	OC(C/1)(CO)CC(NCCCC(C(O)=O)NC2=C(OC)C(CC(CO)(O)C2)=O)=C(OC)C1=N\CCCC(NC3=C(OC)C(CC(CO)(O)C3)=O)C(O)=O	$C_{34}H_{53}N_4O_{15}$	756.00	322	*Nostoc commune*	(Shang *et al.*, 2018)
Usujirene	COC1=C(CC(O)(CO)CC1=NCC(O)=O)NC=CC	$C_{13}H_{20}N_2O_5$	284.31	357	/	(Rozema *et al.*, 2002)

6.4 Biophysicochemical properties of MAAs

Exceptionally high natural extinction coefficients of MAAs, ranging from 20,000 to 50,000 M^{-1} cm^{-1} allow for effective absorbtion UV radiation in the range of 280 to 400 nm (de la Coba *et al.*, 2019; Dunlap and Shick, 1998; Shick and Dunlap, 2002; Heidorn *et al.*, 2012; Wada *et al.*, 2013). Both MAAs and gadusolate variants also demonstrate a high degree of photostability (Whitehead and Hedges, 2005; Reza *et al.*, 2023; Moliné *et al.*; Arbeloa *et al.*, 2011). MAAs employ a unique UV absorption mechanism that allows the release of up to 90 % of absorbed radiation as heat without the generation of ROS (Conde *et al.*, 2004, 2007, 2000). It seems upon photoexcitation, MAAs are able to shift from the excited-state to ground-state at a rapid rate, referred as "ultrafast deactivation" in a non-radiative pathway, and thus avoiding generation of ROS and fluorescence and maintaining stability (Conde *et al.*, 2000, 2004, 2007; Losantos *et al.*, 2015; Sampedro, 2011; Whittock *et al.*, 2022; Hatakeyama *et al.*, 2019; Hatakeyama and Nakamura, 2022; Koizumi *et al.*, 2017). The mechanism of energy release in porphyra-334 was investigated through computational simulation, revealing that upon excitation, hydrogen bonds rapidly form and break between the MAA (solute) and water (solvent), while maintaining a stable overall number of hydrogen bonds (Koizumi et al., 2017). While this preserves a hydration shell around the MAA, additional channels form to release excess energy into the solvent, aided by specific structural relaxation pattern from the excited state (Koizumi *et al.*, 2017; Hatakeyama *et al.*, 2019).

In addition, MAAs are also resistant to heat, various solvents, and pH fluctuations (Rastogi and Incharoensakdi, 2014a; Nishida *et al.*, 2022; Wada *et al.*, 2015; Nishida *et al.*, 2020). Majority of MAAs were shown to be stable at temperatures up to 80 °C and across a pH range of 4.5 to 10.5 (Rastogi and Incharoensakdi, 2014b; de la Coba *et al.*, 2019). Notably, porphyra-334, shinorine, porphyra-334 in combination with mycosporine-serinol were exceptionally stable across all tested pH and temperature conditions, whereas asterina-330 was noted to be the least stable among others (de la Coba *et al.*, 2019; Zhang *et al.*, 2005). Although MAAs can remain stable in such acidic conditions, studies showed that this can lead to a slight blue shift in λ_{max} (Matsuyama *et al.*, 2015)

Consistently, different MAA structural variants were also shown to have considerable antioxidant activity, effectively scavenging singlet oxygen, superoxide anions, hydroperoxyl radicals, and hydroxyl radicals alongside their UV absorbtion capabilities (Rastogi *et al.*, 2023; Moliné *et al.*; Matsui *et al.*, 2011; Misonou *et al.*, 2003; Wada *et al.*, 2015; Wang *et al.*, 2023a; Cheewinthamrongrod *et al.*, 2016). The antioxidant capacity of MAAs however varies significantly among different structural variants due to structural differences (Kageyama and Waditee-Sirisattha, 2019; Torres *et al.*, 2018; Wada *et al.*, 2015). Moreover, MAAs have been shown to have anti-inflammatory, anti-aging, wound-healing and potential tyrosinase-related protein 1 inhibitor properties (Ryu *et al.*, 2014; Choi *et al.*, 2015; Lee *et al.*, 2015; Orfanoudaki *et al.*, 2019; Tarasuntisuk *et al.*, 2019, 2018; Becker *et al.*, 2016). All of these combined,

along with having no adverse effects or cytotoxicity, make MAAs remarkable candidates for various cosmetic and pharmaceutical applications (Rosic *et al.*, 2023).

7 Biosynthesis of mycosporine-like amino acids

7.1 MAA biosynthetic gene clusters and biosynthetic pathway for Shinorine

Studies on *Nostoc punctiforme* ATCC 29133 and *Trichormus variabilis* ATCC 29413, known for producing shinorine, have revealed two types of gene clusters responsible for MAA production: *mysABCD* or *mysABCE* (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011a). These genes encode for five enzymes in total, including a phosphate cyclase homolog (MysA), O-methyltransferase (MysB), ATP-grasp ligase (MysC), and either a D-alanine D-alanine ligase/mycosporine-glycine-amine ligase (MGA-ligase) (MysD), or a non-ribosomal peptide synthetase (NRPS) (MysE). (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011a).

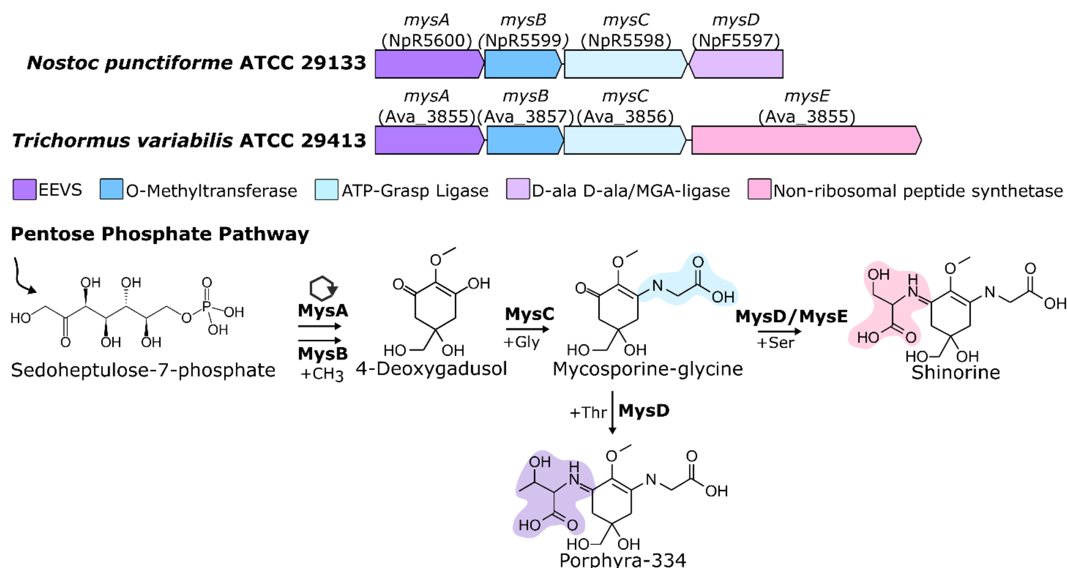

Figure 2. MAA biosynthetic gene clusters from *Nostoc punctiforme* ATCC 29133 and *Trichormus variabilis* ATCC 29413 and the established shinorine and porphyra-334 biosynthetic pathway. Figure drawn based on the descriptions of Balskus and Walsh 2010 & Gao and Garcia-Pichel 2011a.

In vitro studies of the MAA biosynthetic gene cluster genes of *Nostoc punctiforme* ATCC 29133 revealed that MysA enzyme acts on sedoheptulose-7-phosphate into 2-epi-5-epi valiolone which gets methylated by MysB to form the 4-deoxygadusol core (Balskus and Walsh, 2010). MysC then catalyses the addition of Gly onto the 3rd carbon of the 4-deoxygadusol core, forming the intermediate mycosporine-Gly. Finally, MysD (Mycosproine-Gly-amine ligase, MGA-ligase) or MysE (NRPS) catalyses the addition of Ser to the 1st carbon, resulting in the formation of mycosporine-Gly-Ser, also known as shinorine (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011a).

Following the seminal work of Balskus and colleagues, in 2021 Chen and colleagues demonstrated the involvement of 2-oxoglutorate dioxygenase enzymes (MysH) in palythine variants' synthesis. However, the exact biochemistry of this reaction is yet to be elucidated (Chen *et al.*, 2021a). The first three steps of this biosynthetic pathway, from MysA to MysC seems to be tightly conserved among all MAA-producing organisms (Balskus and Walsh, 2010; Peng *et al.*, 2023; Gao and Garcia-Pichel, 2011b, 2011a). Following bioinformatic analyses of the MAA biosynthetic gene clusters also show that the BGC organisations are not limited to the minimum four genes and there are various other genes encoding for putative enzymes which also might be involved in MAA synthesis based on their recurrent presence nearby the clusters such as transporters, and methyltransferases (D'Agostino *et al.*, 2019), (unpublished results).

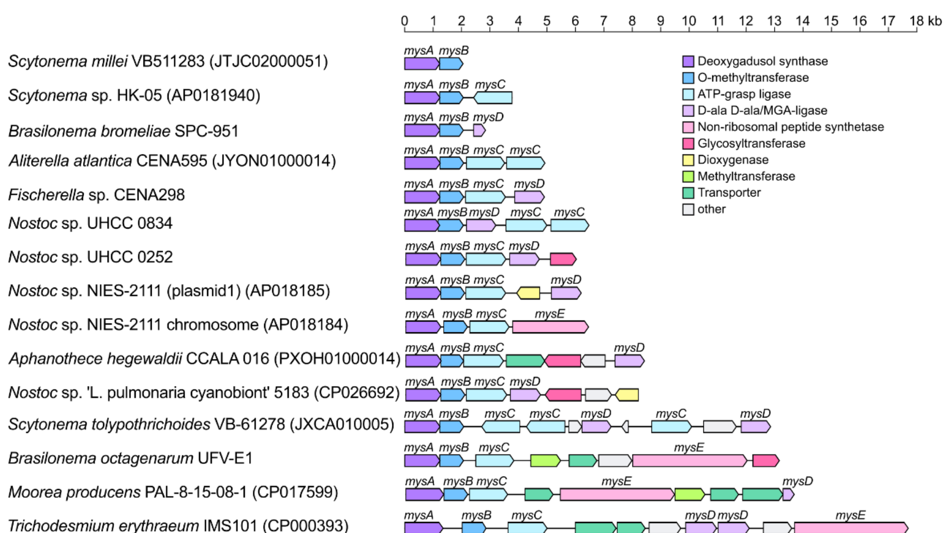

Figure 3. Different types and organisations of MAA BGCs and other putative enzymes.

7.2 MAA biosynthetic enzymes

7.2.1 MysA: EEVS and DHQS-like phosphate cyclase

Sugar phosphate cyclase enzymes deal with the formation of carbon sugar containing cyclic compounds (Osborn *et al.*, 2017; Asamizu *et al.*, 2012). This reaction is the first step of the MAA biosynthetic pathway and is catalysed by the MysA enzyme. In the established shinorine biosynthetic pathway, MysA of *Nostoc punctiforme* ATCC 29133 acts like a 2-epi-5-epivaliolone synthase (EEVS) that converts sedoheptulose-7-phosphate (S7P) into 2-epi-5-epi-valiolone which then gets methylated to form the conserved 4-deoxygadusol chromophore (Balskus and Walsh, 2010). However, these results meant MAA biosynthesis is linked to the primary pentose phosphate pathway and this clashed with the long-standing hypothesis that MAA precursor is obtained from the shikimate pathway via a dehydroquinate synthase (DHQS) enzyme homolog, that is MysA (Balskus and Walsh, 2010; Portwich and Garcia-Pichel, 2003; Favre-Bonvin *et al.*, 1987; Rosic and Dove, 2011). This was further tested by others in MAA

producing *Trichormus variabilis* ATCC 29413 and *Sphaerospermopsis torques-reginae* ITEP-024 via gene knockout and pathway inhibition experiments (Geraldes *et al.*, 2020a; Pope *et al.*, 2015). For *S. torques-reginae* ITEP-024 glyphosate addition indeed inhibited MAA production revealing that 3-dehydroquinate intermediate of the shikimate pathway was the main precursor to MAA biosynthesis (Geraldes *et al.*, 2020a). In *Trichormus variabilis* ATCC 29413 combined knockout and inhibition experiments showed that both shikimate and pentose phosphate pathways were involved in the MAA biosynthesis (Pope *et al.*, 2015). In addition, in halotolerant *Euhalothece* sp. MAA biosynthetic gene cluster, two MysA enzymes; one EEVS-like (MysA1) and another DHQS-like ($MysA_2$) were identified (Mogany *et al.*, 2022). Based on these works, convergent evolution seems to be at play ensuring the synthesis of MAA via both pathways. Also, EEVS and DHQS enzymes overall seem to be closely related and share a similar catalytic mechanism that involve alcohol oxidation, phosphate β-elimination, carbonyl reduction, ring opening and aldol condensation. However, their structures and differences between catalytic residues in relation to their mechanisms require further examination (Asamizu et al., 2012; Kean et al., 2014).

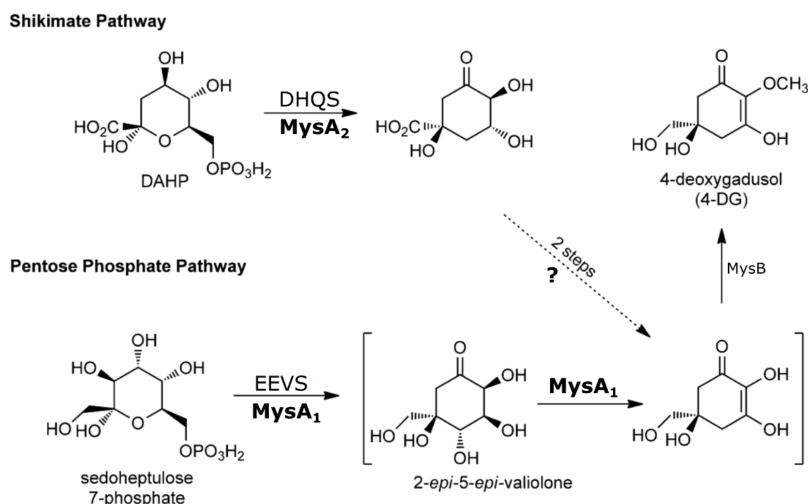

Figure 4. DHQS-like and EEVS-like MysA activities described in literature to form the 4-deoxygadusol core. The scheme is edited and adapted from Pope *et al.*, 2015.

7.2.2 MysB: Class-I SAM O-methyltransferase

Methyltransferases (MT) are a large group of enzymes that are involved in numerous different biological processes that include biosynthesis, metabolism, detoxification, signal transduction, protein sorting and repair, and nucleic acid processing (Martin 2002; Liscombe et al. 2012). Several MT classes exist but S-adenosyl-L-methionine (SAM)-dependent MTs are the largest with around 120 members based on their substrate preferences and targeted atoms (Martin 2002; Liscombe et al. 2012). These enzymes are categorized into three classes, where class 1 and class 2 deal with methylation of phenolic hydroxyl residues and class 3 is involved

in the methylation of carboxyl groups (Parajuli et al. 2018). The N-terminal of the β sheet of SAM-MTs is the SAM-binding region and the C-terminal part of the β sheet is the substrate-binding region. The substrates range from small molecules, nucleic acids, proteins to lipids depending on the O-MT. Due to this substrate-binding region of MT tend to vary immensely in structure and topology among individual members of the family (Martin, 2002; Frick *et al.*, 2001).

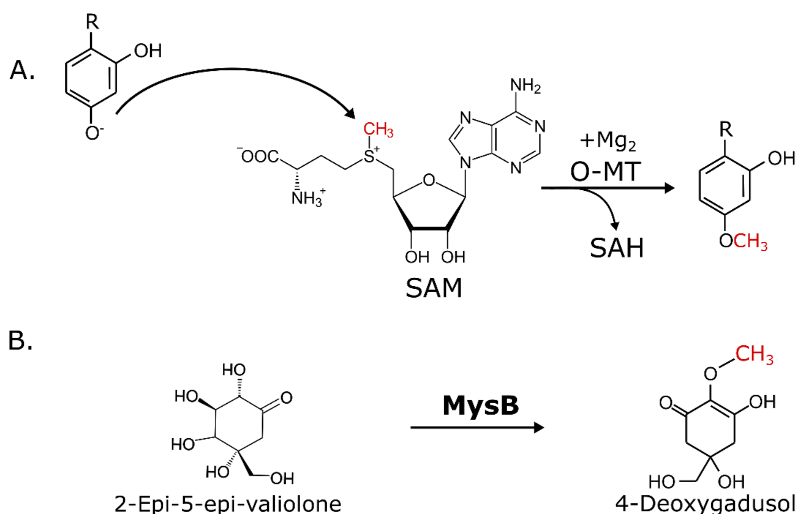

Figure 5. SAM O-MT activity scheme based on descriptions of Lee at al., 2019 (A). MysB activity forming the 4-deoxygadusol core likely working in a similar way based on known enzymatic activity and descriptions by Balskus and Walsh., 2010 and Pope *et al.*, 2015 (B).

In MAA biosynthesis MysB is a class-I SAM O-MT, that transfer methyl-group from SAM to oxygen atoms of the substrates, mostly to the hydroxyl groups (Figure 5.) (Liscombe *et al.*, 2012; Martin, 2002; Balskus and Walsh, 2010). This reaction is commonly found in secondary metabolite biosynthetic pathways, such as many plant metabolites like pigments, antioxidants, phenolic compounds where methylation can contribute to structural diversity (Frick *et al.*, 2001; Kim *et al.*, 2010; Liscombe *et al.*, 2012). The reaction involving the transfer of the methyl group from SAM to the 2-epi-5-epivaliolone or 2-demethyl-4-deoxygadusol by MysB is vital to form the 4-deoxygadusol chromophore as the deletion of MysB enzyme disables the MAA biosynthesis completely (Pope *et al.*, 2015). Despite class-I SAM-dependent O-MT enzymes commonly having relaxed substrate specificities, in MAA biosynthetic pathways activity of the MysB is strictly conserved (Lashley *et al.*, 2022; Martin, 2002; Liscombe *et al.*, 2012; Pope *et al.*, 2015). Similarly in eukaryotic organisms such as the ray-finned fish, reptiles, birds and amphibians, the *mysAB*-like gene cluster involved in gadusol synthesis, is also conserved (Osborn *et al.*, 2015).

7.2.3 MysC: ATP-grasp ligase

ATP-grasp enzymes are a superfamily of 21 different proteins (Fawaz *et al.*, 2011).These enzymes contain a distinct ATP-grasp fold of two α + β domains which "grasp" the ATP molecule that forms the central conserved domain (Fawaz *et al.*, 2011; Murzin, 1996). While the ATP-binding site is highly conserved with thirteen characteristic fingerprint residues, between different enzymes there can still be very low sequence identity (Galperin and Koonin, 1997; Fawaz *et al.*, 2011). ATP-grasp superfamily enzymes are involved in many metabolic pathways such as fatty acid synthesis, glucogenesis and purine synthesis. The mechanism involves an ATP-dependent formation of amide bonds between carboxylate and amino groups with a phosphate intermediate. First steps of the reaction involve the interaction of ATP molecule with the target carboxylic acid to convert it into an electrophile. This leads to the formation of the acylphosphate intermediate which then reacts with the nucleophilic substrate to release the phosphate group and form the final compound (Figure 6.) (Fawaz *et al.*, 2011; Galperin and Koonin, 1997).

In the established shinorine biosynthetic pathway the ATP-grasp homolog Ava_3856 generates a new type of electrophile using vinylogous acid activation. Balskus and colleagues describe this type of reaction as unprecedented in natural product biosynthesis and provides a new biosynthetic logic for imine construction (Balskus and Walsh, 2010). Here the ATP-grasp ligase enzyme, MysC, catalyses the synthesis of a glycine residue onto the 4-deoxygadusol core to form mycosporine glycine in the presence of ATP and Mg^{2+} cofactors (Figure 6.). The reaction was investigated with an in vitro assay using oxygen-18 (^{18}O) isotope labelled Gly released (Balskus and Walsh, 2010). As a result, the unchanged number of the oxygen18 isotope at the end of the assay meant that the MysC phosphorylates the 4-deoxygadusol and not the Gly as otherwise one oxygen atom would have been lost along with the phosphate released (Balskus and Walsh, 2010). Although ATP-grasp ligase activity of MysC is unique amongst its family of enzymes, in cyanobacterial MAA biosynthetic pathways mysC gene and its role in 1, 4 glycine addition onto the 4-deoxygadusol core is conserved (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011).

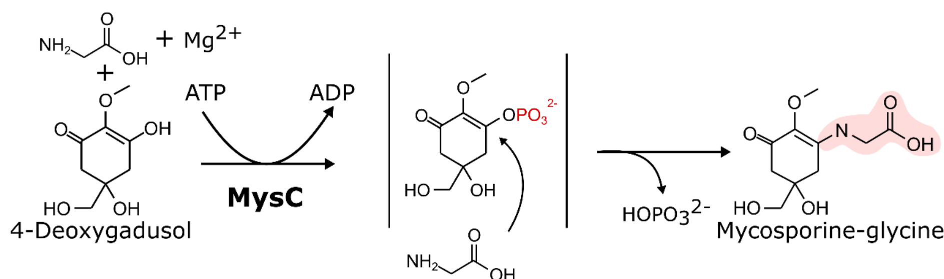

Figure 6. MysC activity in catalysing Gly addition onto 4-deoxygadusol core to form mycosporine-glycine as tested and described by Balskus and Walsh 2010.

7.2.4 MysD: D-alanine D-alanine/ Mycosporine-glycine-amine ligase

MysD enzyme is also a type of an ATP-grasp enzyme, one of the first types to be characterized for its role in bacterial peptidoglycan cell-wall biosynthesis (Walsh, 1989; Kitamura *et al.*, 2009). For its vital function cell wall synthesis, D-alanine D-alanine ligase (Ddl) has been studied as an antibiotic target and specifically targeted as variants can lead to vancomycin resistance (Walsh, 1989; Kitamura *et al.*, 2009; Roper *et al.*, 2000). In peptidoglycan biosynthesis Ddl catalyses attachment of two D-Alanine molecules using ATP. Initial analyses of the MAA biosynthetic pathways identified MysD as a Ddl-like enzyme based on domain similarities and thus named it as D-alanine D-alanine ligase and to this day MysD is annotated as such (Kitamura et al., 2009; Gao and Garcia-Pichel, 2011). However, recent works on MysD and its role in MAA biosynthesis reveal that this naming convention is incorrect as both in it is molecular function and phylogenetic placement MysD differs from the well characterized Ddl enzyme and renamed as mycosporine-glycine-amine-ligase (MGA-ligase) (Chen et al., 2021; Dextro et al., 2023).

In MAA biosynthesis, MysD enzyme acts on mycosporine-glycine and catalyses the attachment of an additional amino acid onto the cyclohexanone core to form an iminomycosporine (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011). In cyanobacteria, these are most commonly Thr and Ser which lead to formation of porphyra-334 and shinorine respectively (Gao and Garcia-Pichel, 2011a; Chen *et al.*, 2021a). Both in vivo and in vitro analyses of the MysD activity reveal a level of promiscuity in amino acid substrates where attachment of alanine, cysteine, arginine and glycine have also been described (Chen et al., 2021; Geraldes et al., 2020; Miyamoto et al., 2014). This was thought to be due to the mechanism of action as it activates the mycosporine-glycine chromophore rather than the amino acids (Chen et al., 2021; Gao and Garcia-Pichel, 2011). The activity of MysD therefore is distinct from that of Ddl which deals with linkage of D-alanine residues in peptidoglycan synthesis (Gao and Garcia-Pichel, 2011; Chen et al., 2021; Kitamura et al., 2009). In addition, distant phylogenetic placement of MysD and Ddl along with differences in predicted tertiary protein structures led Dextro and colleagues to propose the name; MGA-ligase (Dextro *et al.*, 2023). Due to the loose substrate selectivity of MGA-ligase, it has been accepted as a major contributor of the structural variation we observe in cyanobacterial MAAs (Dextro et al., 2023; Gao and Garcia-Pichel, 2011; Chen et al., 2021). Soon after, a study investigating efficient ways of producing MAA variants with engineered *Saccharomyces cerevisiae*, cleared a decade long confusion, revealing that the omega loop made up of 43-45 amino acids in MysD enzymes effectively determine the substrate specificity, demonstrating how MysD enzymes can be modified to catalyse the addition of desired amino acid substrates (Kim *et al.*, 2023).

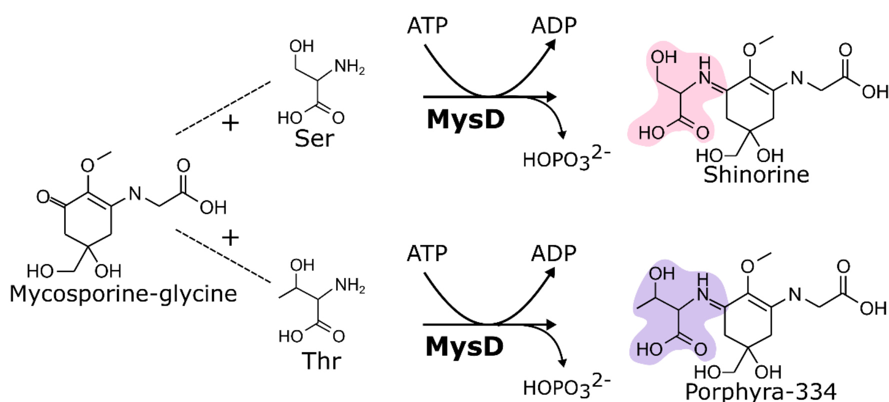

Figure 7. MysD activity scheme in forming shinorine and porphyra-334, based on proposed mechanism for MysD enzyme activity by Chen *et al.*, 2021.

7.2.5 MysE: Non-ribosomal peptide synthetase

Non-ribosomal peptide synthetases (NRPSs) are large modular and multifunctional enzymes which can utilize non-proteinogenic amino acids for the biosynthesis of peptides with diverse structures and functions (Schwarzer *et al.*, 2003). They play a crucial role in the synthesis of many secondary metabolites, including antibiotics, siderophores, and toxins in cyanobacteria and fungi (Oide and Turgeon, 2020; Welker and Von Döhren, 2006).

NRPS enzymes' modular structure is composed of a combination of domain subdivisions which deal with individual steps of the synthesis. The main ones are adenylation (A), peptidyl carrier protein (PCP)/thiolation (T) and the condensation (C) domains (Miller and Gulick, 2016; Schwarzer *et al.*, 2003). These deal with the substrate recognition, activation, formation of the peptide bond and the transport of the peptide molecule. A-domain is about 550 amino acids long and it deals with the activation of the chosen amino acid via adenylation using ATP (Miller and Gulick, 2016; Schwarzer *et al.*, 2003). Next, the activated amino acid is taken by PCP/ T domain which allows the elongation and the movement of the amino acids between catalytic centres, such as to the C domain (Miller and Gulick, 2016; Schwarzer *et al.*, 2003). Here, the nucleophilic amino acid bound to the cofactor of the PCP domain is linked with the downstream electrophilic amino acid or peptide substrate (Miller and Gulick, 2016; Schwarzer *et al.*, 2003). Once the synthesis of the peptide is finalised, the thioesterase (TE) domain that functions similarly to hydrolase or cyclases to release the synthesized peptide (Schwarzer *et al.*, 2003; Miller and Gulick, 2016; Walsh, 2016).

In the established shinorine biosynthesis, MysE enzyme is an NRPS enzyme that contains adenylation (A), thiolation (T), and thioesterase (TE) domains (Balskus and Walsh, 2010). Using HPLC assays, the authors confirmed MysE's role in catalysing the synthesis of serine onto the mycosporine-glycine to make shinorine. This was confirmed with an assay using carboxy-^{18}O-serine. Here, the shift of two mass units on LCMS revealed a shift of two mass units, indicated that MysE catalyses the activation of the serine carboxylate: adenylation and loading by the A and T domains, leading to

the loss of ^{18}O as adenosine monophosphate (AMP) (Balskus and Walsh, 2010). The MysE was strictly selective for Ser and no reactions were observed for the alternative amino acid, Thr. Authors hypothesize that the final stage of shinorine synthesis likely includes TE domain activity, which could catalyse an enol ester intermediate, leading to O-N rearrangement via 1,4-addition of serine nitrogen onto the activated mycosporine-glycine core (Balskus and Walsh, 2010).

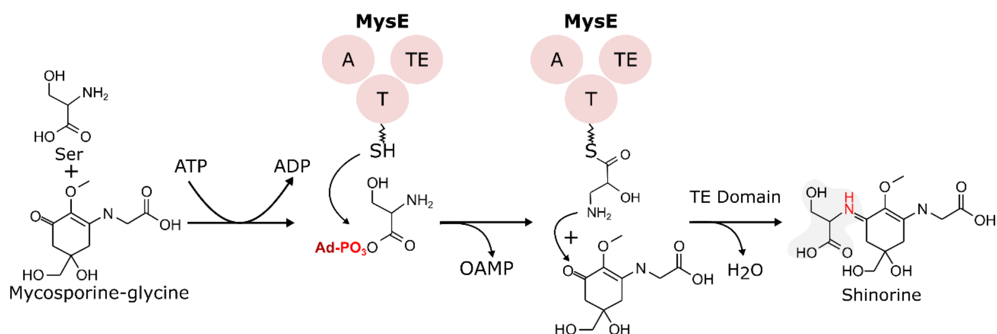

Figure 8. Simplified scheme for MysE activity in catalysing the attachment of a Ser residue on mycosporine-glycine to form shinorine based on the reaction described in Balskus and Walsh, 2010 study.

7.2.6 MysH: Dioxygenases

Dioxygenases are enzymes that catalyse the incorporation of molecular oxygen into substrates. MysH is a phytanoyl-CoA dioxygenase which is a member of the Fe (II)/2OG-dependent oxygenase family characterized by their distorted double-stranded β-helix, also known as jellyroll core folds (McDonough *et al.*, 2005; Martinez and Hausinger, 2015). These enzymes can be found in almost all organisms as they catalyse a variety of oxygenation reactions which is important for many biological processes ranting from fatty acid metabolism, DNA and RNA repair and in secondary metabolite synthesis (Martinez and Hausinger, 2015). Elucidation of the role of MysH enzyme in MAA biosynthetic pathways, specifically in palythine biosynthesis is very recent (Chen et al., 2021). Although its activity is yet to be established with *in vitro* assays, heterologous expression experiments in *E. coli* transformed with *Nostoc linckia* NIES-25 MAA biosynthetic enzymes provided clear evidence for its involvement in the synthesis of palythine variants (Chen et al., 2021). Like the MysD enzyme, MysH enzyme also demonstrates a level of substrate promiscuity as the authors were able to detect three different variants as palythine-Thr, - Ser and -Ala (Chen et al., 2021). The mechanism of action suggested for MysH enzyme seems to be similar to that of taurine:2OG dioxygenase which converts -Tau into an unstable intermediate that spontaneously decomposes into aminoacetalaldehyde and sulfite (Eichhorn *et al.*, 1997; Martinez and Hausinger, 2015). Although this reaction mechanism is yet to be tested and confirmed, MysH seems to act on the glycine residue bound to C3 of the chromophore, destabilizing it upon OH addition and leading to its breakage to leave an amide residue in its stead (Figure 9.).

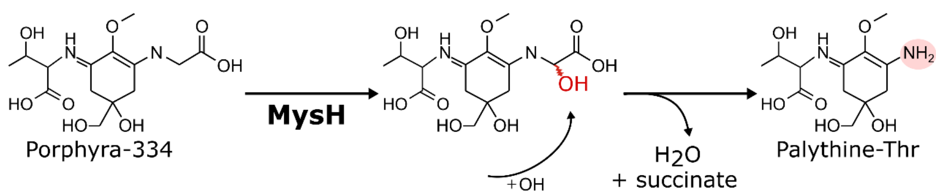

Figure 9. Proposed reaction for MysH based on the known enzymatic activities and the scheme proposed by Chen *et al.*, 2021.

8 Synthetic biology for upscaling MAA production

Commercialised MAA sunscreen formulation Heliogard[365] utilizes extracts from the red algae, *Porphyra umbilicalis* which naturally accumulates around 10 mg·g^{-1} dried biomass of porphyra-334 and shinorine (Hartmann et al., 2017; Schmid et al., 2006). Although they report effective UV protection and damage mitigation to prevent skin aging from their *in vitro* and human studies (Schmid *et al.*, 2006), their products have not been commercially available for purchase neither online nor the via the listed distributors, likely due to the low stocks. Red algae are among the highest natural MAA producers yet, the extraction yields from native producers in general are not enough for large-scale commercial applications (Rosic *et al.*, 2023; Hartmann *et al.*, 2017).

Total synthesis of MAAs had also been attempted by White and colleagues in 1989, wherein a 19-step process to synthesize mycosporine-glycine only resulted in an overall yield of 1 % (White *et al.*, 1989). Later attempts made with a different approach for the synthesis of other structural variants like asterina-330 and palythine had also failed (White *et al.*, 1995). As a result, synthetic biology approach with genetically modified microorganisms is deemed one of the most feasible options to obtain industrial scale MAA production (Wang *et al.*, 2023b; Rosic and Thornber, 2023). Therefore, understanding the genetic and molecular background of different MAA structural variants is crucial to be able to design and genetically engineer microbial factories.

An impressive level of MAA production was achieved in an engineered gram-positive *Streptomyces avermitilis* SUKA22, transformed with the MAA BGC of *mysABCD* from *Actinosynnema mirum* DSM 43827, yielded in 13.9 mg·g^{-1} of MAAs per dry cell weight. 11.4 mg·g^{-1} of this yield was shinorine, and the rest was comprised of porphyra-334, mycosporine-glycine, and mycosporine-glycine alanine (Miyamoto *et al.*, 2014). Other successful attempts include heterologous production of mycosporine-2-glycine, in recombinant *E. coli* and *Synechococcus elongatus* expressing MAA BGC of *Aphanocapsa halophytica* (Waditee-Sirisattha *et al.*, 2014; Patipong *et al.*, 2017).

Considerable progress has also been made in MAA production in metabolically engineered yeast species *Yarrowia lipolytica* and *S. cerevisiae* (Yang *et al.*, 2018; Jin *et al.*, 2021; Park *et al.*, 2019; Hengardi *et al.*, 2024; Kim *et al.*, 2023; Jin *et al.*, 2023) Researchers successfully increased MAA production via metabolic engineering of pentose phosphate pathway to enable higher accumulation of the MAA precursor, S7P, leading to the production of 1.53 g·L^{-1} of shinorine (Kim *et al.*, 2023). This was mainly achieved by the deletion of the transaldolase activity which reduced S7P breakdown and thus accumulation (Park et al., 2019). Improving upon this Hengardi and colleagues demonstrated how shinorine production could be further improved by also the deletion of phosphofructokinase enzymes allowing increased fructose-6-phosphate accumulation to drive further increase of S7P production (Hengardi *et al.*, 2024).

9 Study Aims

In this thesis, we employed a multidisciplinary approach to investigate the distribution and diversity of MAA biosynthetic pathways in cyanobacteria. The objectives of this thesis are listed as follows:

- Characterize the chemical structures of novel structural MAA variants detected.
- Obtain whole genomes for the strains studied and identify the corresponding BGCs.
- Investigate the distribution of MAA BGCs in other cyanobacterial genomes.
- Combining bioinformatic analyses with molecular tools, study the enzymatic reactions involved in MAA biosynthesis to provide a biosynthetic scheme for the characterized MAA structural variants.
- Investigate the distribution and prevalence of MAAs and MAA biosynthetic pathways in the surface bloom forming bacteria sampled from the Baltic Sea and Finnish lakes.

Overall, we set out to bridge gap in our understanding of the genetic and molecular diversity that leads to the observed structural diversity of MAAs in cyanobacteria. We hope that our results will help future research as well as work involving construction of engineered microbial cell factories for large scale production of MAAs for litany of potential biotechnological applications. By also investigating prevalence of MAAs in bloom forming cyanobacteria we hoped to assess their potential as natural reservoirs. Additionally, studying diversity and distribution of MAA biosynthetic pathways in cyanobacterial genomes we also attempted to gain a deeper understanding of the evolutionary processes that shape secondary metabolite biosynthetic pathways.

10 Methods and Materials

Table 2 Methods utilized for the results presented in this thesis as described in the following studies.

Method	Article
Cell culture growth and maintenance	I, II, III
DNA extraction	I, II
Genome sequencing	I, II
Genome mining	I, II
Plasmid engineering	II
Designing synthetic constructs	II
Cloning/ transformation	II
Heterologous expression in *E. coli*	II
Production optimization	II
Extraction of MAAs	I, II, III
HR-LCMS	I, II, III
Flash column chromatography	I, II, III
HPLC/UPLC	I, II, III
NMR	I, II

11 Summary of Results

11.1 Plasticity of MAA biosynthetic pathways in Cyanobacteria (I)

In this study we focused on the MAA biosynthetic pathway of lichen-symbiont *Nostoc* sp. UHCC 0926 maintained in our culture collection. Initial HR-LCMS screening of the *Nostoc* sp. UHCC 0926 biomass extracts revealed the production of two distinct MAA structural variants (Arsın *et al.*, 2023). To follow this, we purified both variants and utilized NMR analysis to characterize them as Aplysiapalythine E ($C_{23}H_{38}N_2O_{15}$) and Tricore B ($C_{34}H_{53}N_4O_{15}$) (Figure 10.). At the time of this study Aplysiapalythine E was a new structural MAA variant whereas the MAA variants with three chromophores such as the Tricore B had been previously reported (Arsın *et al.*, 2023).

To study the biosynthetic pathway corresponding to the synthesis of two such distinct variants, we obtained a whole genome sequence of 8.3 Mb for *Nostoc* sp. UHCC 0926. Our genome mining analysis revealed the presence of previously established MAA biosynthetic enzymes encoded in three separate locations of the genome (Arsın *et al.*, 2023). While the $mysABC_1$ and $mysDC_2C_3$ were located on the chromosome, *mysFIE* gene cluster, which encoded for an additional putative methyltransferase (MysF), a TauD/TfdA family dioxygenase (MysI) and an NRPS (MysE) enzyme, encoded on a plasmid (Arsın *et al.*, 2023). Based on the previously established MAA biosynthetic pathway, predicted enzyme activity and the MAA structural variants we had characterized we proposed a branched biosynthetic pathway involving the cooperation of the three distant MAA biosynthetic gene clusters for the synthesis of the two distinct MAA variants (Figure 10.) (Arsın *et al.*, 2023).

Further bioinformatic analyses of publicly available complete cyanobacterial genomes possessing an MAA biosynthetic gene cluster revealed that 12 % had a similarly discontiguous organisation (Arsın *et al.*, 2023). Including the publicly available genomes, we also analysed the distribution of the individual MAA biosynthetic enzymes via the construction of phylogenetic trees. Phylogenetic distribution of methyltransferase, dioxygenase and NRPS enzymes involved in MAA biosynthesis helped solidify our predictions for their activity in the proposed scheme. Additionally, our analysis of NRPS enzymes also revealed a cryptic diversity among their substrate preferences in MAA biosynthesis, showing they can have substrate specificities for also Gly and Pro along with previously known Ser (Arsın *et al.*, 2023). Overall, our results suggest that MAA biosynthetic pathways are subject to rapid evolutionary processes and highlight the subsequent diversity and complexity beyond previous assumptions (Arsın *et al.*, 2023).

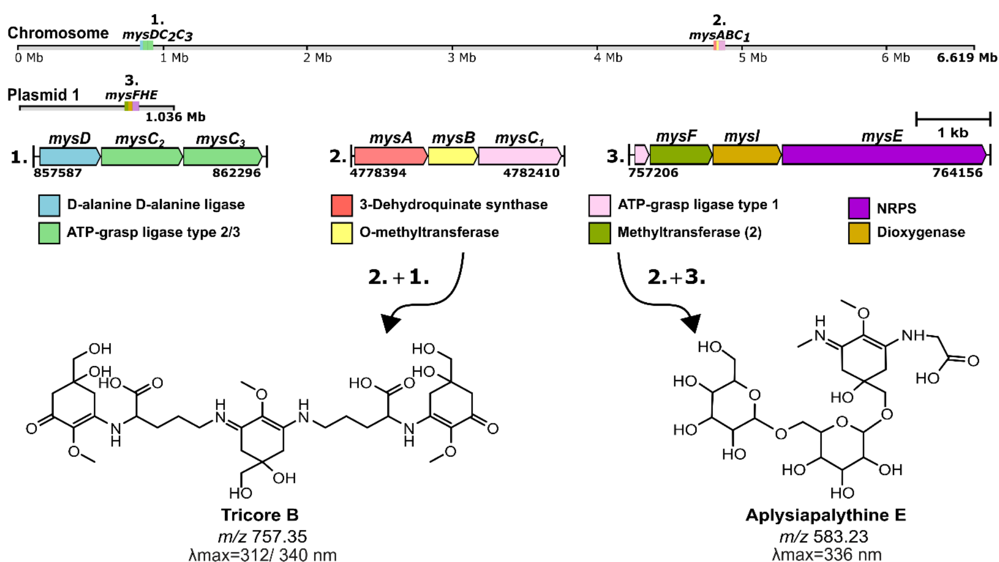

Figure 10. Discontiguous MAA biosynthetic gene clusters identified in different sections of *Nostoc* sp. UHCC 0926 genome coordinating for the synthesis of two distinct MAA structural variants: Tricore B and Aplysiapalythine E. Figure taken from publication I, (Arsın *et al.*, 2023).

11.2 Genetic and molecular insights into biosynthesis of glycosylated MAAs (II)

HR-LCMS screening revealed that the benthic cyanobacterium *Nostoc* sp. UHCC 0302 was able to produce two novel diglycosylated MAA structural variants constitutively. These were the major hexosyl-pentosyl-palythine-Ser and the minor hexosyl-pentosyl-palythine-Thr variants (Arsın *et al.*, 2024). We purified and characterized the chemical structure of the major MAA structural variant by nuclear magnetic resonance (NMR) and confirmed its novel structure as 568 Da, ($C_{22}H_{36}N_2O_{15}$),11-(β-d-galactopyranosyl)-7-(α-d-xylopyranosyl)-palythine-Ser (Figure 11.). We then obtained a whole genome sequence for *Nostoc* sp. UHCC 0302 and identified the 8.3 kb MAA BGC of *mysABCJD$_1$G$_1$H*, and a distant partial cluster of *mysD$_2$J$_2$G$_2$*, encoding additional copies of the MysD, MysJ and MysG (Arsın *et al.*, 2024). Here, the MysJ and MysG enzymes are two distinct types of glycosyltransferases which we thought might be involved in the attachment of hexose and pentose sugar moieties onto the identified MAA structural variants (Arsın *et al.*, 2024). We tested this via heterologously expressing the refactored 8.3 kb *mysABCJD$_1$G$_1$H* gene cluster in *E. coli*. This yielded mainly in the production of 450 Da, ($C_{18}H_{30}N_2O_{11}$) hexose bound palythine-Thr (7-(α-d-glucopyranosyl)-palythine-Thr), and this result pointed towards two main facts. Firstly, MysG$_1$ enzyme, homologous to a glycosyltransferase family 4 enzymes, consistently catalysed the addition of a hexose sugar onto the palythine-Thr intermediate in *E. coli* (Arsın *et al.*, 2024). Secondly, and interestingly, the dominant variants produced in *E. coli* are Thr bound, meaning the MysD$_1$ enzyme is selecting for Thr in *E. coli* but somehow not in *Nostoc* sp. UHCC 0302, where the dominant MAA structural variant is Ser bound (Arsın *et al.*, 2024). This led us to designing a

modifiable synthetic MAA BGC construct including the enzymes encoded in the distant, partial, $mysD_2J_2G_2$ alongside the main gene cluster genes to test whether the enzymes encoded in the distant and incomplete cluster are involved in MAA biosynthesis as well (Arsın *et al.*, 2024).

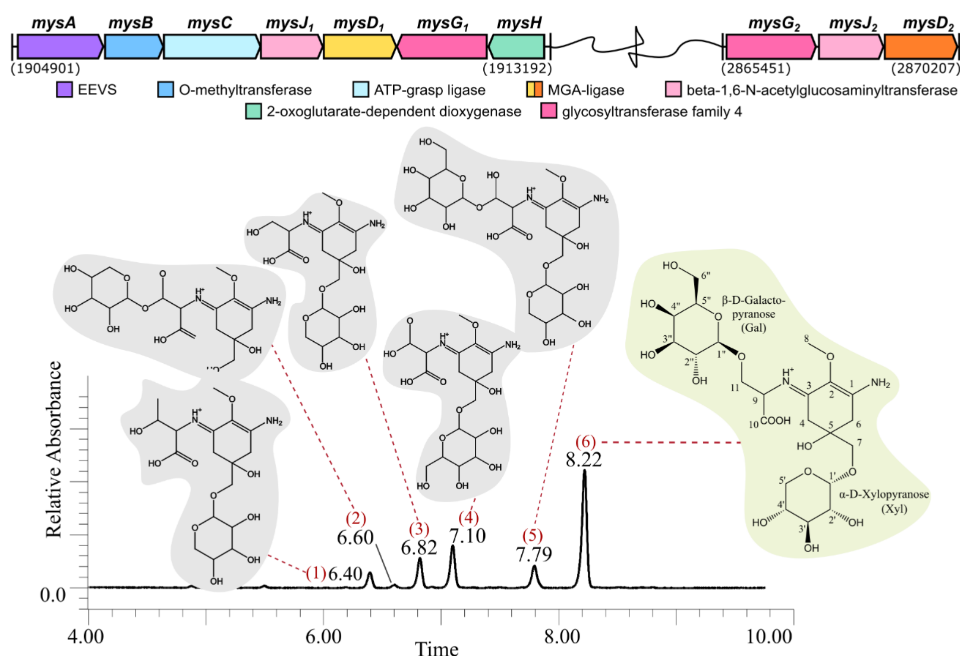

Figure 11. Discontiguous MAA BGC of *Nostoc* sp. UHCC 0302 which includes pairs of two distinct glycosyltransferase enzymes and the structural MAA variants and intermediates detected by HR-LCMS-UV. The major 568 Da galactosyl-xylosyl-palythine-Ser variant chemical structure characterized by NMR is highlighted in lime-green. Figure adopted from publication II, (Arsın *et al.*, 2024).

The heterologous expression of $mysABCD_1$ and $mysABCD_2$ in *E. coli* clarified the substrate specificities where MysD$_1$ indeed was specific towards Thr while MysD$_2$ had a higher preference for Ser while also being more promiscuous (Figure 12.) (Arsın *et al.*, 2024). Like our first study, this meant that the two distant gene clusters in *Nostoc* sp. UHCC 0302 are also required to collaborate to synthesize major galactosyl-xylosyl-palythine-Ser and the minor hexosyl-pentosyl-Thr variants. Additionally, we also investigated the occurrence and distribution of similar MysD variants as well as glycosyltransferases in other 336 publicly available complete cyanobacterial genomes including a MAA BGC as well (Arsın *et al.*, 2024). This revealed a random, scattered phylogenetic distribution for MysD$_1$ and MysD2 enzymes in cyanobacterial genomes (Figure 12.). In the case of glycosyltransferases, MysG seems to be more commonly associated with MAA biosynthetic clusters while MysJ enzyme's occurrence is limited to a single clade of 7 cyanobacterial strains out of the 70 analysed (II, Supplementary Figure S21.) (Arsın *et al.*, 2024). Although previously, many studies have reported glycosylated MAA variants, no putative glycosyltransferase enzymes had been identified neither near MAA BGCs nor elsewhere in genome. Therefore, MysG and MysJ presented in this study are the first glycosyltransferases implicated in MAA

biosynthesis. Our results provided further insights into the dynamic nature of MAA biosynthetic pathways while also introducing new set of putative enzymes that contribute to the structural diversity of MAAs (Arsın *et al.*, 2024).

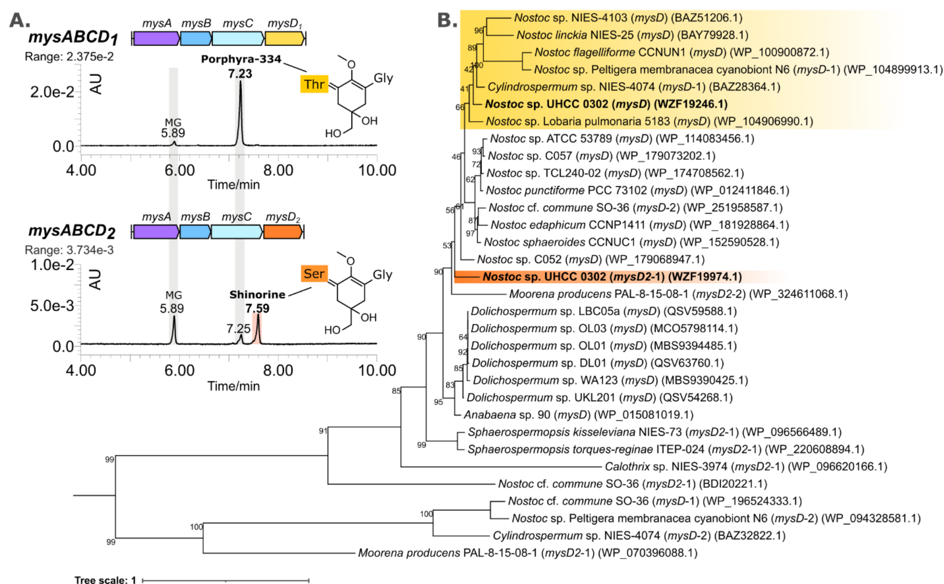

Figure 12. Comparing MAA intermediates and variants detected by HR-LCMS-UV at 320 nm, extracted from *E. coli*colonies expressing MAA biosynthetic gene cluster constructs *mysABCD₁*, and *mysABCD₂* (A). Phylogenetic distribution of the MysD enzymes selected from complete cyanobacterial genomes encoding for multiple MysD homologs in and around MAA biosynthetic gene clusters (B). Figure taken from publication, II (Arsın *et al.*, 2024).

11.3 Prevalence of MAA biosynthetic pathways in cyanobacterial blooms from Baltic Sea (III)

MAAs have been reported from all orders of cyanobacteria inhabiting various types of environments including blooms in freshwater lakes, reservoirs, and other water bodies. Cyanobacterial bloom formations are common occurrences in Baltic Sea region and in Finnish lakes (Vuori *et al.*, 2025). Cyanobacterial blooms can also be found forming surface scums due to various environmental factors, leaving the cells exposed to constant and high levels of UV radiation. To investigate the prevalence of MAA production in surface scums of cyanobacteria, we analysed 59 environmental samples collected from the southern coast of Finland during the summer months of 2021 and 2022 (Figure 13.) (Vuori *et al.*, 2025). HR-LCMS and UPLC screening of these samples revealed presence of MAAs in almost all samples, containing mostly shinorine along with varying amounts of porphyra-334 in combination (Figure 1.) (Vuori *et al.*, 2025).

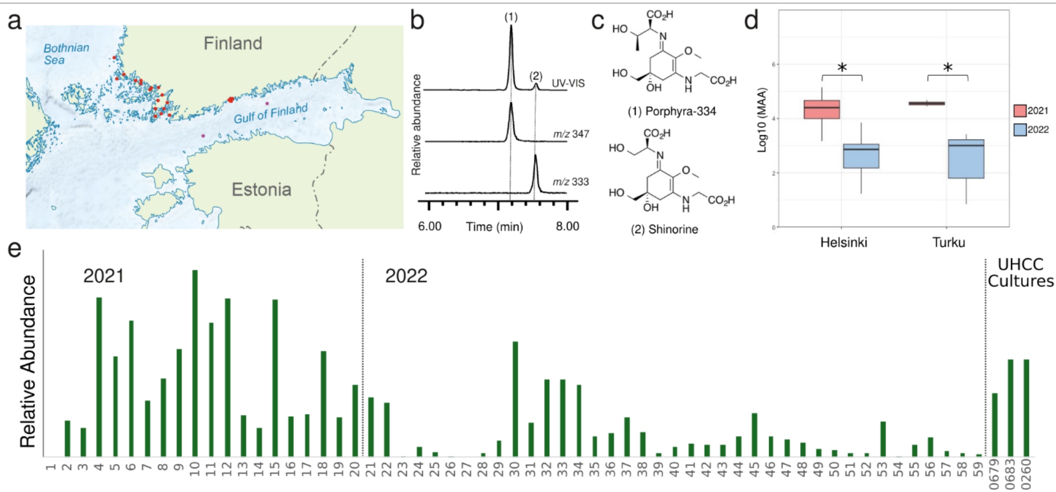

Figure 13. Relative total MAA abundance of 59 surface bloom samples (e) collected from the southern coast of the Baltic Sea in 2021 and 2022, sampling locations marked by red dots (a). Example of HR-LCMS-UV spectra with peaks 1 and 2 corresponding to porphyra-334 and shinorine (b). Chemical structures of porphyra-334 and shinorine (c). Boxplot of Log10-transformed total relative amounts of MAAs where we noted significant differences at $p = 0.05$ (*) between the years 2021 and 2022 (d). Figure adopted from publication III, (Vuori *et al.*, 2025).

Scum samples were further investigated by DNA extraction and screened for MAA biosynthetic pathways using the conserved *mysB* gene sequence (Vuori *et al.*, 2025). This revealed that all samples tested possessed a form of an MAA biosynthetic gene cluster. We then focused on the high MAA producing scum sample no. 4 and by sequencing its *mysB* gene we found out that its sequence was most like the *mysB* of *Aphanizomenon flos-aquae* FACHB-1171. Phylogenetic tree constructed using MysB protein sequences, placed sample no.4 in a clade with the *Aphanizomenon flos-aquae* and *Dolichospermum* sp. which are a part of the ADA species complex commonly found in blooms (Vuori *et al.*, 2025). Based on these, we extended our bioinformatic analyses to look into the distribution of the MAA biosynthetic enzymes in the ADA species complex, using a phylogenomic tree constructed using 120 bacterial single copy conserved genes from 101 publicly available genomes of the cyanobacteria. The analysis revealed that 48 % of the species within the ADA species complex encode MAA BGCs, most commonly in *mysABCD* organisation and this was most prominent in ADA-2 and ADA-4 complexes (Vuori *et al.*, 2025).

Additionally, we compared the five MAA producing *Dolichospermum* sp. strains isolated from the surface waters of the Gulf of Finland between 2004-2007 and maintained under constant low-light conditions (Vuori *et al.*, 2025). Based on UV absorbtion peak area and extinction coefficients we estimate that the *Dolichospermum* sp. UHCC 0684 produces the highest amount of porphyra-334, at around 7.4 mg·g^{-1} dry weight. Although quantification of the MAAs from the scum samples were out of the scope of this study our collective findings highlight the prevalence of MAA production in varying amounts in bloom forming ADA complex species (Vuori *et al.*, 2025).

12 Discussion

12.1 Discovery of novel structural MAA variants (I, II)

Our work on the *Nostoc* sp. UHCC 0926 (I) and UHCC 0302 (II) aimed to investigate and broaden our understanding of genetic and molecular diversity underlying MAA biosynthesis alongside the chemical characterization of new MAA structural variants. Utilizing HR-LCMS, in total we identified three novel glycosylated MAA structural variants; Aplysiapalythine E (I) and the hexosyl-pentosyl-palythine-Ser/-Thr (II) (Arsın *et al.*, 2024, 2023). Using NMR, we managed to provide the detailed chemical structures for Aplysiapalythine E (I) and 568 Da galactosyl-xylosyl-palythine-Ser (II) (Arsın *et al.*, 2024, 2023).

Aplysiapalythine A, B and C variants were first identified in sea hare, *Aplysia californica* (Kamio *et al.*, 2011). Aplysiapalythine C and D variants are the only other two types that have been identified from cyanobacteria (Geraldes *et al.*, 2020b; Werner *et al.*, 2021) (Table 1.) Aplysiapalythine E identified in our study from *Nostoc* sp. UHCC 0926, has a core structure matching to Aplysiapaltyhine C (N-methylpalythine) but also possesses two hexose moieties attached to C7 of the chromophore, making it a novel variant (I) (Arsın *et al.*, 2023). Similarly, monoglycosylated palythine-Thr/Ser and 2 x hexose-palythine-Thr variants were previously reported from cyanobacteria (Table 1.) (Nazifi *et al.*, 2013; D'Agostino *et al.*, 2016). The two MAAs variants we identified from the benthic cyanobacterium *Nostoc* sp. UHCC 0302 were novel as both the palythine-Thr and palythine-Ser structural variants possessed one hexose and one pentose moiety (II) (Arsın *et al.*, 2024). Many glycosylated MAAs have been reported to have antioxidant properties, and it is also thought that glycosylation allows for integration into EPS layers of cyanobacteria to provide effective protection against harmful effects of UV-R (Matsui *et al.*, 2011; Nazifi *et al.*, 2013; Ishihara *et al.*, 2017). Further work on the novel variants we characterized would have involved testing for antioxidant capabilities and covalent addition strategies in nanocellulose hydrogel formulations that could potentially have numerous cosmetic and pharmaceutical applications (Heise *et al.*, 2021; De France *et al.*, 2017). However, due to limitations of low production and purification yields this was not possible.

12.2 Capturing biosynthetic diversity and complexity using manual genome mining and annotation methods (I, II)

In 2010, seminal work on the biosynthetic pathway of shinorine provided the first molecular and genetic understanding of mycosporine-like amino acid (MAA) biosynthesis, identifying the *mysABCD/E* MAA BGC organization (Balskus and Walsh, 2010; Gao and Garcia-Pichel, 2011a). This discovery remained the foundational knowledge for MAA biosynthesis for almost over a decade. Meanwhile, the literature expanded with reports of novel MAA structural variants from diverse organisms and environments (Table 1; Peng *et al.*, 2023; Oren and Gunde-Cimerman, 2007). This thesis aimed to connect the growing

structural diversity of MAAs with the underlying molecular and genetic mechanisms of their biosynthetic pathways through a multidisciplinary, hands-on approach. By employing a manual genome mining strategy, we explored beyond the well-characterized, compact MAA BGC organization (*mysABCD/E*) and uncovered the hidden complexity of these pathways (I, II) (Arsın *et al.*, 2024, 2023). At the start of the thesis project, the widely used secondary metabolite genome mining tool, antiSMASH v5.0, although effective in identifying general secondary metabolite pathways, was insufficient for detecting MAA BGCs with more intricate architectures (Blin *et al.*, 2019; Micallef *et al.*, 2015). Thus, by manually screening 10 kb flanking regions of homologous sequences to known MAA biosynthetic enzymes, we identified more complex MAA BGCs with additional putative enzymes and discontiguous gene cluster organizations (I, II, unpublished) (Arsın *et al.*, 2024, 2023).

The biosynthesis of the two distinct MAA structural variants, Tricore B and Aplysiapalythine E variants produced by the lichen-symbiont *Nostoc* sp. UHCC 0926's could not have been linked to the initially identified MAA BGC of *mysABC*$_1$ (I). Based on the works done on *Nostoc flagelliforme* CCNUN1 which possessed a MAA BGC of *mysABDC*$_2$*C*$_3$ (Zhang *et al.*, 2021; Shang *et al.*, 2018), and phylogenetic analyses of each of the enzymes, we were able to propose that the distantly located *mysDC*$_2$*C*$_3$ in *Nostoc* sp. UHCC 0926 should be involved in the synthesis of Tricore B variant (I) (Arsın *et al.*, 2023). Similarly, the Aplysiapalythine E variant (I) required the likely involvement of *mysFIE* located as its synthesis necessitates the activity of an additional methyltransferase (MysF), dioxygenase (MysI) which likely acts on the amino acid residue bound on C1, unlike the previously identified MysH, acting on C3 (Chen *et al.*, 2021a), and an enzyme for the addition of an amino acid on C1, which in this case was an NRPS (MysE) (Arsın *et al.*, 2023). Based on the bioinformatic analyses we revealed a cryptic diversity among MysE enzymes predicting additional substrate specificities other than the most common -Ser (Balskus and Walsh, 2010), such as -Gly in the MAA biosynthetic pathway of *Nostoc* sp. UHCC 0926 (I) (Arsın *et al.*, 2023). In addition, the novel Aplysiapaltyhine E variant contains two hexose sugars, yet, like many other previous studies, we could not identify any putative glycosyltransferase enzymes in the UHCC 0926 genome (Matsui *et al.*, 2011; Nazifi *et al.*, 2013; Ishihara *et al.*, 2017; Nazifi *et al.*, 2015; D'Agostino *et al.*, 2016). Although the proposed biosynthetic pathway involving the discontiguous MAA BGCs of *Nostoc* sp. UHCC 0926 (I) is a hypothetical one that warrants further work to establish the reactions suggested, this work revealed that the concept of discontiguous BGCs was not an uncommon one for MAA biosynthesis (I, II) (Arsın *et al.*, 2024, 2023).

12.3 Heterologous expression for investigation of MAA biosynthetic pathway (II)

In the case of *Nostoc* sp. UHCC 0302 MAA biosynthetic pathway, we were able to show that the distantly located *mysABCJ*$_1$*D*$_1$*G*$_1$*H* and *mysD*$_2$*J*$_2$*G*$_2$ clusters were involved in MAA biosynthesis via heterologous expression systems in *E. coli* (II) (Arsın *et al.*, 2024). This work provided first glances into the glycosylation of MAAs as the MysG$_1$, a glycosyltransferase family 4, consistently catalysed the addition of a hexose sugar in the

recombinant *E. coli* expressing the refactored *mysABCJ$_1$D$_1$G$_1$H* gene cluster (II) (Arsın *et al.*, 2024, 2023). Although we could not detect any activity for the MysJ$_1$ and the MysJ$_2$/G$_2$ pair encoded in the distant cluster, we could suggest activities based on the bioinformatic predictions and the chemical structures we characterized (II) (Arsın *et al.*, 2024). Glycosyltransferase enzymes are involved vast in a range of biological processes and cyanobacterial genomes are known to encode for multiple copies with no clear function discerned so far (Kehr and Dittmann, 2015; Schmid *et al.*, 2016; Potnis *et al.*, 2021). While biochemical characterisation is required to confirm the activity of these enzymes, we hope that with this work we could give clear pointers for future research (II) (Arsın *et al.*, 2024). Heterologous expression in *E. coli* also yielded in curious results regarding the MysD derivatives where MysD$_1$ is involved in synthesis of palythine-Thr and MysD$_2$ in palythine-Ser (Figure 11, 12.) (II) (Arsın *et al.*, 2024). This meant that the distantly located MysD2 was the active enzyme in synthesis of the major 568 Da galactosyl-xylosyl-palythine-Ser variant and not the MysD$_1$ encoded in the main MAA BGC (II) (Arsın *et al.*, 2024). To understand the regulatory elements orchestrating the expression of discontiguous BGCs in both *Nostoc* sp. UHCC 0926 (I) and UHCC 0302 (II) a transcriptomic approach is necessary (Arsın *et al.*, 2024, 2023).

Overall, our collective findings from articles I and II predict a highly dynamic nature for MAA biosynthetic pathways, that are likely subject to regular horizontal gene transfer (HGT) events in cyanobacterial genomes, mixing and shuffling existing MAA BGCs to create new ones (I, II) (Arsın *et al.*, 2024, 2023). HGT events are major contributors of prokaryotic genome evolution and secondary metabolite pathways are generally known to be more prone to HGT events, especially smaller pathways such as that of MAAs (Jain *et al.*, 1999; Shi and Falkowski, 2008; Chevrette *et al.*, 2020; Chen *et al.*, 2021c). Unlike the conserved primary metabolic pathways and the photosynthetic machinery, there are no immediate detrimental consequences for losing, mutating or gaining secondary metabolite biosynthetic pathways and diversifying the end products (Fischbach *et al.*, 2008). Although MAAs, due to their effective and efficient multifunctional nature are likely very advantageous for the producing organism (Oren and Gunde-Cimerman, 2007), loss or diversification of its pathways are not likely to reduce the fitness of the organism drastically thereby retaining the diversifying pathway within the gene pool (Fischbach *et al.*, 2008). This can allow for further evolution and distribution of the mobile, rapidly shifting, mosaic biosynthetic pathways leading to the structural diversity we are observing.

12.4 Bloom forming cyanobacteria as a source of MAAs (III)

MAAs have been reported from various species of cyanobacteria including well studied bloom formers such as *Microcystis aeruginosa* and *Aphanizomenon flos aquae* (Hu *et al.*, 2015; Zhang *et al.*, 2022a). Previous findings reported low MAA concentrations from these strains and suggested the likelihood of MAAs as accessory UV screening compounds alongside caretenoids and polysaccharides (Hu *et al.*, 2015; Zhang *et al.*, 2022a). Our results add to these findings with the Baltic Sea surface bloom (scum) samples (III), wherein almost all tested samples contained some amount of shinorine and porphyra-334 (Vuori *et al.*,

2025). Although the exact amounts were not possible to calculate, comparing the relative amounts based on UV absorbtion peak area we could identify samples that were high MAA producers such as the scum sample no. 4, 10, 12 and 15 (Figure 13.). Phylogenetic analysis based on the MysB sequence show that the representative sample no. 4 has high similarity to *Aphanizomenon* sp. which belong to ADA complex species. Our extended bioinformatic analysis also shows ADA complex species, especially species belonging to ADA-2 and ADA-4 clades commonly possess MAA BGCs (III) (Vuori *et al.*, 2025). These cyanobacteria species are prolific bloom formers and known to produce variety of toxins (Dreher *et al.*, 2021; Österholm *et al.*, 2020). Mechanisms of surface blooms are still being studied however, it has been suggested that increased polysaccharide formation might be playing an important role in formation and maintenance (Yang *et al.*, 2024; Aparicio Medrano *et al.*, 2016; Wu *et al.*, 2024) MAAs are multifunctional compounds and their incorporation into these polysaccharide would make sense (Wu *et al.*, 2024; Aparicio Medrano *et al.*, 2016; Zhang *et al.*, 2022a). Although this statement warrants further experimentation, synergistic regulatory mechanisms for MAA and EPS upregulation would also support this hypothesis, wherein MAAs in EPS may provide physical support while also absorbing UV radiation and acting as antioxidants and thus help form and maintain the surface blooms (Yang *et al.*, 2024; Jacinavicius *et al.*, 2021; Wright *et al.*, 2005; Potnis *et al.*, 2021).

Further experimentation would also be required in elucidating regulatory mechanisms of high MAA producing strains such as the *Dolichospermum* sp. UHCC 0684 which constitutively produces and accumulates around 7.4 $mg{\cdot}g^{-1}$ dry weight of MAAs (III) (Vuori *et al.*, 2025). Difference is striking when compared to other cyanobacteria we worked on, such as benthic *Nostoc* sp. UHCC 0302 that constitutively accumulates only about 0.29 $mg{\cdot}g^{-1}$ dry weight of MAAs (II) (Arsın *et al.*, 2024). Even in the engineered *E. coli* maximum MAA concentration we managed to achieve was only 10x more with 2.9 $mg{\cdot}g^{-1}$ dry weight of MAAs (II) (Arsın *et al.*, 2024). These results point at blooms as potential sources of MAAs. Even though the MAA content varies in different bloom forming strains as well, with extensive nationwide bloom harvests the extracts could potentially be enough for biotechnological applications, while also helping mitigate ecological damage they might cause otherwise (Gu *et al.*, 2022).

12.5 Conclusions

Our research provides valuable insights into genetic and molecular diversity of the cyanobacterial MAA biosynthetic pathways, shedding some light on the dynamic evolutionary processes underlying them. Combining structural chemistry, molecular biology and bioinformatics we presented novel biosynthetic pathways for the novel MAA structural variants characterised from the two *Nostoc* strains we studied. Through this work we demonstrated the intricate complexities found in MAA biosynthetic pathways and the encoded enzymes, providing blueprints of the structural diversity we observe. The discovery of discontiguous biosynthetic pathways found in both *Nostoc* sp. UHCC 0926 and UHCC 0302, show the dynamic nature and rapid evolution of MAA BGCs. The diversity is further enhanced by the cryptic variations of MAA biosynthetic enzymes, such as MysE with broader

substrate specificities as well as additional accessory enzymes like MysF and MysI. While these enzymes' molecular mechanisms need further work to uncover, for the *Nostoc* sp. UHCC 0302, heterologous expression of its refactored MAA biosynthetic pathway provided some answers. Particularly implication of the glycosyltransferase enzyme $MysG_1$ in hexose addition as well as involvement of two MysD derivatives in the synthesis different MAA structural variants. All in all, these results manage to address decades long gaps in our understanding of MAA biosynthesis and the genetic and molecular diversity of its pathways (Arsın *et al.*, 2023, 2024).

In addition to the *Nostoc* sp. that are rich in more complex MAA structural variants and biosynthetic pathways, we show that bloom forming cyanobacteria can be a potential reservoir of MAAs such as shinorine and porphyra-334. Particularly the cyanobacteria belonging to ADA complex species that are prolific toxic bloom formers, are also rich in MAAs. MAA production might have a role in establishing persistent surface blooms during summer months. While this is a major ecological problem and a topic for further investigation to better understand bloom formation mechanisms, this could also be viewed as an opportunity. Tackling cyanobacterial blooms by harvesting them in large scales as a form of alleviating their environmental damage, may also provide useful in sourcing of MAAs and other secondary metabolites for industrial applications (Vuori *et al.*, 2025).

Ultimately, this thesis work advances the field by characterizing novel structural variants of MAAs and their complex biosynthetic pathways. By exploring the diversity and distribution of MAA biosynthetic pathways, this study provides insights into the evolutionary forces shaping natural product biosynthesis. Beyond its fundamental biological significance, the findings presented in this thesis also support future biotechnological and synthetic biology efforts to optimize and upscale MAAs for commercial applications.

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14 Original Scientific Articles

I. “A Plastic Biosynthetic Pathway for the Production of Structurally Distinct Microbial Sunscreens” - Sıla Arsın, Endrews Delbaje, Jouni Jokela, Matti Wahlsten, Zoe M. Farrar., Perttu Permi, David Fewer., 15 Sept 2023, In: **ACS Chemical Biology**. 18, 9, p. 1959-1967 9 p. DOI: 10.1021/acschembio.3c00112

II. “A refactored biosynthetic pathway for the production of glycosylated microbial sunscreens” - Sıla Arsın, Endrews Delbaje, Maija Pollari, Jouni Jokela, Matti Wahlsten, Perttu Permi, David Fewer., 20 Aug 2024, In: **RSC Chemical Biology**. DOI: 10.1039/D4CB00128A

III. “Direct evidence for production of microbial sunscreens by scum-forming cyanobacteria from the Baltic Sea” - Inkeri Vuori, Greta Gaiani, Endrews Delbaje, Sıla Arsın, Jouni Jokela, Matti Wahlsten, Perttu Permi, David Fewer., 16 Jan 2025, In: **Environmental Microbiology Reports** DOI: 10.1111/1758-2229.70056