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Detection of Ribosomal Peptides in Cyanobacterial Strains

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TESE DE MESTRADO APRESENTADA

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Detection of Ribosomal Peptides in Cyanobacterial Strains

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Abstract

Cyanobacteria produce a variety of secondary metabolites of peptidic nature, many of which with potent biological activities. These can be produced either ribosomally or non- ribosomally. Cyanobactins are a group of recently described cyclic peptides with low molecular weight, synthesized by ribosomal pathways that exhibit important bioactivities, such as antitumor, cytotoxic or multi-drug-reversing activities.

Anacyclamides are a set of cyclic cyanobactins, which have been described from strains belonging to the *Anabaena* genus.

The main objective of this work was the detection of anacyclamide- related genes in cyanobacteria from the culture collection of LEGE (LEGE CC) in an attempt to expand the diversity of anacyclamides know to date. In addition, we tried to characterize the cyanobacterial strains using both morphological and molecular data, with the ultimate goal of making phylogenetic inferences regarding cyanobactins production.

For this effect, we conducted a molecular screening among *Anabaena* strains, for a gene involved in the synthesis of the ribosomal peptides cyanobactins (patE). As a result, we detected a putative new anacyclamide in two of the ten *Anabaena* strains. Sequencing of the patE gene allowed us to predict the amino acid sequence of a new anacyclamide. The presence of the new compound in the cultures was checked using LC-MS, which additionally suggested that the cyanobactin was post-translationally modified through two prenylation events.

By expanding the known genetic and chemical diversity of anacyclamides this work can help us to know more about the cyanobactins. In particular, this new anacyclamide can aid in investigations on post-translational processing of cyclic peptides derived from these pathways.

Resumo

As cianobactérias produzem uma variedade de metabolitos secundários de natureza peptídica, muitos dos quais apresentam forte bioactividade. Estes biocompostos podem ser produzidos quer por via ribossomal quer por via não ribossomal. As cianobactinas são um grupo de péptidos cíclicos recentemente descobertos, com baixo peso molecular, sintetizados por ribossomas e que apresentam importantes bioactividades, tais como actividade citotóxica, anti tumoral ou reversora de multi drogas.

Neste sentido, o objectivo principal deste trabalho foi a detecção dos genes relacionados com a produção de anaciclamidas em cianobactérias provenientes da colecção de culturas LEGE (LEGE CC), com a consequente identificação de novas anaciclamidas. Da mesma forma, foi feita a caracterização morfológica e o rastreio molecular das estirpes de cianobactérias, tendo como ultimo objectivo a análise filogenética, atendendo à produção de cianobactinas por parte das mesmas.

Para este efeito, o rastreio molecular teve como alvo o gene envolvido na síntese dos péptidos ribossomais (cianobactinas) – patE. Como resultado, detectámos uma nova anaciclamida em duas das dez estirpes analisadas.

A sequenciação do gene patE permitiu-nos prever a presença da anaciclamida, bem como a sua sequência de amino ácidos, em duas das dez estirpes em estudo. A presença desta nova anaciclamida nas culturas foi confirmada usando a LC-MS. A utilização desta técnica também demostrou que a nova cyanobactina apresentava modificações pós tradução bem como dois grupos -prenil na sua constituição.

Assim, este trabalho ajudou a aumentar o conhecimento sobre este grupo de péptidos, de baixo peso molecular e com grandes variações estruturais. O estudo da nova anaciclamida pode estender-nos o conhecimento em relação às modificações pós tradução, que caracterizam estes péptidos, particularmente sobre prenilação.

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Abbreviations

Ala (A) Alanine

Arg (R) Arginine

Asn (N) Asparagine

Asp (D) Aspartic acid

ATP- Adenosine-5'-triphosphate

BLAST- Basic Local Alignment Search Tool

bp- base pair

Cys (C) Cysteine

DNA- Deoxyribonucleic acid

Gln (Q) Glutamine

Glu (E) Glutamic acid

Gly (G) Glycine

HIV- Human immunodeficiency vírus

Ile (I) Isoleusine

LC- liquid chromatography

LEGE- Laboratório de Ecologia, Genómica e Evolução

Leu (L) Leusine

Lys (K) Lysine

Met (M) Methionine

MS- mass spectrometry

NMR- nuclear magnetic resonance

NRPS- non-ribosomal peptide synthetase

ORF- Open Reading frame

PCR- polymerase chain reaction

Phe(F) Phenylalanine

Pro (P) Proline

PRPS- post-ribosomal peptide synthesis

PS- photosystem

RiPP- ribosomally synthesized post-translationally modified peptide

rRNA- Ribosomal ribonucleic acid

S.D. - Standard Deviation

Ser (S) Serine

sp- species

Thr(T) Threonine

Trp (W) Tryptophan

Tyr(Y) Tyrosine

Val (V) Valine

1. Introduction

Many aquatic organisms are able to produce bioactive secondary metabolites, some of which with the potential of being used in biotechnology (Arnison et al. 2013; Schmidt et al. 2005; Sivonen et al, 2010). Cyanobacteria are a particularly rich source of bioactive secondary metabolites (Sivonen et al., 2008). These include biomedically interesting compounds, such as the anticancer drug lead cryptophycin (Magarvey et al., 2006) and environmentally problematic hepatotoxic peptides, such as microcystins , nodularins and many other toxins produced by bloom-forming cyanobacteria (Sivonen et al.,2008). Many of these compounds are peptides containing nonproteinogenic amino acids, produced by nonribosomal peptide synthesis (Sivonen et al., 2008; Welker et al., 2006). Additionally, modified peptides produced by ribosomal pathways – cyanobactins - have recently been found in cyanobacteria (Donia et al., 2008; Donia and Schmidt, 2010; Leikoski et al., 2009, 2010, Schmidt et al., 2005; Sivonen et al., 2010). This study was carried out within this topic and here we report a novel low-molecular-weight peptide produced ribosomally by *Anabaena* strains.

1.2. Cyanobacteria

Cyanobacteria are a phylum of oxygenic photosynthetic prokaryotes that have two photosystems (PSII and PSI) and use H_2O as a photoreductant in photosynthesis. All known cyanobacteria are photoautotrophic, using primarily CO_2 as the carbon source. (Castenholz, 2001). Many cyanobacterial species are also capable of atmospheric nitrogen fixation.

Cyanobacteria have a gram-negative cell wall, which includes two distinct membranes, the plasma membrane and an outer membrane, and a peptidoglycan layer, which is thicker than in other Gram-negative bacteria, between these two membranes (Hoiczyk and Hansel 2000). External to the cell wall are different layers that protect the cells from desiccation and presumably from phages and predators. Cyanobacteria contain extensive internal thylakoid membranes, which are the site of photosynthetic reactions (Woese, 1987; Hoiczyk and Hansel, 2000; Liberton, 2011).

These organisms are morphologically very diverse, with unicellular, colonial, and multicellular filamentous forms and can inhabit a wide range of environments including

extreme conditions such as hot springs, the poles and, desert soils. Cyanobacterial species can occupy terrestrial habitats and, more commonly, aqueous environments (Sivonen et al., 2010). According to the botanical system of classification, cyanobacterial diversity has been traditionally grouped into five orders, Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales, which generally correspond to the five subsections proposed in the Bergey's Manual of Systematic Bacteriology (Knoll, 2008; Whitton, 2008). Cyanobacteria form a distinct group in bacterial tree of life, based on bacterial 16S rRNA genes (Castenholz, 2001). They are responsible for the production of many toxins, including the most studied group of cyanobacterial secondary metabolites, the hepatotoxic microcystins (Sivonen and Jones, 1999). Cyanobacteria are also a rich source of other secondary metabolites, some of which have interesting antimicrobial, anti-HIV, anti-malaria and anticancer activities (Burja et al., 2001, Welker and von Döhren 2006, Linington et al., 2007). Of particular interest for the present work, we emphasize the cyanobactins group of secondary metabolites, which are a group of cyclic peptides recently found in cyanobacteria (Donia et al., 2008).

1.2.1. Cyanobacteria toxic effects

Some cyanobacteria frequently form mass occurrences (blooms) in aquatic systems (freshwater as well as marine environments). This phenomenon occurs under favorable environmental conditions, such as salinity, light, temperature and nutrient concentration and usually results in the release of the cells' constituents (which sometimes includes potent toxins, termed cyanotoxins) to the water body (Béchemin et al., 1999; John and Flynn, 2000). At this level, eutrophication (natural or anthropogenic) and global warming have a strong influence in ecosystem health, but also in the economic development and in public heath, because the population is a consumer of aquatic resources that can be contaminated with cyanotoxins (John and Flynn, 2000; Paul, 2008).

Studies on planktonic cyanobacteria from freshwaters and marine ecosystems became more common since the first report of a toxic episode caused by cyanobacteria in 1878 (Francis, 1878; Sivonen and Jones, 1999). Eventually, the study of toxic compounds produced by cyanobacteria revealed the biotechnological potential of these organisms, in terms of their production of bioactive compounds. These biocompounds can be of different chemical families, including lipids, terpenes, glycosides, polyketides and peptides

or hybrids of some of these classes (Metting and Pyne, 1986, Rouhiainen et al., 2000; Tan et al., 2001, Donia et al., 2008, Donia and Schmidt, 2010).

A significant fraction of these cyanobacterial compounds are linear and cyclic peptides that are produced by either ribosomal or nonribosomal biosynthetic pathways. The first, ribosomal pathway, was described in 2005 for the cyanobactin patellamide (Schmidt et al., 2005) while the first nonribosomal gene cluster had been reported earlier for the cyanotoxin microcystin (Tillet et al., 2000). Many cyanotoxins are partially or fully produced by nonribosomal pathways and released to the environment, such as microcystin; nodularin; saxitoxin and cylindrospermopsin. On the other hand, cyanobactins have been discovered as novel peptides produced ribosomally and their ecosystem-level impact is currently unknown (Schmidt et al., 2005; Arnison et al., 2013).

1.3. Cyanobactins

The cyanobactins are small peptides with low molecular weight, produced by various strains of free-living or symbiotic cyanobacteria, in terrestrial, marine or freshwater environments. (Schmidt et al., 2005; Donia et al., 2006; Sivonen., 2010). In this group are included compounds with anti-malarial, anti-tumoral and multidrug reversing action (Burja et al., 2001, Welker and von Döhren, 2006, Linington et al., 2007, Salvatella et al., 2003). Therefore, cyanobactins are compounds of potential interest to the pharmaceutical industry. These compounds possess versatile structures and are produced by proteolytic cleavage and cyclization of hypervariable peptide precursors, coupled with other post-translational modifications such as heterociclization, prenylation of amino acid or oxidation (Sivonen et al., 2010; Leikoski et al., 2010). Cyanobactins are ribosomally synthesized and post-translationally modified peptides (RiPPs), which are produced by a pathway now designated as post-ribosomal peptide synthesis (PRPS) (Arnison et al., 2013). In PRPS, an unmodified precursor peptide produced by normal translation on the ribosome, includes the sequence that will correspond to the end-product peptide, termed core sequence (Oman and van der Donk, 2010 and Arnison et al., 2013).Subsequently, the precursor peptide is cleaved and modified to form the final product (Figure1).

The cyanobactin gene cluster usually encodes two proteases responsible for the cyclization and cleavage of the precursor peptide (Sivonen et al., 2010).

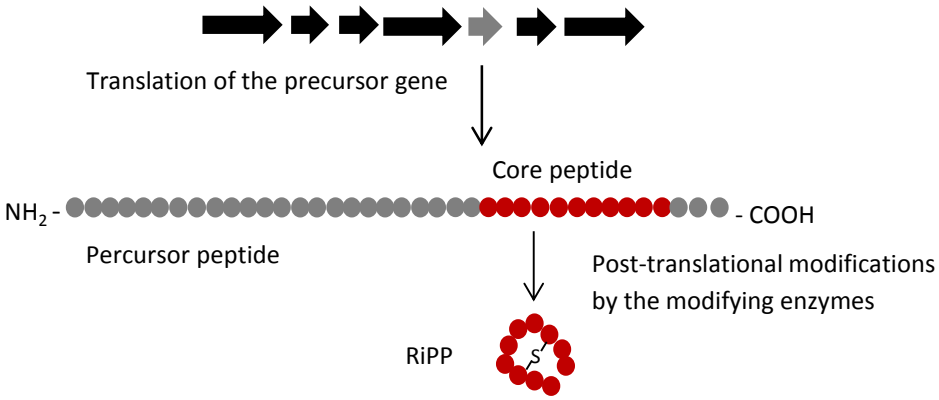


Figure 1- Schematic of post-ribosomal peptide synthesis (PRPS) (Adapted from Arniston et al., 2013 and Leikoski et al., 2013)

Initially, cyanobactins were proposed as a group of cyclic peptides containing oxazolines, thiazolines, or their oxidized derivatives oxazoles and thiazoles (Sivonen et al., 2010). This definition was modified to include cyclic peptides which consist solely in proteinogenic amino acids (Leikoski et al., 2010). Sometimes, isoprenoid amino acids derivatives are also found, for example in trunkamide, patellin, and anacyclamides, although they are rare (Sivonen et al., 2010). Hence, cyanobactin was proposed as a collective name for cyclic peptides which can contain heterocyclized amino acids or isoprenoid amino acid derivatives (Donia et al., 2008; Schmidt and Donia, 2009). Very recently it has been shown that some cyanobacteria produce short linear cyanobactins with a chain length ranging from three to five amino acids (Leikoski et al., 2013). According with the Leikoski et al. (2013) study, these linear cyanobactins were *N*-prenylated and *O*-methylated on N and C termini, respectively.

1.3.1. Analogous pathways

Similarly to cyanobactins, another cyanobacterial peptide class, microviridins, was recently shown to be ribosomally produced in *Microcystis aeruginosa* and *Planktothrix agardhii*, but their biosynthetic machinery differs that of cyanobactins (Ziemert et al., 2008a; Philmus et al., 2008; Sivonen et al., 2010). Initially, these compounds were thought to be products of nonribosomal peptide biosynthesis. However, microviridins are synthesized from precursor peptides that are converted into tricyclic depsipeptides through the action of ATP grasp ligases and a transporter peptidase (Ziemert et al., 2008a; Philmus et al., 2008). The work of Philmus et al. (2008) reported similar gene clusters in the genomes of *Anabaena variabilis, Nostoc punctiforme,* and *Nodularia*

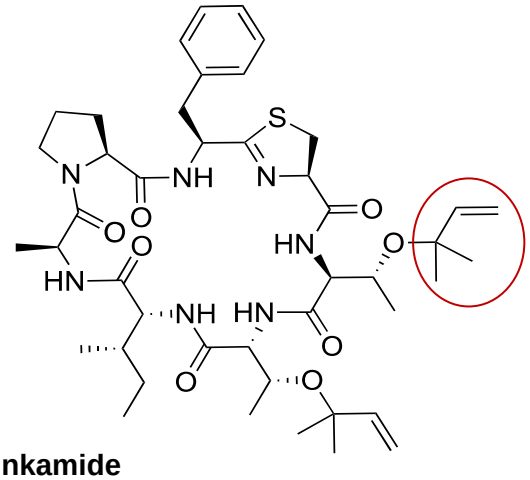
spumigena as well as in genomes of other bacteria. The biosynthetic gene clusters encoding the production pathway of ribosomal peptides with oxazoles and thiazoles are present in a broad range of bacteria (Lee et al., 2008). Bacteria distantly related to cyanobacteria are known to produce bacteriocins by posttranslational modification. Cyanobactin biosynthesis is analogous in many ways to the biosynthesis of bacteriocins (Franz et al., 2007; Nolan and Walsh, 2009). The leader-peptide-guided biosynthesis is common in many ribosomally synthesized natural products where the precursor peptide is synthesized and cleaved, and in some cases the core peptide is posttranslationally modified (Oman and van der Donk, 2010). Bacteriocins can also present cyclization as cyanobactins, and also similar posttranslational modifications for example in case of thiazoles and oxazoles (Jack and Jung, 2000; Maqueda et al., 2008; Martin-Visscher et al., 2009).

Many of cyanobacterial bioactive compound classes are synthesized on nonribosomal peptide synthetases (NRPS) or combined NRPS and polyketide synthases (Welker and von Döhren, 2006; Sivonen and Börner, 2008). However, the cyanobactins have been shown to be produced by posttranslational modification of the gene-encoded precursor peptides (e.g., Schmidt et al., 2005; Donia et al., 2008). The gene clusters responsible for ribosomal peptide production are small compared to the large nonribosomal peptide synthetase gene clusters. In NRPS, variation in the chemical structure of the peptide is achieved by utilization of more than 200 nonproteinogenic amino acids (Nolan and Walsh, 2009) whereas ribosomal peptides are restricted to 20 proteinogenic amino acids which may be posttranslationally modified. In NRPS, the enzymes seem to have relaxed substrate specificity and thus allow simultaneous production of a number of structural variants in the same strain of a cyanobacterium (Welker and von Döhren, 2006).

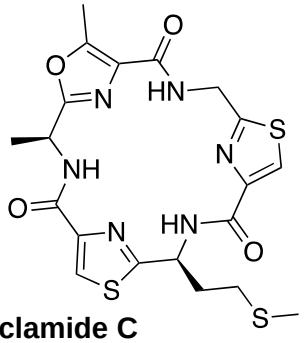
1.3.2. Chemical diversity and Occurrence

A large part of cyanobactins, more than hundred as suggested by Sivonen et al. (2010) and Donia et al. (2008), have been identified from symbiotic associations formed between cyanobacteria and ascidians or from free-living cyanobacteria. This renders cyanobactins one of the largest classes of cyanobacterial peptides produced (Donia et al., 2006; Schmidt and Donia, 2009). In general, cyanobactins produced by ascidians and sponges, for example, usually contain from six to ten amino acids and varying numbers

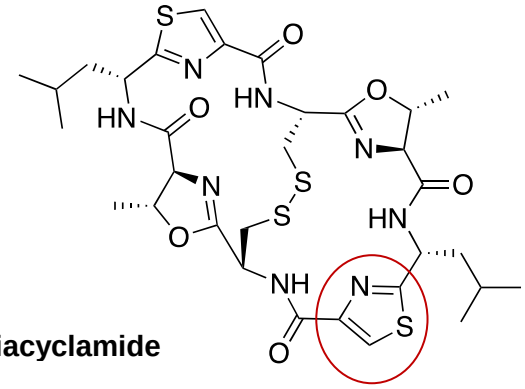
and combinations of oxazoles, oxazolines, thiazoles, and thiazolines (Sivonen et al., 2010). A few cyanobactins, such as ulithiacyclamides, containing prenylated amino acids, have disulfide bridges between two cysteine residues. It is unclear if the cyanobactins are produced by the filter feeding organisms themselves, heterotrophic bacteria, or cyanobacteria associated with these organisms, but some cyanobactins have now been shown to be produced by cyanobacteria (Schmidt et al., 2005; Donia et al., 2006; Sivonen et al., 2010). For the majority of the analogous cyclic peptides reported from cyanobacteria, a biosynthetic origin is currently unknown. Hitherto, only ribosomal biosynthetic pathways have been described to produce these cyclic peptides (Schmidt et al., 2005; Donia et al., 2006, 2008; Sudek et al., 2006; Ziemert et al., 2008b; Leikoski et al., 2010). Still, a nonribosomal peptide synthetase pathway could be an alternative route for the biosynthesis of these compounds (Sivonen et al., 2010). In general, cyanobactins that contain heterocyclized amino acids range in size from six to eleven amino acids. Cyanobactins without heterocyclized amino acids have a length from seven to twenty amino acids. An interesting fact is that cyanobactins without heterocyclized amino acids, in addition to occasional prenylations, can present a conserved proline residue. Oxazoles and thiazoles are more common, while oxazolines or thiazolines occur with a lower frequency (Sivonen et al., 2010).



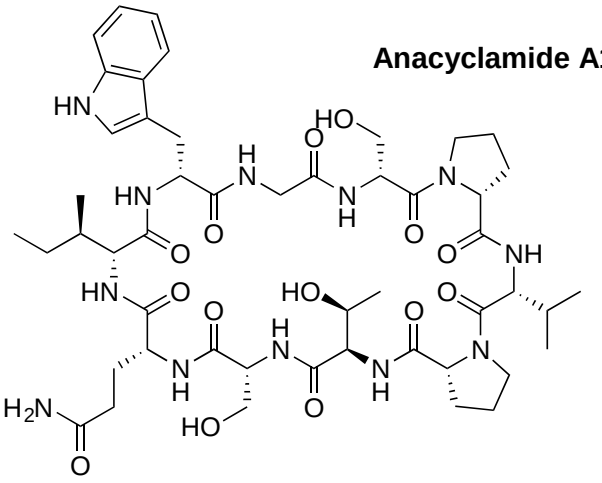
Trunkamide



Tenuecyclamide C



Ulithiacyclamide



Anacyclamide A10

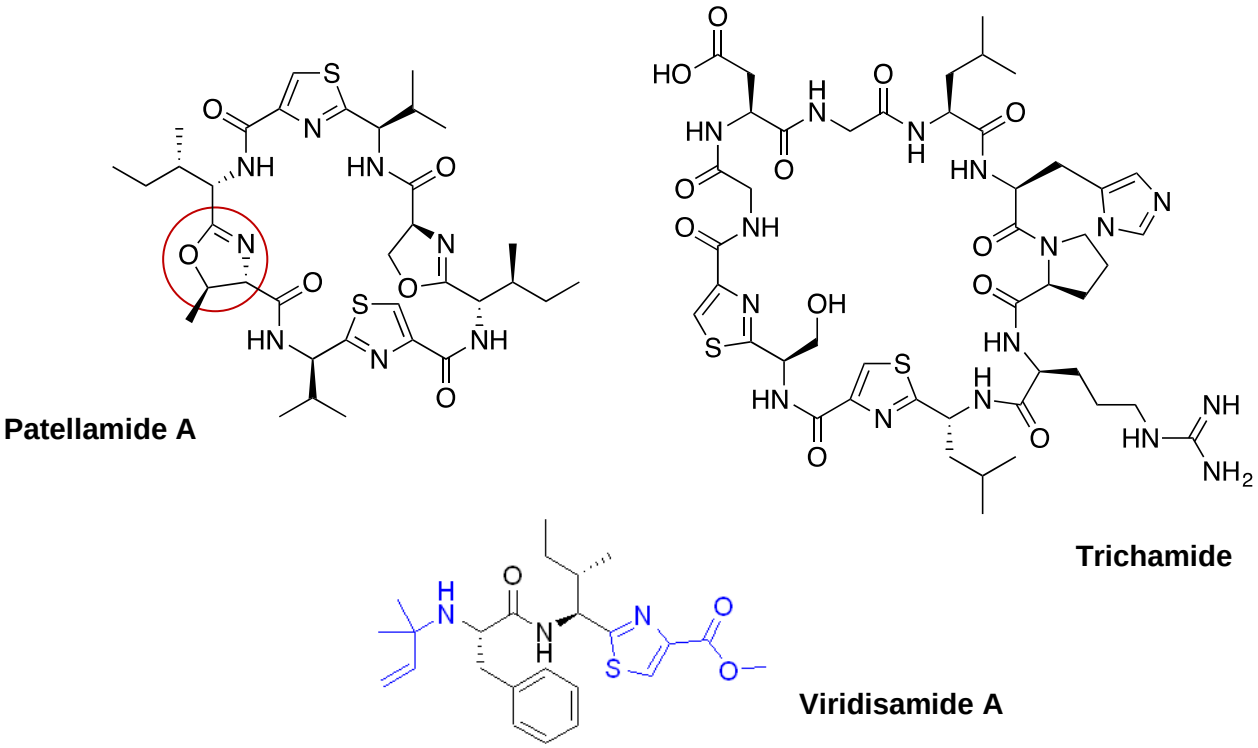


Figure 2- The chemical structures of a selection of cyanobactins. Trunkamide was isolated from *L. patella*, tenuecyclamide from *N. spongiaeforme*, anacyclamide from *Anabaena*, trichamide from *T. erythraeum*, ulithiacyclamide and patellamide from *Prochloron*(Originally from *L. Patella*); Viridisamide from *Oscillatoria viridis* PCC7112.(Adapted from Sivonen el al., 2010 and Leikoski et al., 2013). The post-translational modifications; oxazoline, thiazole, and reverse *O*-prenyl are highlighted with circles. In the linear cyanobactin, the identical prenylated *N*-termini and methylated *C*-termini bound to thiazoles are in blue. (Adapted from Sivonen et al., 2010; Leikoski et al., 2013)

1.3.3. Biosynthesis of cyanobactins

Cyanobactins are made through PRPS (Arnison et al., 2013). It means that a gene-encoded precursor peptide is first transcribed. The precursor peptide is 50-150 amino acids long and the final peptide is modified and cleaved (Donia and Schmidt, 2010; Sivonen et al., 2010). The cleavage of the cyanobactin precursor peptide takes place at a minimum of two sites. In adition to the cleavage, the cyanobactin precursor is N-to-C macrocyclized and some amino acids can be modified (Donia and Schmidt, 2010; Oman and van der Donk, 2010; Sivonen et al., 2010). A hypervariable amino acids core sequence inside these will ultimately form the cyanobactin (Figure 3) (Donia et al., 2006,

Donia et al., 2008). Commonly, the cysteines, threonines and serines are heterocyclized to thiazolines and oxazolines, which can be oxidized to thiazoles and oxazoles (Figure 2). The core sequence may also be prenylated (Donia et al., 2008; Donia and Schmidt, 2011).

Thus, cyanobactins results from the proteolytic cleavage and head-to-tail (N–C) cyclization of precursor peptides coupled with modification of specific amino acids. (Sivonen et al.,2010; Oman and van der Donk, 2010). This cyclic structure results from the amide linkage of the α-carbonyl of C-terminal amino acid and α-amino group of the N-terminal amino acid.

In cyanobactins biosynthesis, the precursor peptide, designated with the letter “E”, directly encodes one or more cyanobactins flanked by the putative recognition sequences at which the precursor peptide is cleaved by two proteases, A and G (Schmidt et al., 2005; Lee et al., 2009; Donia and Schmidt, 2010; Sivonen et al., 2010). The fact that the precursor peptide can encode more than one cyanobactin represents a mechanism to generate chemical diversity in peptide production.

The cyanobactin gene cluster is approximately 10 kb (contained between 7 and 12 genes) and consists of the genes A into G, however the gene arrangement can vary. This gene cluster encodes two proteases (A and G), which are involved in the cleavage of the precursor peptide and cyclization of cyanobactin (Lee et al., 2009; Sivonen et al., 2010). In the biosynthetic gene cluster of patellamide it was shown that the encoded PatA proteases is responsible for cleavage the precursor peptide at the N-terminal recognition sequence while the PatG protease cleaved the precursor peptide at the C-terminal recognition sequence (Lee et al., 2009). However, only the G- protease has a macrocyclase domain which N-to-C cyclizes the cyanobactin (Lee et al., 2009). As stated above, the gene order is not strictly conserved but generally biosynthetic genes are organized as in the patellamide *pat* gene cluster (Figure 4). All of the cyanobactin gene clusters contain two the two tandem-acting proteases, a short precursor peptide as well as proteins involved in the maturation of the cyanobactins (Sivonen et al., 2010). Thiazoles and oxazoles are formed through the heterocyclization and subsequent oxidation of cysteine, serine, and threonine amino acids. In general, cyanobactin gene clusters contain a gene encoding a PatD homolog which is predicted to heterocyclize cysteine, serine, and threonine to thiazolines and oxazolines (Schmidt et al., 2005). The oxidase domain of the bimodular PatG protein is believed to catalyze the oxidation of thiazolines and oxazolines to thiazoles and oxazoles (Schmidt et al., 2005). A PatF homolog is often encoded in

cyanobactin gene clusters and thought to be involved in the heterocyclization and/or prenylation of cyanobactins (Schmidt and Donia, 2009). In general, the PatB and PatC proteins are encoded in the majority of cyanobactin gene clusters but were found to be nonessential - in *Escherichia coli* the patellamides were produced by heterologous expression of the *pat* gene cluster, even in the absence of the *patB* and *patC* genes (Figure 3).

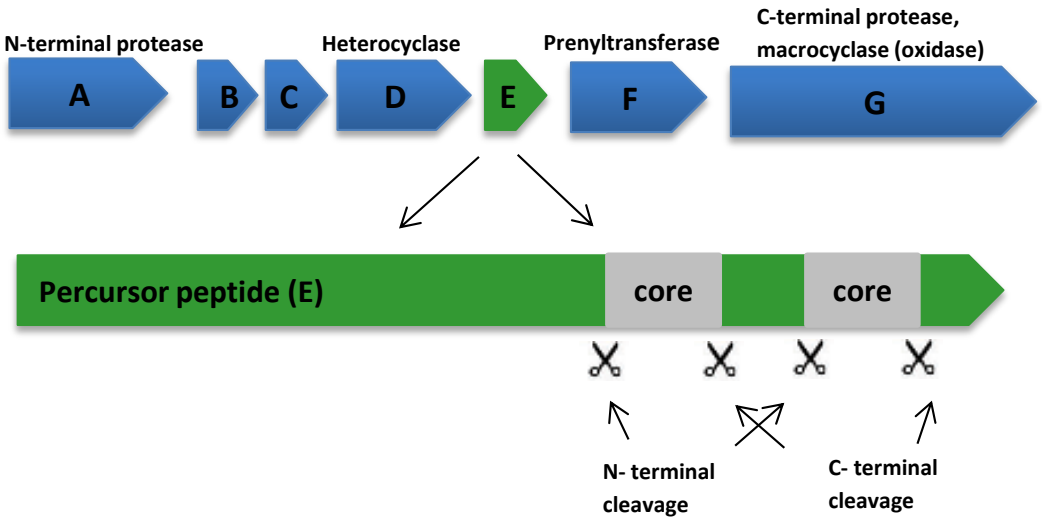


Figure 3- Schematic figure of the cyanobactin gene cluster (homologous to the *pat* gene cluster[1]) and the structure of the precursor peptide (E). The functions of the genes are shown. The genes B and C are of unknown function. The cyanobactin structures are formed from the core region shown in the precursor peptide. (Adapted from Houssen el al., 2012; [1] Donia et al., 2008; [1]Schmidt et al., 2005)

The biosynthetic genes for cyanobactin production have been described in distantly related cyanobacteria *Prochloron, Trichodesmium, Microcystis, Prochlorococcus, Synechococcus, Nostoc, Lyngbya, and Anabaen*a (Figure 4) (Schmidt et al., 2005; Donia et al., 2006, 2008; Sudek et al., 2006; Ziemert et al., 2008b; Leikoski et al., 2010; Shih et al., 2013). One of the protease genes responsible for cleavage of the cyanobactin precursor peptide was shown to be common among planktonic freshwater cyanobacteria and present in 48 out of 132 strains studied (Leikoski et al., 2009; Sivonen et al., 2010). These planktonic cyanobacteria included fresh and brackish water strains from filamentous heterocystous (*Anabaena, Aphanizomenon, Nodularia*), filamentous (*Planktothrix*), as well as colony-forming (*Microcystis* and *Snowella*) cyanobacteria. The biosynthetic pathway appears to be relatively common among these strains (Leikoski et al., 2009).

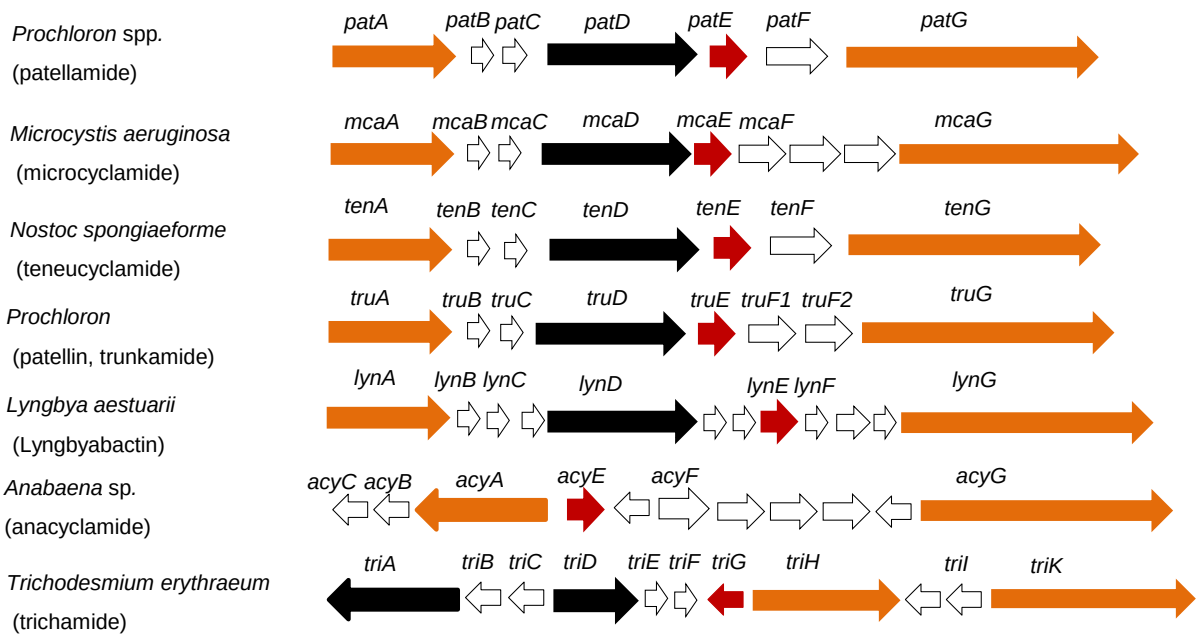


Figure 4- Cyanobactin gene clusters published from seven distantly related cyanobacteria. These gene clusters are typified by genes encoding proteases (yellow), a short precursor peptide (red), proteins involved in the maturation of the cyanobactin (black), as well as conserved and hypothetical open reading frames (white). (Adapted from Sivonen et al., 2010)

1.3.4. Bioactivities

Cyanobactins and cyclic peptides with analogous structures have diverse reported bioactivities. This diversity is derived from their variable structures. However, until this moment, this bioactivity has not been studied systematically for the cyanobactins.

A large fraction of cyanobacterial metabolites have been found to be anticancer compound. For example, trunkamide (Salvatella et al., 2003) has multidrug reversing (Ogino et al.,1996) as well as anti-viral activities (Burja et al., 2001, Welker and von Döhren, 2006, Linington et al., 2007) or acting against tropical parasites such as *Plasmodium falciparum* (Linington et al., 2007; Portmann et al., 2008b).

According to Nolan and Walsh (2009), in Bacteria many of the ribosomally produced peptides are antibiotics or bactericides are produced to kill or inhibit growth of competing microbes. Some compounds that were isolated from cyanobacteria present antibiotic (Ishida et al., 1997) or antiviral (Boyd et al., 1997; Bokesch et al., 2003) effects; hence, a strong possibility is that cyanobactins are defense molecules.

1.4. Anacyclamides

Anacyclamides are a group of cyanobactins with alike structural features. Leikosky et al. (2010) identified several anacyclamides from strains of genus *Anabaena.* This study revealed the *acy* gene cluster in *Anabaena sp. 90,* which encodes 11 open reading frame (ORFs), and is arranged in an 11kb operon which is bidirectionally transcribed (Sivonen et al., 2010). The *acy* gene cluster shows a limited degree of homology with the *pat* gene cluster. Some hypothetical ORFs present in the *acy* gene cluster were also absent in the *pat* gene cluster (Leikoski et al., 2010). The precursor peptide AcyE encodes a single copy of the cyanobactin anacyclamide A10- flanked by putative recognition sequences which differ substantially from other cyanobactin precursors. The anacyclamide gene cluster lacks a PatD-homolog in conformity with the anacyclamides lack of posttranslationally heterocyclized amino acids (Leikoski et al., 2010). Likewise, the AcyG protein also lacks an oxidase domain (Leikoski et al., 2010).

The length of the anacyclamides is highly variable, as is the amino acid sequence, with only one proline being conserved (Sivonen et al., 2010).

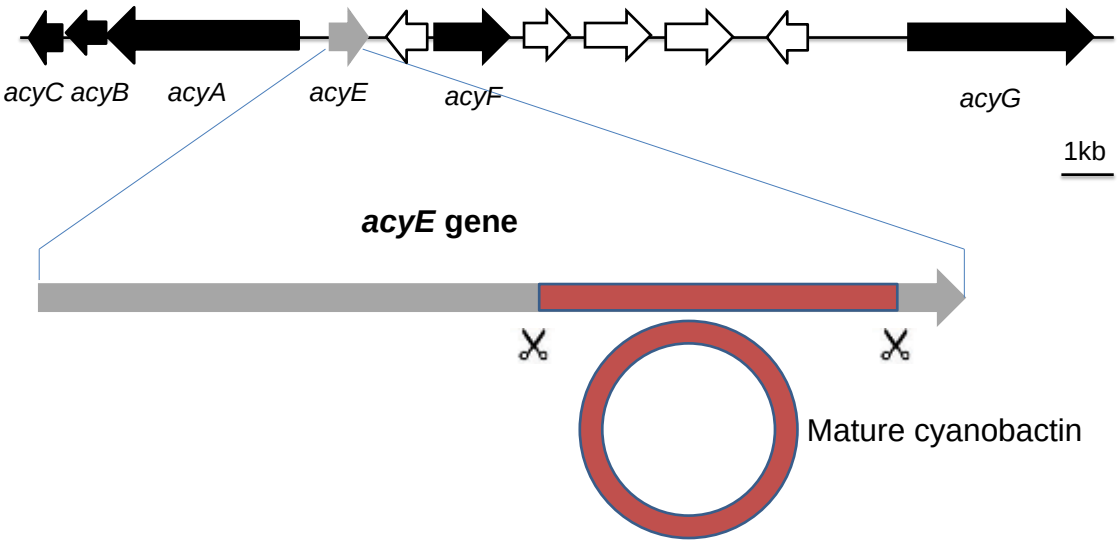


Figure 5- Arquitecture of the anacyclamide gene cluster (Houssen el al., 2012).

1.5. Anabaena

The nostocalean genus *Anabaena* includes filamentous, heterocyst-forming cyanobacteria that can have gas vacuoles (Rippka et al., 2001). The presence of heterocysts in *Anabaena* coupled with a fast growth rate has rendered *Anabaena* one of the most studied cyanobacterial genera. Heterocysts are specialized nitrogen-fixing cells formed during nitrogen starvation by some filamentous cyanobacteria of the orders Nostocales and Stignomatales. These cells fix nitrogen from dinitrogen (N_2) in the air using the enzyme nitrogenase, which is then used in the cells for biosynthesis. Nitrogenase is inactivated by oxygen, so the heterocyst must create a microanaerobic environment. An additional envelope surrounds heterocysts, helping to protect the enzyme nitrogenase from oxygen (Fay, 1992). Heterocysts are typically distinguishable from vegetative cells due to their somewhat larger and rounder shape, diminished pigmentation, thicker cell envelopes, and usually prominent cyanophycin granules at poles adjacent to vegetative cells (Figure 6). Mature heterocysts provide the ideal environment required for nitrogen fixation, especially separating the oxygenic photosynthesis in vegetative cells (Golden and Yoon., 1998)

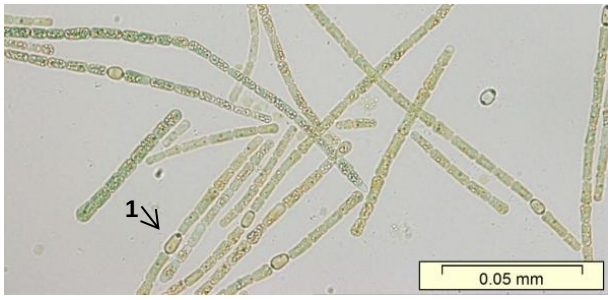


Figure 6- Heterocysts development (1) in Anabaena LEGE 00259, one of the strains used in this work.

Anabaena sp. can produce neurotoxic and cytotoxic alkaloids and hepatotoxic cyclic peptides (Sivonen and Jones, 1999). This variety of toxins has a strong impact on ecosystems and, consequently, several actions on terrestrial vertebrates, especially mammals. Some examples of saxitoxin and cylindrospermopsin from *Anabaena* have been reported (Sivonen and Jones, 1999). Microcystin is one of the most frequently cyanobacterial toxins found in *Anabaena* blooms and act as an inhibitor of protein phosphatase. Similarly, anatoxin-a and anatoxin-a(S) have been characterized from this genus and their mechanism of action is binding irreversibly to the nicotinic acetylcholine receptors and the inhibition of acetylholinesterase activity, respectively.

This study was carried out using only A*nabaena* strains.

1.6. Biotechnological Aspects

The biotechnological exploitation of cyanobactins will require detailed studies on the enzymes involved in the biosynthesis as well as mechanisms of action of these peptides.

The small size of the cyanobactin gene clusters and their amenability to be fully heterologously expressed (Schmidt et al., 2005; Donia et al., 2006, 2008; Leikoski et al., 2010) will provide new possibilities to create compound libraries and novel compounds (Donia et al., 2006, 2008). In the study of cyanobactins pathway, the heterologous expression gives options to study the role of individual genes in biosynthesis as well as produce novel peptides. The core sequence of the precursor peptide directly encodes the resulting cyanobactin and this sequence can be changed easily by genetic engineering in heterologous hosts and recombinant peptides can be produced (Donia et al., 2006; Tianero et al., 2012).

According to Donia et al. (2006) the cyanobactin pathway was utilized in *E.coli* to synthesize an engineered cyclic peptide (eptidemnamide) similar to an anticoagulant in clinical use. This approach demonstrates a means to exploit cyanobacterial pathways and produce novel compounds by the rational design of peptides (Donia et al., 2006; Sivonen et al., 2010).

According to the work by Oman and van der Donk (2010) the peptide precursor-directed synthesis allows manipulations directly to the precursor gene and enables production of engineered peptides in heterologous hosts. In addition, the enzymes in the cyanobactin pathways could be used as catalysts to assist the chemical synthesis of the desired compounds.

The work by Lee et al. (2009), showcased not only the important role of proteases in cyanobactin biosynthesis but also was demonstrated the potential of the enzymes as general catalysts for cyclization of peptides. The PatG protease was shown to require no exogenous energy for the cleavage and cyclization, and to be tolerant to different substrate lengths and sequences as long as the C-terminal recognition sequence was present (Sivonen et al., 2010; Lee et al., 2009). This is important, for example, in synthetic peptide manufacture where the head-to-tail cyclization restricts peptide production in bulk amounts (Sivonen et al., 2010). It is important to note that whole-genome information has

already led to the discovery of cyanobactin biosynthesis as well as several new compounds and compound classes, for example novel patellamides (Schmidt et al., 2005), trichamide (Sudek et al., 2006), and anacyclamides (Leikoski et al., 2010).

The number of genome sequencing projects involving cyanobacteria and metagenomic studies applied to various environments is increasing. This should allow new discoveries in the near future, including new ribosomal pathways and cyanobactins.

1.7. Objective

The main objectives of this work were the detection of anacyclamide- related genes in cyanobacteria from the culture collection LEGE (LEGE CC) and, the corresponding identification of new anacyclamides. As a secondary objective, we tried to characterize the cyanobacterial strains using both morphological and molecular data, with the ultimate goal of making phylogenetic inferences regarding cyanobactins production.

For this purpose, a PCR-screening of ten freshwater cyanobacterial strains for cyanobactin-related genes was carried out. The resulting amplicons were then cloned and sequenced in order to identify potential producers of new members of this class of compounds. Finally, investigations on the production and the nature of the new cyanobactins were performed using analytical methodologies.

2. Material and Methods

The methods used are outlined in the workflow shown in figure 7.

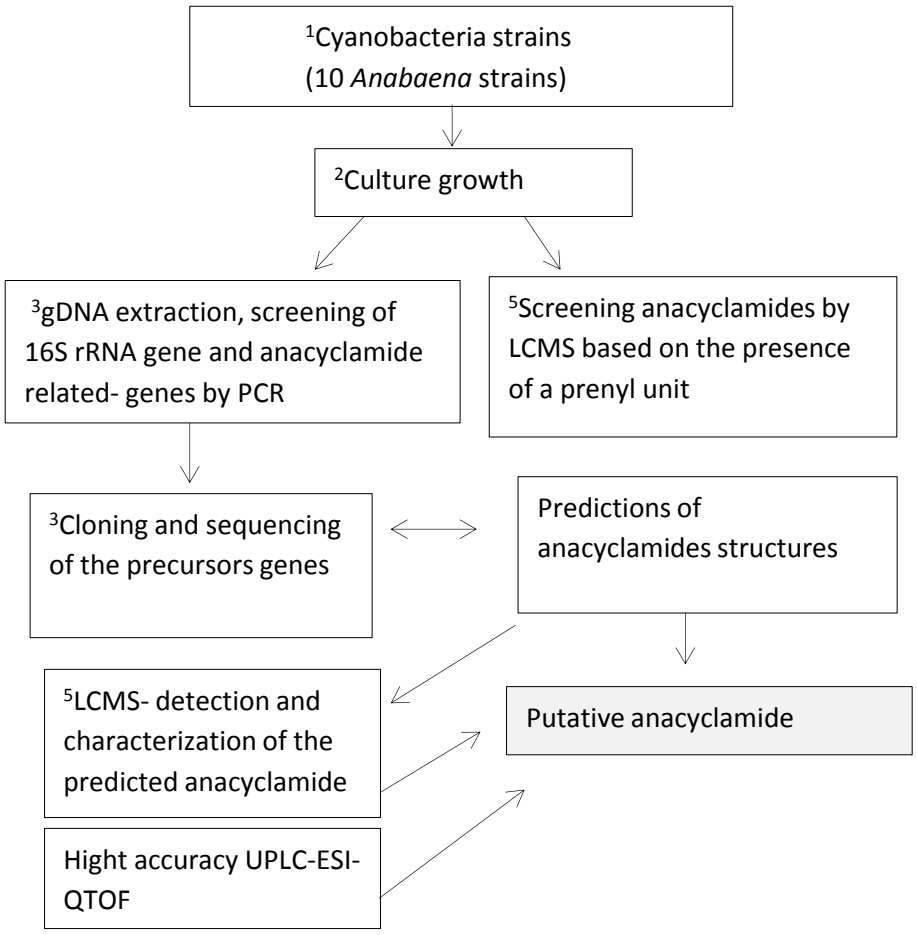


Figure 7- Schematic drawing of the workflow.

2.1. Source of strains

Cyanobacteria used in this study are part of the LEGE culture collection and had been isolated from water samples collected from freshwater supplies, located in north and central regions of Portugal (Table1). Additionally, for comparison purposes, the strain *Anabaena* sp.*90*, isolated in Finland from Lake Vesijärvi, in 1986 and maintained at the University of Helsinki was used (Sivonen et al., 1992).

Table 1- Identification of the ten strains in study, from the LEGE culture collection

Anabaena **strain**	**Origin/source**	**Isolator**	**Year of harvest**	**Co-identification**
LEGE00233	Maranhão reservoir	Joana Osswald	2000	J1
LEGE00241	Maranhão reservoir	Joana Osswald	2000	J14
LEGE00243	Maranhão reservoir	Joana Osswald	2000	J16
LEGE00245	Maranhão reservoir	Joana Osswald	2000	J18
LEGE00246	Maranhão reservoir	Joana Osswald	2000	J19
LEGE00248	Maranhão reservoir	Joana Osswald	2000	J21
LEGE00250	Maranhão reservoir	Joana Osswald	2000	J27
LEGE00253	Chaves reservoir	Joana Osswald	2000	J37
LEGE00259	Maranhão reservoir	Joana Osswald	2000	J46
LEGE04289	Marco de Canaveses	Joana Osswald	2004	J83

2.2. Cyanobacterial strains and culturing

The ten *Anabaena* sp. strains were grown in sterile Z8 medium (Kotai, 1972), in 40-ml cultures under a 14:10 h light: dark cycle (Martins et al., 2005), with a photon irradiance of 10-30 µmol m^2 s^{-1} at 25°C.

Regarding large scale biomass production, for the chemical analyses, two isolates were grown in sterile 16L culture vessels, with aeration, under 14:10h light: dark cycle, with a photon irradiance of 10-30 µmol m^2 s^{-1} at 25°C.

Figure 8- Cyanobacterial strains and Culturing
***Anabaena sp.* Strains were grown in z8 medium, with aeration, under 14:10 light:dark at 25°C.**

2.3. DNA extraction, PCR amplification, Cloning and sequencing

Genomic DNA (gDNA) extraction was carried out either with the Purelink genomic DNA mini kit (Invitrogen) or with the Dneasy Plant Mini kit (Qiagen). PCR amplification of the 16S small ribosomal subunit gene (16S rRNA gene) was carried out using the gDNAs as templates. Primers 27F, 359F and 1491R (Neilan et al., 1997; Jungblut et al., 2005), targeting the 16S rRNA gene, were used.

All PCR reactions were prepared in a 20µl of volume containing 10x PCR buffer for Super Taq (HT Biotechnology Ltd.). PCR conditions were as described previously. Amplicons were cloned into a PGEM-T vector and transformed into Oneshot *E.coli* TOP10 cells (Invitrogen). Plasmid DNA was isolated from the transformed cells using the GenElute Plasmid MiniPrep kit (Sigma-Aldrich) and sequenced (Macrogen) using M13 primers.

Table 2- Primers used for the 16S rRNA gene amplification.

Primer[a]	Sequence (5'»3')	Reference
27F	AGAGTTTGATCCTGGCTCAG	Neilan et al., 1997
1494R	TACGGCTACCTTGTTACGAC	Jungblut et al., 2005
CYA359F	GGGGAATYTTCCGCAATGGG	

[a]Gene primers are named according to their locus

The amplification of the patA gene was made using a pair of primers that amplified a 1.4 kb section, PatAR and PatA F (Lee et al., 2009). These amplifications, using these primers, was already made by Leikoski et al. in previous studies to screen many cyanobactia strains, including *Anabaena* sp.90. The annealing temperature used in the PCR program was of 52°C.

Anabaena sp.90 (Sivonen et al., 1992) was used as a positive control in the screening.

In order to identify an *Anabaena* strain producing an anacyclamide, the acyE peptide precursor gene was amplified using primers preRNAF (5'-GAAGAACATCCGCCCCCAACAAGTTG-3') and preRNAR (5'-CTCCGCGTCGTC GCCTGCAAAAGG-3') and primers PreF (5'-GCCTTCACCAAACCAGTCT TCTTCAT-3') and PreR (5'-CATCGAGGCGAACCGTGCGCCAAGGGAT- 3') (Leikoski et al., 2009) from the genomic DNA of all the *Anabaena* strains. The expected size band, for the last primer pair is about 312 pb and for the PCR program the Annealing Temperature was calculated at 53°C; using the first primer pair the expected size band is about 160 bp and the Annealing Temperature was calculated at 56°C. Following cloning and sequencing as described above, the amplified fragments were compared to those of known acyE precursor to infer the novelty of the encoded anacyclamide.

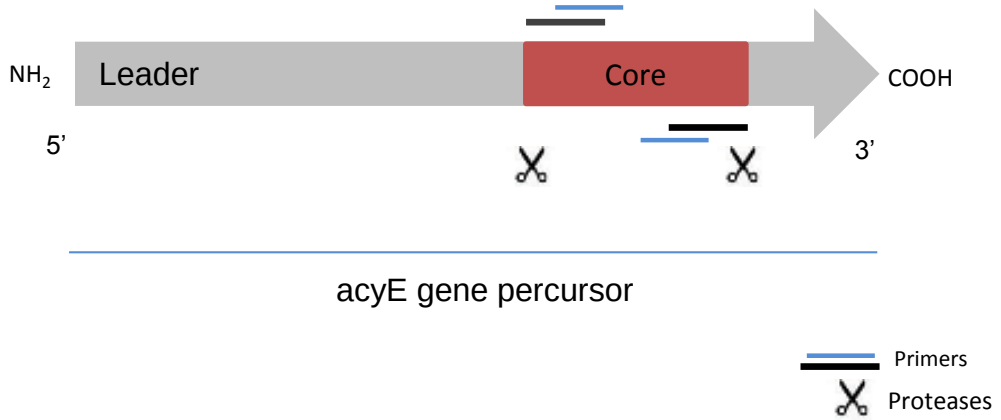


Figure 9- The acyE peptide precursor gene with the protease cleaving sites highlighted and the primer annealing regions shown.

Table 3- PCR reaction for each primers pair

Pair of primers	PCR reaction [a]					
	Inicial Denaturation	Denaturation	Annealing	extension	Final extension	
27F/1494R 359F/1494R	94°C 4min	35 cycles 94°C 45sec		 50°C 45sec 72°C 1min20sec	72°C 8min	8°C ∞
preRNAF/preRNAR	94°C 2min	94°C 30sec	56°C 30sec	72°C 10sec	72°C 7min	8°C ∞
PreF/PreR	94°C 2min	94°C 30sec	53°C 30sec	72°C 20sec	72°C 7min	8°C ∞

[a]The PCR reactions were calculated according with the specific primers used and they are not publish.

2.4. Cloning the acy biosynthetic gene cluster

In order to amplify the entire 12 kb gene cluster from genomic DNA of *Anabaena sp.* LEGE 00259 by PCR , the patex2f (5'-ATGGATCCTGATGGACTGTAGTGTGAG-3') and patex5r (5'- TACTCGAGAGGTTTTGGGACTCTTTAG-3') primer pair (Leikoski et al.,

2009) was used, in three 60-µL reaction mixtures, containing 10× PCR buffer for Super Taq Plus (HT Biotechnology Ltd.), 200 µmol of each nucleotide (Finnzymes), 0.75 µM of each primer, 0.8 U Super Taq Plus proofreading polymerase (HT Biotechnology Ltd.), and 100 ng of *Anabaena sp.* LEGE 00259 gDNA. The thermocycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56.4°C for 30 s, and 68°C for 9 min and then a final extension at 68°C for 20 min. The PCR products were separated on a 0.7% agarose gel containing 0.5× Tris-acetate-EDTA and run for 30 min at 100 V. The gel was stained using SYBR Safe DNA gel stain (Invitrogen) and was visualized using a Dark reader (Clare Chemical Research Inc.) to avoid DNA damage from UV light during gel extraction. The 12-kb PCR product was gel extracted with a MinElute gel extraction kit (Qiagen) and cloned into the PCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) with an insert-to-vector molar ratio of 3:1. The vector was used to transform chemically competent *E.coli* One Shot TOP10 cells according to the manufacturer's instructions. The resultant RC_c1, RC_c2 and RC_c3 plasmids were analyzed by PCR and restriction digestion to ensure that the integrity of the insert was maintained during the cloning and amplification in *E. coli*. The transformants containing the 12-kb insert in the plasmid were grown overnight with shaking at 120 rpm at 28°C in 50 ml of LB medium containing 150 µg ml $^{-1}$ of ampicillin (sodium salt; Sigma-Aldrich) for liquid chromatography (LC)-mass spectrometry (MS) analysis. The cells were harvested by centrifugation at 10,000 × g for 10 min (Ep- pendorf 5804R centrifuge), dried with a Heto vacuum centrifuge (Heto- Holten A/S), which yielded ca. 43 mg (dry weight) and sent to sequencing (Macrogen; Seoul, Korea).

2.5. Biomass collection of LEGE 00248 and LEGE 00259 and preparation of extracts

The biomass of 16L cultures of LEGE 00248 and LEGE 00259 was collected following approximately one month of growth under the conditions detailed in the "Cyanobacterial strains and cultivation" section. The collection was carried out by filtration using a 41 µm plankton net. The filtered cells were then centrifuged at 4600rpm for 10min and the pellet was frozen and freeze-dried.

Figure 10- Biomass collection of 16L cultures of LEGE 00248 and LEGE 00259 strains

2.5.1. Biomass extraction

The lyophilized biomass was weighed and then methanol extraction was made (Leikoski et al., 2010) using 60ml of solvent (ACS grade). The biomass with methanol was macerated and then the supernatant was transferred into a 45ml falcon and centrifuged at 4600×g for 10min. This procedure was repeated six times. The pooled supernatants were then concentrated in *vacuo* and the mass of the resulting crude extract determined.

2.6. Chemical analysis

Cells of *Anabaena* strains were collected from the 40 mL cultures by centrifugation at 7,000 g for 7 min. The collected cells were freeze-dried with Supermodulyo (Edwards High Vacuum International) or dried with a Heto vacuum centrifuge, which yielded 5 to 12 mg (dry weight). The dried *Anabaena* cells, as well as *E. coli* transformants, were extracted with 1 mL of methanol (HiperSolv, HPLC grade; BDH Laboratory Supplies) in 2 mL plastic tubes containing glass beads (cell disruption medium; 0.5-mm-diameter glass beads; Scientific Industries Inc.).

Each mixture was homogenized by shaking with a FastPrep cell disrupter (Bio 101, Thermo Electron Corporation, Qbiogene, Inc.) for 30 s at a speed of 5 ms^{-1}. The mixture was centrifuged at 10000 x *g* for 5 min, and the supernatant was used for LC-MS analysis.

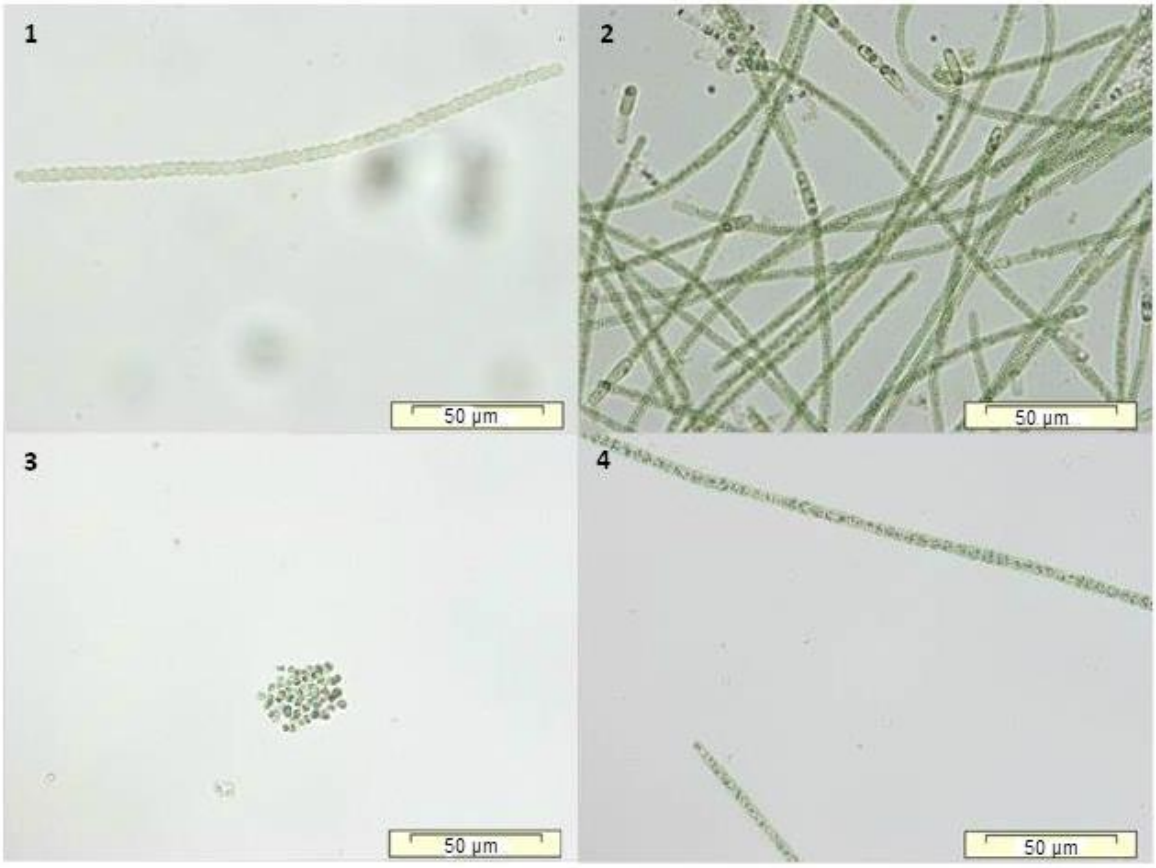
The methanol extracts were analyzed with a high-performance liquid chromatograph combined with a mass spectrometer (Agilent 1100 series LC/MSD with Ion Trap XCT Plus and an electrospray ion source) in order to detect low-molecular-weight peptides. Peptides were separated from the extracts by HPLC using a Phenomenex C18(2) column (2.0 mm x 150 mm; particle size, 5µm). The mobile phase gradient consisted of 0.1% aqueous (water purified with Milli-Q Plus; Millipore) formic acid (Fluka) (solvent A) and 0.1% formic acid in isopropyl alcohol (Sigma-Aldrich) (solvent B). Two different settings were used; one setting was used for screening methanolic extracts of *Anabaena* cells, and the other setting (values in parentheses below) was used for further structural characterization of natural and synthetic peptides. The percentage of solvent B was increased from 5 to 50% in 60 min. A flow rate of 0.15 mL min^{-1} was used, and the columns were heated to 40°C during separation. The positive-ion mode of electrospray ionization was used. The pressure of the nebulizer gas (N_2) was 30 lb/in^2 (35 lb/in^2), the drying gas flow rate was 8 L min^{-1}, and the temperature was 350°C. The capillary voltage was set at 5,000 V (4,500 V), and the capillary offset value was 300 V. A skimmer potential of 85 V (100 V) and a trap drive value of 144 (111) were used. Spectra were recorded using a scan range from m/z 100 to m/z 2200. Identification of the anacyclamides was based on the molecular weights calculated from the predicted peptide AcyE precursor amino acid sequences and the assigned fragment ion patterns of MS^n (n= 1 to 3) spectra. A comparison with the retention time and MS data for a synthetic reference was used in five cases (Beijing SBS Genetech Co., Ltd., China; anacyclamide B7 from JPT Peptide Technologies GmbH, Germany).

3. Results and Discussion

In this study we proposed to detect new cyanobactins among ten *Anabaena* strains from LEGE CC. At the same time, it was our aim to characterize the cyanobacterial strains using both morphological and molecular data, with the final goal making phylogenetic inferences. For this purpose were made a variety of procedures, including genomic DNA extraction, PCR screening, *in silico* analysis and detection by LC-MS.

3.1. Morphological characterization

In the beginning of this work a morphological characterization was made for each strain. Microphotographs for each strain are shown in Figure 11. Morphometric parameters determined for each strain are presented in Table 4.



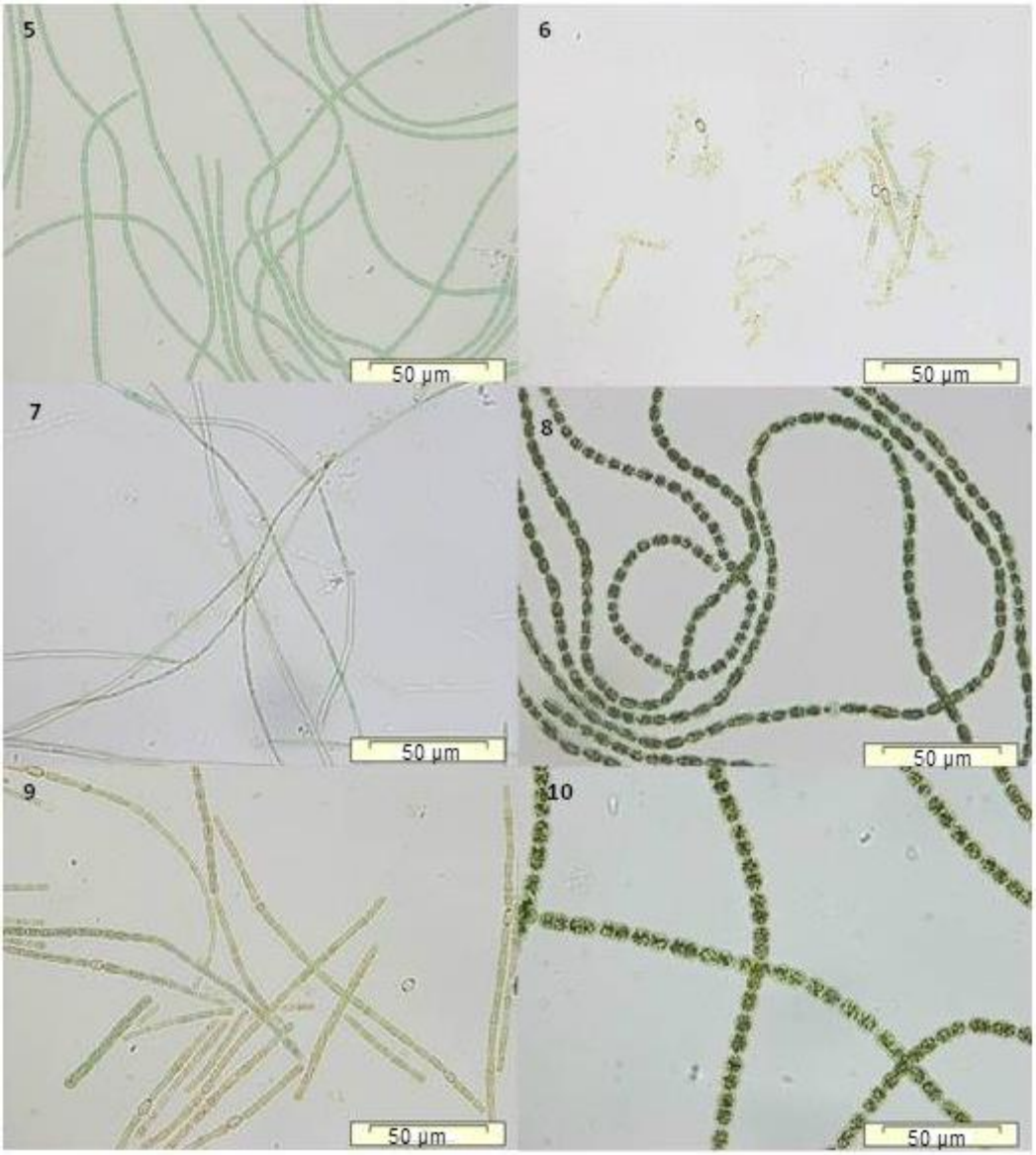


Figure 11- Morphological characterization of the *Anabaena* strains, using an optical microscopy approach. 1- LEGE 00233, 2- LEGE 00241, 3- LEGE 00243, 4- LEGE 00245, 5- LEGE 00246, 6- LEGE 00248, 7- LEGE 00250, 8- LEGE 00253, 9-LEGE 00259, 10- 04289.

3.1.1. Morphometry

Table 4- Morphometric parameters for each strain

Strains	Average cell length (S.D)	Average cell width (S.D)
LEGE 00233	4,84 (±0,84)	3,37 (± 1,20)
LEGE 00241	6,99 (±2,62)	3,91 (±0,35)
LEGE 00243	2,82 (±0,44	
LEGE 00245	4,98 (±1,01)	4,43 (±0,57)
LEGE 00246	5,85 (±0.80)	2,82 (±0,36)
LEGE 00248	7,21(± 1,67)	2,89 (±0,33)
LEGE 00250	7,55 (±1,22)	2,00 (±0,39)
LEGE 00253	7,31 (±2,59)	
LEGE 00259	6,97 (±1,15)	3,52 (±0,57)
LEGE 04289	7,27 (±1,97)	7,60 (±0,90)

In accordance with the figure 11, which showed the microphotographs for each strain, we conclude that the morphological data obtained is consistent with the characteristics of nostocalean genus *Anabaena*, since that the main part of them presented filamentous morphology with heterocysts. The exception was the strain LEGE 00243, which presented non-filamentous morphology.

To support this characterization, the morphometric parameters determined for each strains was presented in Table 4.

3.2. Genomic DNA extraction, PCR amplification, cloning and sequencing

To start the molecular screening of the ten strains, we performed the genomic DNA extraction for each strain. Then, the amplification of the 16S rRNA gene was made, using different primer pairs, 27F-1494R; 359F-1494R (Neilan et al., 1997; Jungblut et al.,2005). The amplification of the 16S rRNA gene, using these primers, resulted in amplicons with different size, since that with the 27F-1494R pair of primers all cyanobacteria strains presented a 1467 bp amplicon, as expected. In the same way, with the 359F-1494R all strains presented a 1135 bp amplicon. The organization of the results is performed according to the following numerical correspondence (1-10):

Strain 1- LEGE 00233
Strain 2- LEGE 00241
Strain 3- LEGE 00243
Strain 4- LEGE 00245
Strain 5- LEGE 00246
Strain 6- LEGE 00248
Strain 7- LEGE 00250
Strain 8- LEGE 00253
Strain 9- LEGE 00259
Strain 10- LEGE 04289

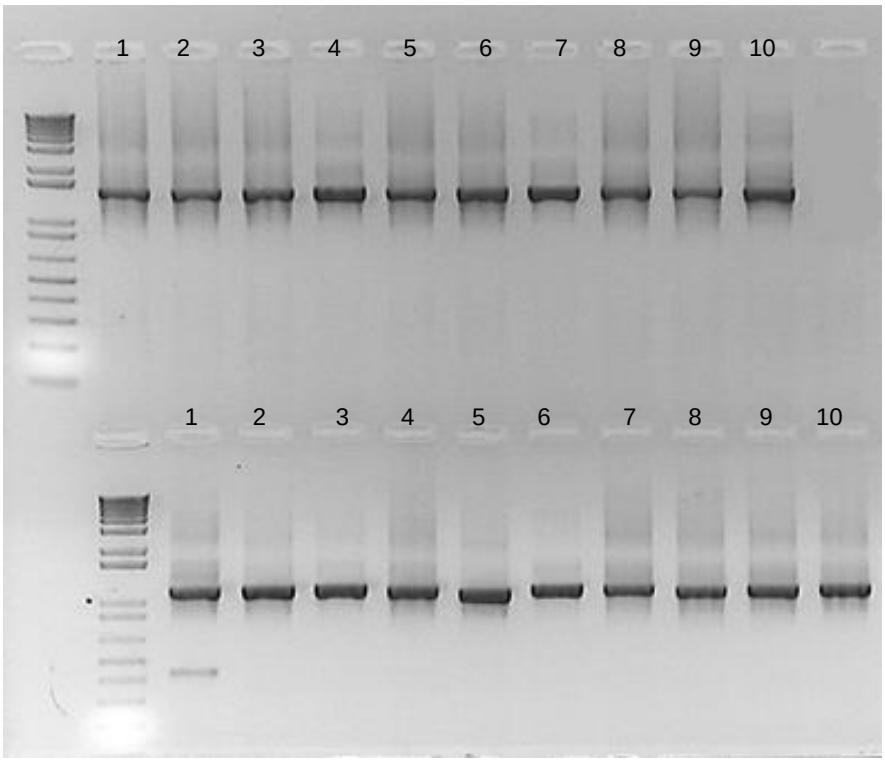


Figure 12- Amplification of the 16S rRNA gene with the primers 359F- 1494R(up); 27F-1494R (down) for all ten strains. Samples: 3µl; 1kb plus DNA ladder: 1,5µl. 1,5% agarose gel; 1- LEGE 00233, 2- LEGE 00241, 3- LEGE 00243, 4- LEGE 00245, 5- LEGE 00246, 6- LEGE 00248, 7- LEGE 00250, 8- LEGE 00253, 9-LEGE 00259, 10- 04289.

All the strains showed amplicons of the expected size, using these sets of universal primers. These results were consistent with the classification of the strains, as members of cyanobacteria group.

These bands were excised and then the purified product was cloned into a pGEM-T vector, according with the protocol and method described above. The amplicons resulting from 27F-1494R was sent to sequencing but until the moment we haven't obtained the sequencing results. The sequencing results of the amplicons resulting from the 359F-1494R will provide more specific information about the strains, since the primers 359F is a cyanobacteria-specific primer.

Following, we performed a screening for the genes involved in the cyanobactins production. In this work, was used a pair of primers, which were designed to amplify the gene encoding PatA protease, which cleaves the precursor peptide at the N-terminal recognition site (Lee et al., 2009). The primers amplified a 1.4 kb section of the patA gene. According with the Leikoski et al. study (2013), the patA gene is especially common in planktonic cyanobacteria. Furthermore, it's also known that the cyanobactin pathway is

widespread and sporadically distributed in cyanobacteria and hinted at the potential chemical diversity of cyanobactins encoded in this pathway.

These results were compared with the *Anabaena* sp.90, as a positive control (Leikoski et al., 2010).

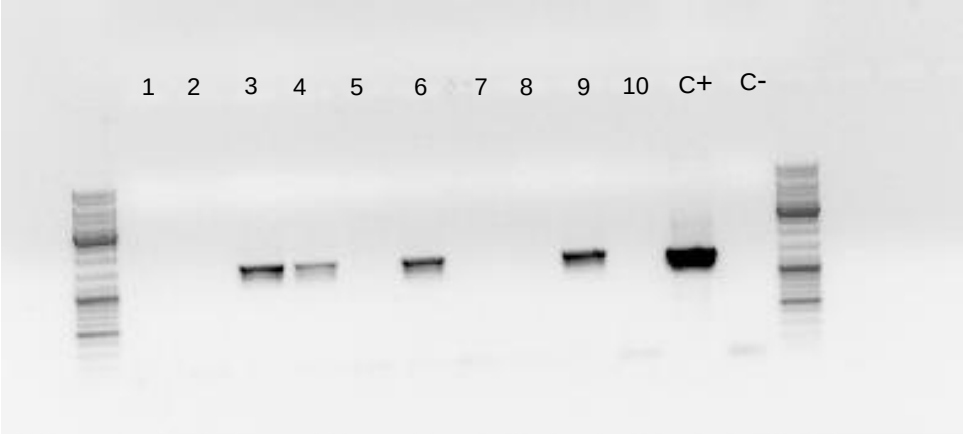


Figure 13- Amplification of the patA gene (1.4kb section) with the PatA pair of primers designed for this effect, by Leikoski et al. All the ten strains are represented. Tann = 52°C. Samples: 4µl; 10kb GeneRuler DNA ladder: 3µl. 0.7agarose gel. Positive control= *Anabaena* sp. 90.

According with Figure 13, showing the electrophoresis results of the patA gene amplification, we can conclude that the strains with 1.4 kb amplicon were LEGE 00243, LEGE 00245, LEGE 00248 and LEGE 00259.

We then proceeded to the amplification of the patE gene, using different pairs of primers (PreF/PreR and PreRNAR/PreRNAR).

At this stage, we worked with only nine strains because one of them (LEGE 00250) showed a deficit of growth. Due to the lag in growth, this strain was not included in amplification of patE genes. We decided to increase the growth time and do the screening for this strain later. When the strain achieved the proper growth the amplifications of the patA genes was made and we verified that it did not result in an amplicon of the expected size (Figure 13).

Regarding the patE genes, in the first screening the PreF/PreR pair of primers were used. The results were in accordance with the patA genes amplification, since we obtained positive result for the strains 3- LEGE 00243, 4-LEGE 00345, 6-LEGE 00248 and 9-LEGE 00259. The exception on these results was the strains 8-LEGE 00253 and

10- LEGE 04289, which also presented band in the expected size. The results are shown in Figure 14 as an example.

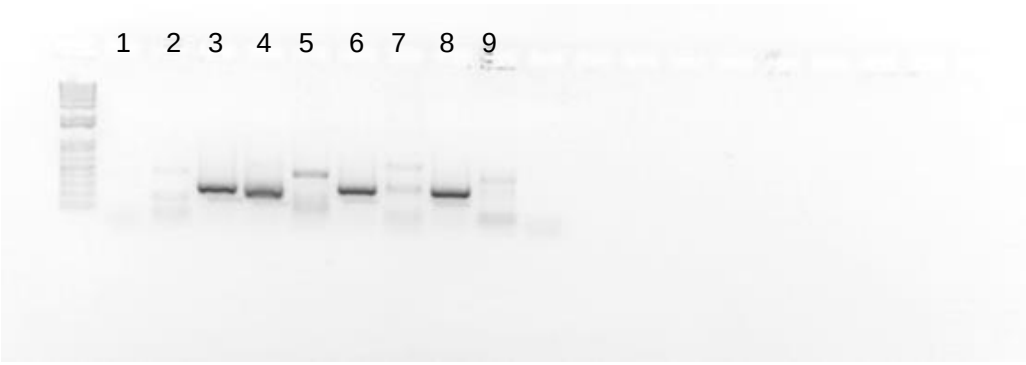


Figure 14- Amplification of patE genes, using primers PreR and PreF, from nine strains. Tann= 53ºC. Samples: 3µl ,1kb plus DNA ladder: 1.5µl. 1,5% agarose gel; 1- LEGE 00233, 2- LEGE 00241, 3- LEGE 00243, 4- LEGE 00245, 5- LEGE 00246, 6- LEGE 00248, 7- LEGE 00253, 8- LEGE 00259, 9-LEGE 04289

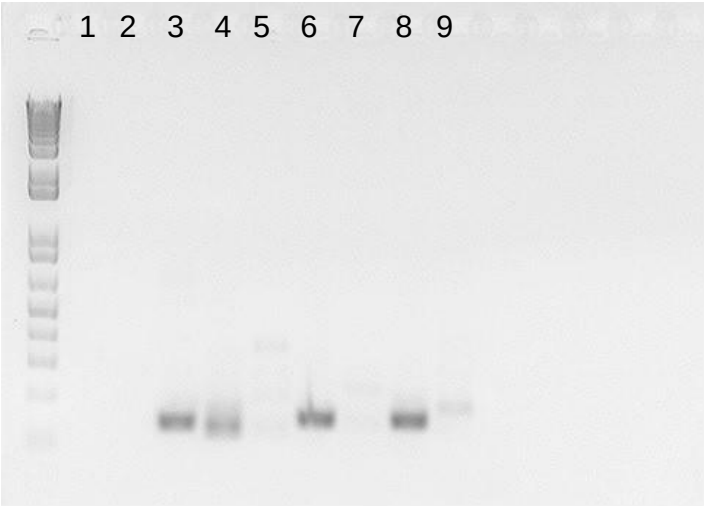


Figure 15- Amplification of the patE genes, using primers PreRNA R and PreRNA F, from nine strians. Tann=56ºC. Samples: 3µl, 1kb plus DNA ladder: 1.5µl. 1,5% agarose gel; 1- LEGE 00233, 2- LEGE 00241, 3- LEGE 00243, 4- LEGE 00245, 5- LEGE 00246, 6- LEGE 00248, 7- LEGE 00253, 8- LEGE 00259, 9-LEGE 04289.

Using the primers PreRNA R and PreRNA F we obtained one more strains with amplicon in the expected size (160 bp) comparing with the first primer pair used for patE gene amplification. This strain was 5- LEGE 00246. After, the excised bands were cloned and sent to sequencing

With the precursor gene sequences in hand we proceeded to the *in silico* analysis. We aligned the sequence of the acy gene precursor for all strains with the acy gene percusor sequence of the *Anabaena* sp.90 (Figure 16). For this alignment, we used two

clones for each strain, each clone for a different set of primers, excluding the strain LEGE 04289, which has only one clone and strain LEGE 00245 that has tree clones. This difference in the number of clones is because in the strain LEGE 04289 one of the sequenced clones was not in accordance with the expected (according to BLAST results), and in case of strain LEGE 00245 the patE gene screening result presented two bands of similar size.

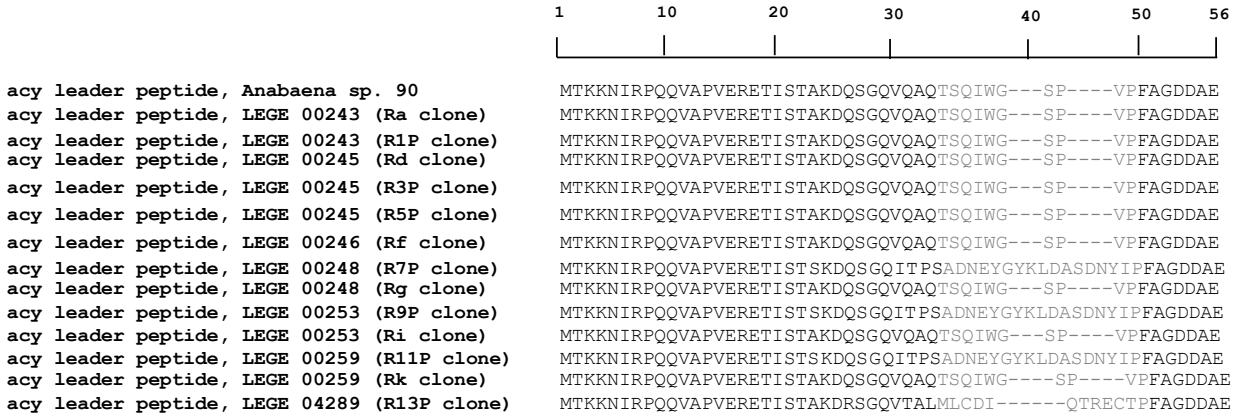


Figure 16- Alignment of the sequences of the acy precursor from all the strains with the acy precursor of the Anabaena sp. 90 (acyE). The areas of the precursor protein which form the mature cyanobactin are in grey

The AcyE core peptide sequences allowed us to put forward putative structures of the anacyclamides found in the cyanobacterial strains, using the ChemDraw software.

The anacyclamide with the amino acids sequence (Thr-Ser-Gln-Ile-Trp-Gly-Ser-Pro-Val-Pro) was found in some of these strains and is already described in literature in several studies (Leikoski et al., 2010; Sivonen et al., 2010). It corresponds to anacyclamide A10 originally described from *Anabaena* sp. 90 (Figure 17). However, in four clones we obtained a different amino acids core sequence, which suggested that we could have a putative new anacyclamide. These four clones were from four different strains. Those are LEGE 00248, LEGE00253, LEGE 00259 and LEGE 04289.The new amino acids core sequence common between the first three strains, Ala-Asp-Asx-Glu-Tyr-Gly-Tyr-Lys-Leu-Asp-Ala-Ser-Asp-Asx-Tyr-Ile-Pro, presents 17 amino acids. While the amino acids core sequence of LEGE 04289, Met-Leu-Cys-Asp-Ile-Gln-Thr-Arg-Glu-Cys-Thr-Pro is smaller with 12 amino acids (Figure 18 and Figure 19).

Percursor peptide AcyE from anabaena sp.90

A

MTKKNIRPQQVAPVERETISTAKDQSGQVQAQ TSQIWGSPVP FAGDDAE

B

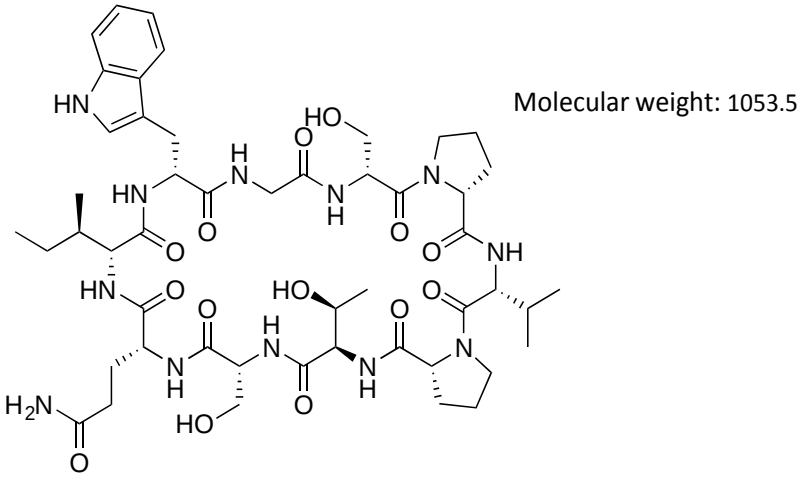


Figure 17- (A) AcyE peptide precursor from Anabaena sp. 90 with the hypervariable region of the 49-aminoacid protein encoding the mature anacyclamide shaded, indicating the position of cleavage and macrocyclization (grey). (B) Putative structure of the decapeptide anacyclamide A10 from *Anabaena* sp. 90 (Leikoski et al., 2010)

In the same way, we designed the possible structure for the putative new anacyclamides based on these results.

Percursor peptide AcyE from LEGE 00259

MTKKNIRPQQVAPVERETISTSKDQSGQITPS ADNEYGYKLDASDNYIP FAGDDAE

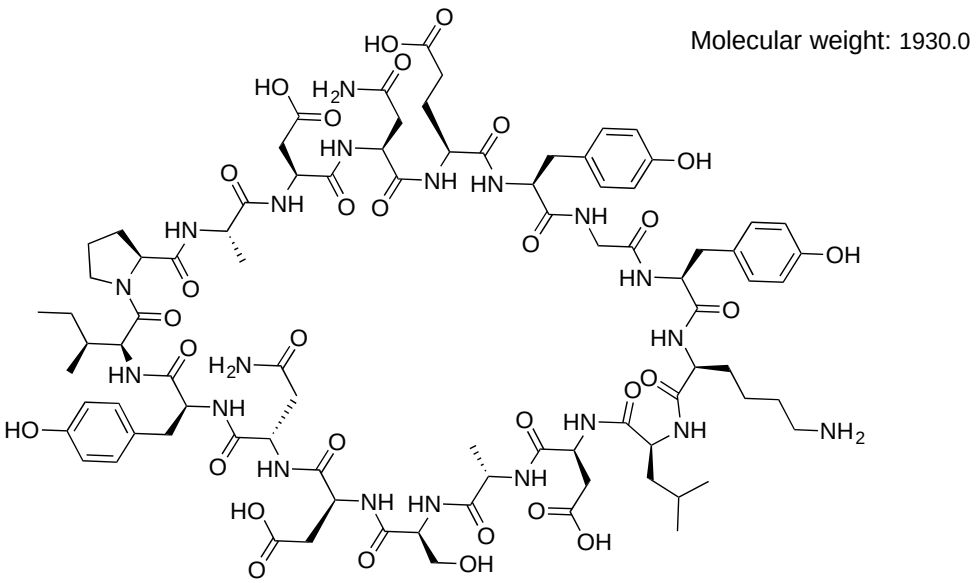


Figure 18- Putative structure of ADNEYGYKLDASDNYIP anacyclamide, using the sequence of AcyE precursor peptide from strain LEGE 00259 (example)

Percursor peptide AcyE from LEGE 04289

MTKKNIRPQQVAPVERETISTAKDRSGQVTAL MLCDIQTRECTP FAGDDAE

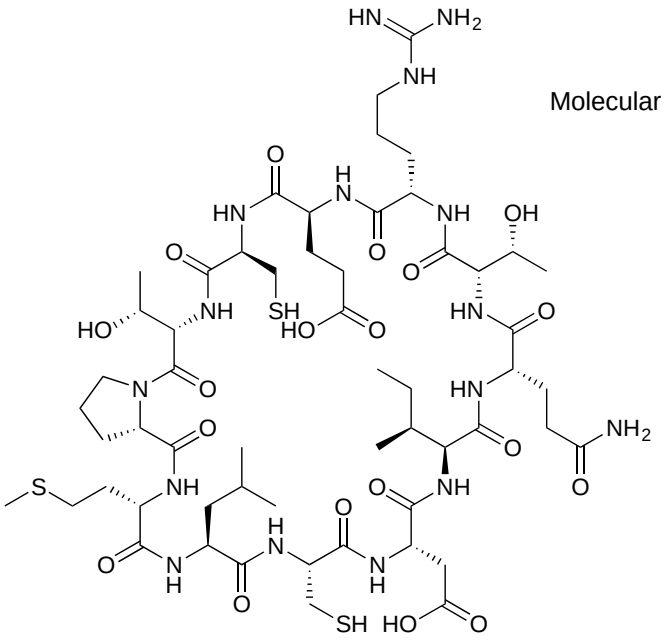


Figure 19- Putative structure MLCDIQTRECTP of anacyclamide, using the sequence of AcyE precursor peptide from strain LEGE 04289 (example)

Based on these results some predictions regarding the linear mass, cyclic mass and post-translational modifications were also made. All the predictions are presented in Table 5.

Table 5- Anacyclamide predictions by each strain in study

Strains	Predictions					Reference
	Core sequence	Amino acids	Linear mass (Da)	Cyclic mass (Da)	Post-translational modifications	
LEGE 00233	No predictions					
LEGE00241	No predictions					
LEGE00243	TSQIWGSPVP	10	1053.5			I.
LEGE 00245	TSQIWGSPVP	10	1053.5			I.

LEGE 00246	TSQIWGSPVP	10	1053.5			I.
LEGE 00248	TSQIWGSPVP	10	1053.5			I.
	ADNEYGYKLDASDNYIP	17	1946.8	1928.8	1996.8, 2064.8 (monoprenyl, diprenyl	
LEGE 00250	No predictions					
LEGE 00253	TSQIWGSPVP	10	1053.5			I.
	ADNEYGYKLDASDNYIP	17	1946.8	1928.8	1996.8, 2064.8 (monoprenyl, diprenyl	
LEGE 00259	TSQIWGSPVP	10	1053.5			I.
	ADNEYGYKLDASDNYIP	17	1946.8	1928.8	1996.8, 2064.8 (monoprenyl, diprenyl	
LEGE 04289	MLCDIQTRECTP	12	1408.6	1390.6	1350.6, 1312.6 (thiazoles, thiazoles+oxazoles) 1388.6 (sulphur bridge)	

I.-This prediction is in accordance with data already published by Leikoski et al., 2010.

3.3. Chemical analysis

We then sought to confirm our predictions by looking for the new cyanobactin-related masses in the *Anabaena* cells. The methanol extracts of all strains were submitted to the LC-MS analysis as described.

Table 6- Anacyclamides and their detection by LC-MS, in two *Anabaena* strains

Strain	Amino acid sequence	$[M+H]^+$	Chemical formula Diprenylated	Experimental mass	Error (ppm)
LEGE 00248	ADNEYGYKLDASDNYIP	2065,975	$C_{96}H_{136}N_{20}O_{31}$	2065.9839	4.16
LEGE 00259	ADNEYGYKLDASDNYIP	2065,975	$C_{96}H_{136}N_{20}O_{31}$	2065.9792	1.88

After the LC-MS analysis of all the strains, only the anacyclamide with the amino acids sequence ADNEYGYKLDASDNYIP was found, in its diprenylated form. This anacyclamide showed presence in two strains 6- LEGE 00248 and 9-LEGE 00259 (Table 6), out of three candidates strains.

In order to compare all the strains regarding the production of this or other prenylated anacyclamides, an analysis of the ten strains using a Neutral loss of 68 Da filter was performed (Figure 20).

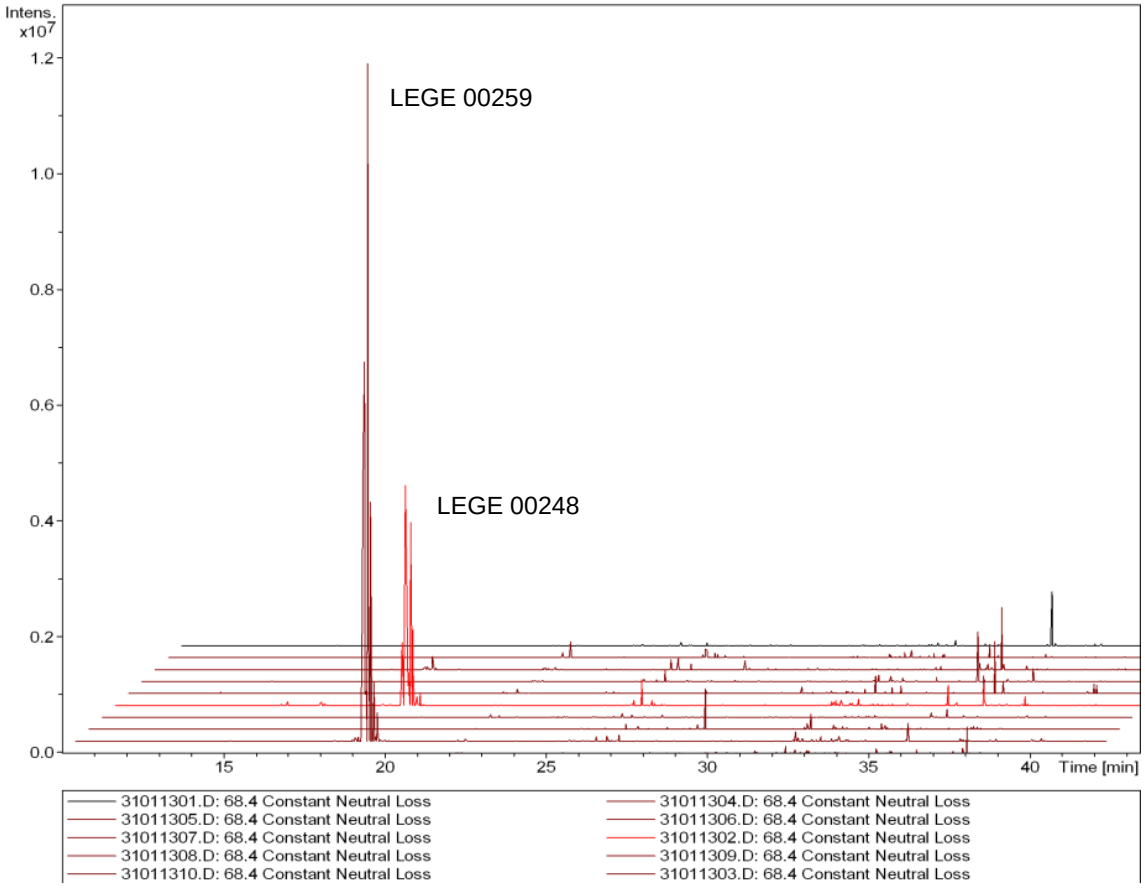


Figure 20- Ion chromatograms of the ten Anabaena strains filtered for Neutral losses of 68 Da. The two large clusters correspond to the two strains containing the diprenylated novel anacyclamide

As seen in Figure 20, the strains LEGE 00259 and LEGE 00248 showed a peak with strong intensity compared with the other strains. This pick appeared in the chromatogram around the same retention time, in both strains. This fact is consistent with the anacyclamide production by both strains.

The ADNEYGYKLDASDNYIP anacyclamide is present in two strains (LEGE 00248 and LEGE 00259), as seen by LC-MS (Figures 21 and 22). The chromatograms show a peak with the retention time 19.0 min, with a mass spectrum containing a cluster with a $[M+H]^+$ pseudomolecular ion at 2067 Da, indicating the presence of the predicted anacyclamide.

LC-MS analysis for the strain LEGE 00248

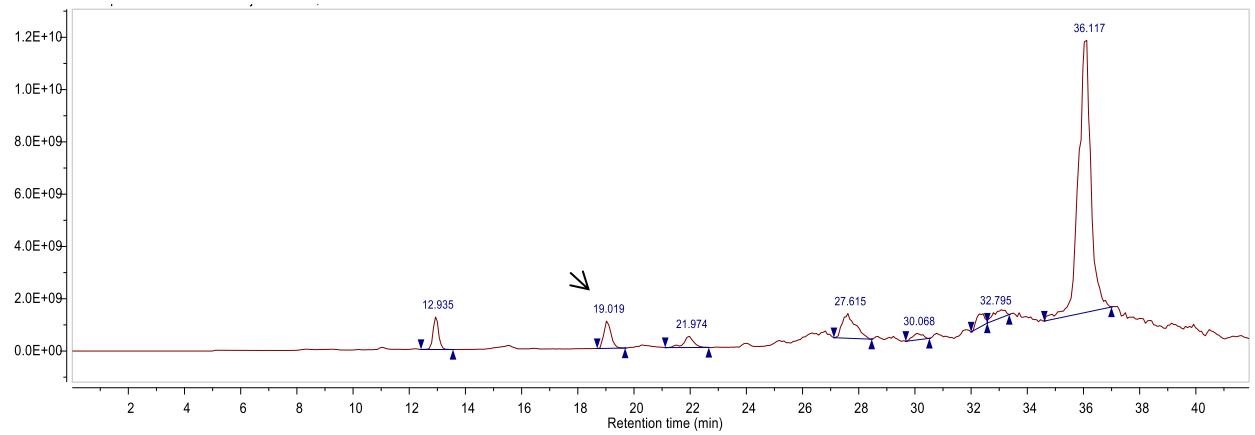


Figure 21- MS profile TIC to the LEGE 00248 strain

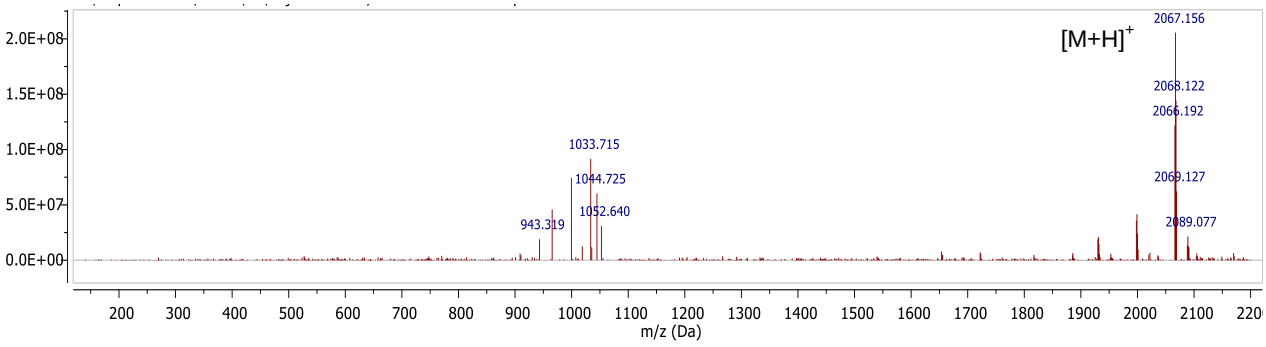


Figure 22- MS profile MS to the LEGE 00248 strain; Retention Time: 18,91min- 19,32min

LC-MS analysis for the strain LEGE 00259

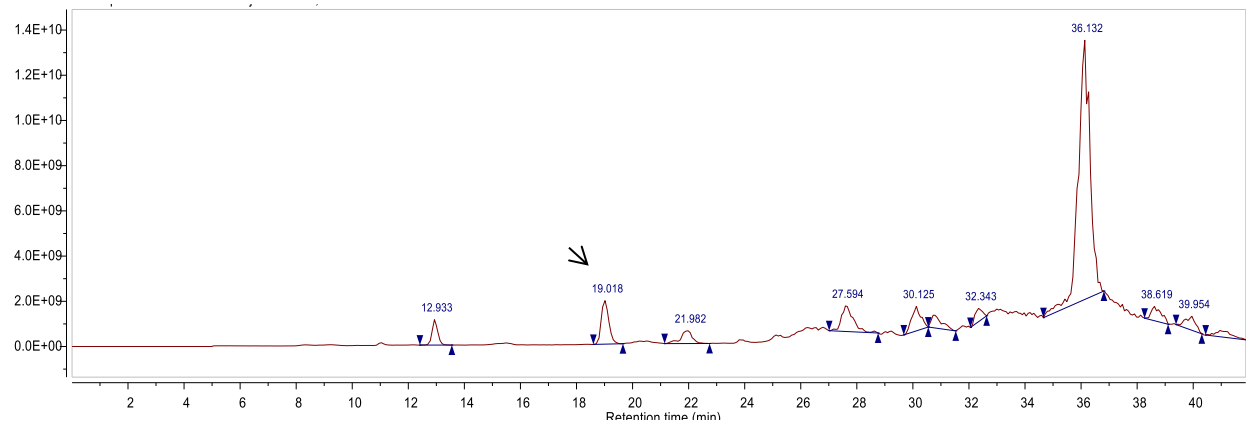


Figure 23- MS profile TIC to the LEGE 00259 strain

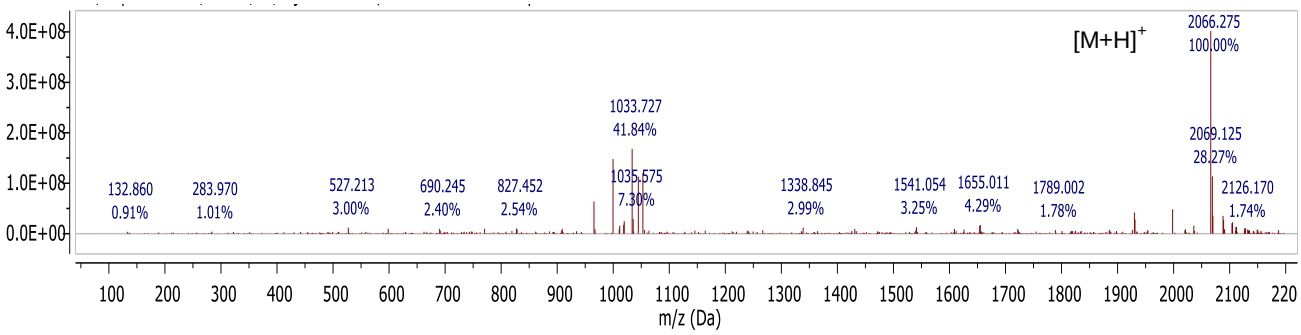


Figure 24- MS profile MS to the LEGE 00259 strain; Retention Time: 18.93min-19.38min

Comparing the LC-MS data with the predictions made, we conclude that the experimental mass of the putative new anacyclamide produced by strains LEGE 00248 ans LEGE 00259 was bigger than the predicted mass by 136 Da. This fact is consistent with two prenylation sites.

According with the literature, the known prenylated amino acids are tyrosine, serine, threonine, and tryptophan (Leikoski et al., 2010; Erickson et al., 2003). So, the probable prenylation sites in that anacyclamide is on Tyrosines (Tyr). However, in amino acids sequence the new anacyclamide presents three Tyrosines, so one of them probably didn't presents prenylation and is unclear which tyrosines are prenylated. We have growth both strains in large-scale and performed a methanol extraction with the goal of purifying the new anacyclamides, as described in the material and methods. However, we have yet to perform the purification steps. Our objective is to perform 1D and 2D NMR experiments in the purified material to clarify the positioning of the prenyl groups, which should be achieved in conjunction with MS^2 data.

In Table 7 we present the extraction yields for both strains

Table 7- Extraction yields for LEGE 00248 and LEGE 00259 strains

Strains	Lyophilized mass	Extract mass	R
LEGE 00248	1,29g	0,28g	21,7%
LEGE00259	1,27g	0,28g	22,0%

We have also strived to characterize the putative cyanobactin cluster in LEGE 00259, which is responsible for the new anacyclamide production. Thus, the acy gene cluster was amplified, with patex2f (5'-ATGGATCCTGATGGACTGTAGTGTGAG-3') and patex5r (5'-TACTCGAGAGGTTTTGGGACTCTTTAG-3') primers in three 60-µl reaction, as described previously, and cloned in *E.coli* TOP10. The three clones obtained were sent to sequencing. This approach was supported in other works, for example in the work involving the Anabaena sp. 90 gene cluster sequencing (Leikoski et al., 2010). By this time, we have yet to obtain the sequencing results and perform the consequent analysis of the entire gene cluster from LEGE 00259 strain.

4. Conclusion and future work

In this study we reported a novel low-molecular-weight peptide produced ribosomally by *Anabaena* strains. The new post-translationally modified anacyclamide, with two prenyl groups, was detected in two of ten *Anabaena* strains screened for. The probable prenylation sites are on Tyrosines. However the anacyclamide presented, in the amino acids sequence, three Tyrosines. It is unclear which Tyrosines are prenylated and further investigations on the prenylation mechanisms may help us gain more insight into these post-translational tailoring events, for example, the basis for the non-prenylation of one of the tyrosine side-chains.

The next step will be to carry out nuclear magnetic resonance (NMR) to fully characterize this new compound, identifying in which Tyrosines the prenylation occurs to make a phylogenetic inference regarding anacyclamides production in these and other strains.

Overall, this study reports a new cyanobacterial secondary metabolite, which can expand our knowledge about this family of compounds and their biological activity.

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