Characterisation of the lectin microvirin from *Microcystis aeruginosa* PCC 7806 and new insights into the role of microcystin

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät I

der Humboldt-Universität zu Berlin

von

Jan-Christoph Kehr

geboren am 21.02.1977 in Osnabrück

Präsident der Humboldt-Universität zu Berlin

Prof. Dr. Christoph Markschies

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I

Prof. Dr. Lutz-Helmut Schön

Gutachter:　　　1. Prof. Dr. Elke Dittmann

2. Dr. Nicole Tandeau de Marsac

3. PD Dr. Hans von Döhren

Tag der mündlichen Prüfung: 11.06.2009

CONTENTS

ZUSAMMENFASSUNG

Sowohl in Süßwasserseen als auch in marinen Gewässern kommt es in den Sommermonaten immer wieder zu Massenentwicklungen von Cyanobakterien, sogenannten "Blüten". In Seen werden diese oftmals von Cyanobakterien der Gattung *Microcystis* dominiert, deren Arten häufig Toxine bilden und somit eine Gefahr für Menschen und Tiere darstellen. Die verbreitesten dieser Toxine sind die leberschädigen Microcystine, die eine Klasse nichtribosomal synthetisierter Peptide darstellen. Nachdem die toxische Wirkung der Microcystine bisher als deren Hauptfunktion angesehen wurde, deuten neuere Forschungsergebnisse darauf hin, dass Microcystine eine andere Primärfunktion für die Produzenten besitzen. Es wird unter anderem angenommen, dass Mircocystine eine Rolle in der Antwort auf oxidativen Stress, wie er z.B. durch Starklicht ausgelöst wird, spielen.

Im Rahmen dieser Studie wurde Microvirin (Mvn), ein putatives Lektin aus *Microcystis aeruginosa* PCC 7806, von dem angnommen wurde, dass es funktional mit Microcystin assoziiert ist, charakterisiert. Zunächst konnte gezeigt werden, dass Mvn tatsächlich zuckerbindende Aktivität besitzt und spezifisch Mannan, ein Oligosaccharid aus Mannoseuntereinheiten, erkennt. Bindestudien mit fluoreszenzmarkiertem Mvn und Antikörpern zeigten, dass Zucker dieses Typs auf der Zelloberfläche von *M. aeruginosa* PCC 7806 lokalisiert sind und eine Bindestelle für das sekretierte Mvn darstellen.

Mit Hilfe fluoreszenzmikroskopiebasierender Methoden wurde gezeigt, dass sowohl Mvn als auch das korrespondierende Mannanoligosaccharid stammspezifisch sind. Weiterhin konnte durch PCR gezeigt werden, dass das *mvn*-Gen in allen getesteten *Microcystis*-Stämmen vorkommt, die auch Gene für die Microcystinbiosynthese besitzen.

Eine direkte Interaktion von Microcystin und Mvn konnte *in vitro* bestätigt werden. Microcystin bindet dabei über seinen *N*-Methyl-Dehydroalaninrest kovalent an die reduzierten Cysteinreste des Proteins. Ein Einfluss auf die Oligomerisierung des Proteins wurde festgestellt. Microcystin scheint unspezifisch Cysteinreste von Proteinen zu binden, und es konnte gezeigt werden, dass dies besonders unter oxidativen Stressbedingungen wie Eisenmangel und Starklichtexposition verstärkt geschieht. Die Daten liefern somit weitere Indizien für eine Rolle von Microcystin in der Stressadaptation.

ABSTRACT

Cyanobacteria frequently appear as so-called "water-blooms" during summer months. Cyanobacteria of the genus *Microcystis*, whose species often dominate freshwater lakes, produce toxins that represent a potential threat for humans and animals. The most prominent toxins are the non-ribosomally synthesised hepatotoxic microcystins. Toxicity has been considered the main function of these peptides, but recent studies propose different primary functions of microcystins for their producers. The involvement of microcystins in the response to oxidative stress was proposed recently.

Within this study the putative lectin microvirin (Mvn), which was suggested to be functionally related to microcystin, was characterised. Initially it was shown that Mvn does indeed possess a carbohydrate binding activity, and specificity for mannan, an oligosaccharide made of mannose subunits, was proven. Binding studies using fluorescence-labelled Mvn and antibodies identified carbohydrates of this type at the cell surface of *M. aeruginosa* being a binding site for the secreted Mvn.

Fluorescence microscopy techniques were employed to show that Mvn as well as the corresponding mannan oligosaccharide are strain-specific. Additionally it was shown by PCR that the *mvn* gene is present in all tested *Microcystis* strains possessing microcystin biosynthesis genes.

A direct interaction of microcystin and Mvn was confirmed *in vitro*. Microcystin covalently binds to the reduced cysteine residues of the protein via its *N*-methyl-dehydroalanine moiety. An impact on the oligomerisation state of Mvn was observed. Microcystin seems to bind cysteine residues in an unspecific manner *in vivo*, and it was shown that this occurs especially under conditions of oxidative stress such as iron depletion and exposition to high light. Hence, the data provide further evidence for an involvement of microcystins in stress adaptation.

1 INTRODUCTION

1.1 Cyanobacteria

Cyanobacteria, also referred to as blue-green algae, are oxygenic photolithotrophic prokaryotes capable of CO_2 assimilation through photosynthesis using the reducing power derived from the light-driven water cleavage (Lengeler *et al.*, 1999). They are the only group among bacteria that perform an oxygenic photosynthesis and they have contributed essentially to the formation of the oxygen atmosphere of the earth (Dismukes *et al.*, 2001). Fossil cyanobacteria findings have been dated to 2.5 to 3.5 billion years (Schopf and Packer, 1987). Due to their photosynthesis activity cyanobacteria are important primary producers (Liu *et al.*, 1998). They occupy a wide range of habitats reaching from aquatic – both marine and freshwater – to terrestrial. These include extreme habitats like hot springs and deserts (Whitton and Potts, 2000). Other species are able to fix molecular nitrogen in heterocysts - specialised cells that are impervious for oxygen, which is toxic for the nitrogenase enzyme (Rippka, 1988). Other species can live in symbiosis with plants or fungi (DePriest, 2004). The progenitor of today's chloroplasts was an ancient cyanobacterium-like endosymbiont that was engulfed by a heterotrophic eukaryote (McFadden, 1999).

Cyanobacteria show considerable morphological diversity and have been classified into five sections based on morphological features (Rippka *et al.*, 1979). Representatives of section I and section II are unicellular and divide by binary fission or multiple fission, respectively. Section III comprises filamentous nonhetercystous cyanobacteria. Members of section IV and V are also filamentous but are capable of cell differentiation. The strains of section V can be distinguished from section IV members by their ability to form branched filaments.

Cyanobacteria are a rich source of secondary metabolites, in particular non-ribosomal peptides (NRPS) and polyketides (PKS) (Welker and von Döhren, 2006). By using degenerate primers Christiansen *et al.* have demonstrated that NRPS genes are present in 75% of 146 axenic strains of the Pasteur Culture Collection, which included members of all cyanobacterial sections (Christiansen *et al.*, 2001).

1.1.1 MICROCYSTIS AERUGINOSA

The genus *Microcystis* is a member of the *Chroococcaceae* in the order *Chroococcales* and belongs to section I of the cyanobacterial classification (Rippka, 1988). *Microcystis* is a colony

forming genus and its different species are determined by distinct colony morphology (**Fig. 1**). However, this taxonomy is not supported by phylogenetic analyses. Comparisons of 16S to 23S ribosomal DNA internal transcribed spacer sequences from five morphospecies revealed a high degree of sequence similarity. Clusters in a phylogenetic tree generated from this data did not correspond to morphological characteristics of the examined strains (Otsuka *et al.*, 1999). Therefore, these strains should be considered as one species regarding them as morphological variants (Otsuka *et al.*, 2001).

Microcystis species produce a variety of secondary metabolites, mainly NRPS (Welker *et al.*, 2004). Because of its toxic impact on eukaryotes the hepatotoxic heptapeptide microcystin (see 1.1.2) is the most prominent.
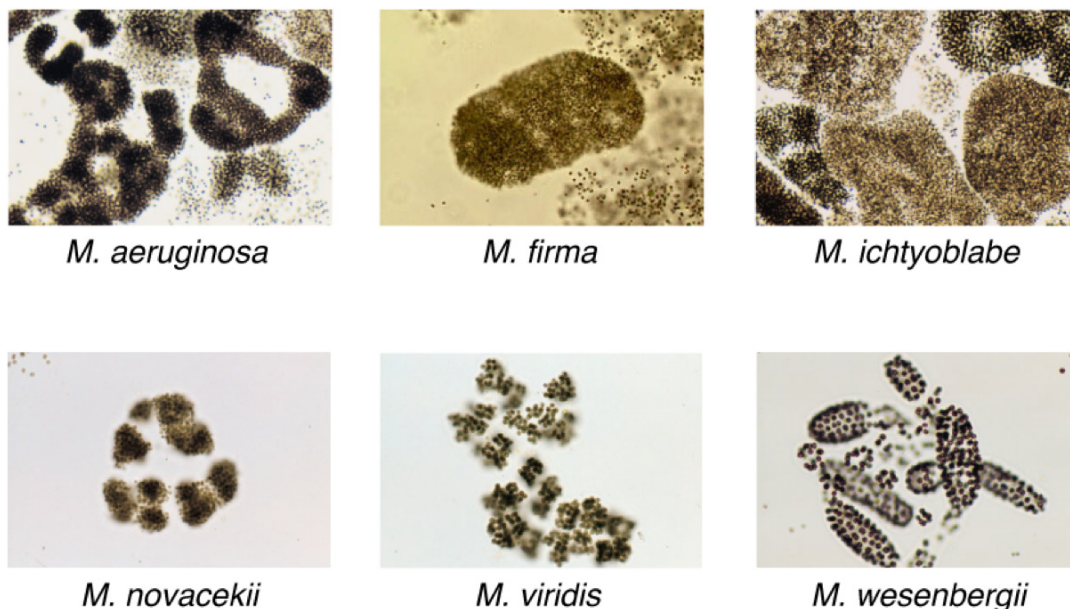
Fig. 1: A selection of common *Microcystis* colony morphotypes (pictures from: http://research.kahaku.go.jp/botany/aoko/aokokids/mycro-pictures.html).

1.1.1.1 HABITAT

Microcystis inhabits freshwater lakes all over the world. In contrast to e.g. *Planktothrix* species that are mainly found in shallow lakes, *Microcystis* commonly occupies deep lakes with a stable stratification, because it performs an extensive buoyancy regulation. During warm summers

Microcystis occurs in so called "blooms" - mass developments of cyanobacteria. Bloom formation is promoted by eutrophication of lakes.

1.1.1.2 COLONY FORMATION AND BUOYANCY REGULATION

A key feature of *Microcystis spp.* is the ability to form characteristic colonies, which seems to play an important role in buoyancy regulation. In stratified lakes *Microcystis* is able to perform vertical migration in the water body. The buoyancy is regulated through gas vesicles and carbohydrate content of the cells. *Microcystis* continues to form gas vesicles at high irradiance (150 μmol m^{-2}s^{-1}), but buoyancy is reduced due to the accumulation of carbohydrates (Thomas and Walsby, 1985). Field studies have confirmed that the relative gas vesicle volume does not change significantly during the day. The loss of buoyancy is mainly driven by the accumulation of carbohydrate and protein ballast (Ibelings *et al.*, 1991). The cell density increases as a result of enhanced photosynthesis activity at the lake surface and the cells become less buoyant. The carbohydrate ballast is reduced as a consequence of respiration in deeper layers of the lake and the cell density decreases again. Under light–limited conditions *M. aeruginosa* was shown to be always buoyant. A loss of buoyancy was only observed when energy was generated faster than it could be utilised for growth (Kromkamp *et al.*, 1988). Furthermore, the gas vesicle volume decreased during phosphorus-limited and nitrogen-limited growth, whereby the role of carbohydrate accumulation was emphasised (Chu *et al.*, 2007). Since nutrient limitation in stratified lakes often occurs in the surface layer, the cells might migrate to deeper layers to meet their nutrient requirements. A further parameter of vertical migration is the colony size. Though it does not influence the buoyancy itself colony size contributes by modulating velocity of the sinking and rising, respectively. Several models of the vertical migration (**Fig. 2**) were computed and showed that, according to Stoke's law, large colonies migrate faster than smaller ones (Rabouille *et al.*, 2005). Hence, colony diameter determines the time the cells are exposed to light or nutrition rich dark layers (Rabouille *et al.*, 2003). The influence of colony size has been verified by field observations where small colonies were most abundant in deeper water (Ibelings *et al.*, 1991).
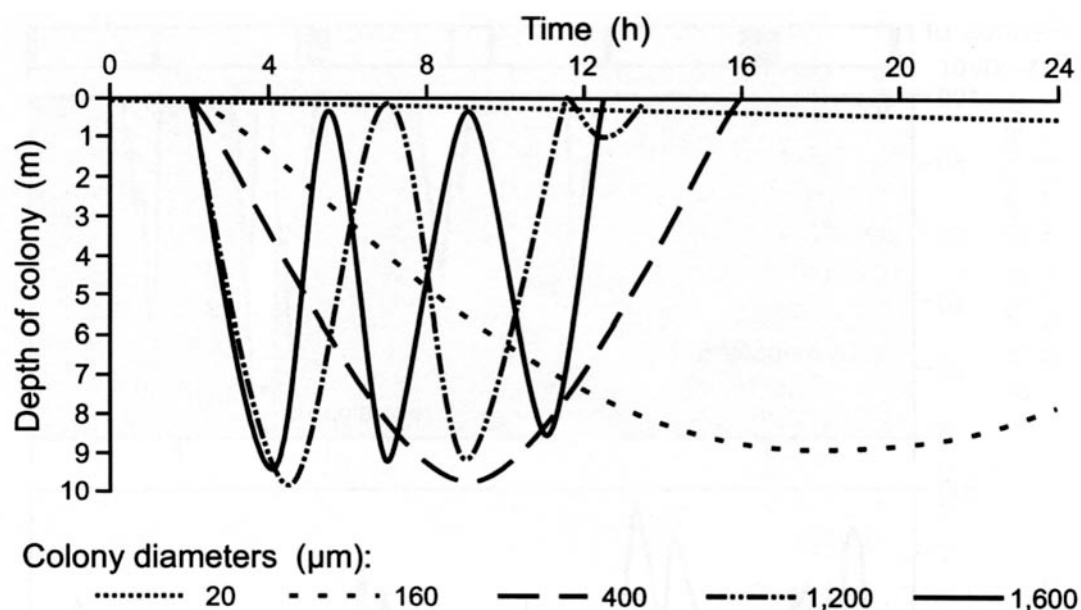
Fig. 2: A model describing the correlation between colony size and vertical migration. According to Stoke's law an increase in colony diameter results in an accelerated movement. Therefore bigger colonies can adjust their position in the water column far more rapidly than smaller ones. From Chorus & Bartram, 1999.

1.1.1.3 CHEMOTYPES OF MICROCYSTIS

Numerous *Microcystis* strains produce a variety of secondary metabolites and the chemotype is defined as the entirety of peptides produced by a particular strain. Although the morphotype of *Microcystis* cannot be deduced from the genotype (Otsuka *et al.*, 2000; Otsuka *et al.*, 1999), several studies tried to find a correlation between the morphotype and the chemotype. A study conducted by Fastner *et al.* (2001) in Lake Wannsee (Berlin, Germany) revealed a correlation between certain peptide combinations and morphotypes (Fastner *et al.*, 2001). Microcystins were chiefly found in *M. aeruginosa*, while colonies of *M. ichtyoblabe* and *M. wesenbergii* did not contain microcystins but anabaenopeptins, microginins and cyanopeptolins. Additionally, the occurrence of microcystin in combination with anabaenopeptins and microginins was mutually exclusive. A second study in lakes around Berlin could not find a clear correlation of morphotype and chemotype, although the production of microcystins was mainly attributed to *M. aeruginosa* while *M. ichtyoblabe* did not produce microcystins (Welker *et al.*, 2004). A survey on distribution of microcystin-producing *Microcystis* in European freshwater bodies revealed that *M. aeruginosa* and *M. botrys* morphospecies have a higher proportion of mc-producers (<70%) than *M. flos-aquae* and *M. ichtyoblabe*, while *M. wesenbergii* did not contain

microcystin at all (Via-Ordorika *et al.*, 2004). In 2000 a PCR-based study on the distribution of microcystin genotypes in Lake Wannsee led to similar results (Kurmayer *et al.*, 2002).

1.1.2 MICROCYSTIN

Microcystins (**Fig. 3**) are a group of hepatotoxic heptapeptides that are predominantly produced by strains of the genera *Anabaena*, *Microcystis* and *Planktothrix*. In addition, occurence of microcystins has been reported for single strains of *Anabaenopsis*, *Hapalosiphon* and *Nostoc* (Sivonen and Jones, 1999). Microcystins (mc) share the common structure of cyclo(-Adda-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z) where X and Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid and Mdha is *N*-methyl-dehydroalanine (Botes *et al.*, 1984; Sivonen and Jones, 1999). Modifications occur at all seven amino acid residues and over 70 variants have been described to date.
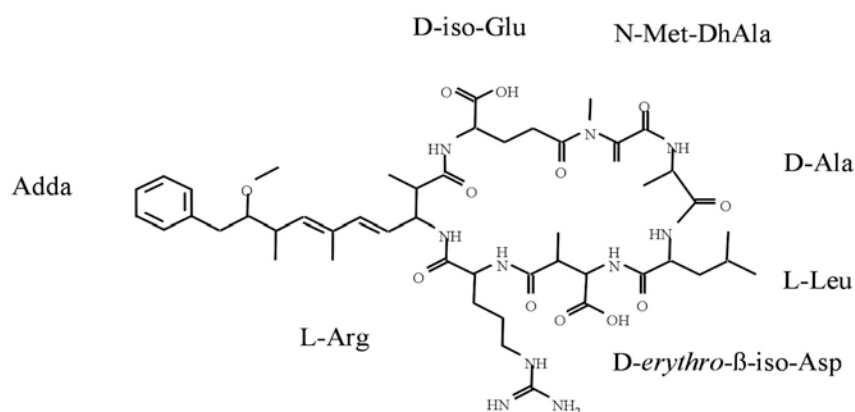
Fig. 3: Microcystin-LR

The toxicity of microcystins originates from their ability to bind eukaryotic protein phosphatases of type 1 and 2A (MacKintosh *et al.*, 1990; Runnegar *et al.*, 1995; Runnegar *et al.*, 1993; Toivola *et al.*, 1994). Protein phosphatases are the antagonists of protein kinases and their inhibition causes a hyper-phosphorylation of their target proteins. Microcystin is taken up by the organic anion transport system of hepatocytes and induces excessive phosphorylation of cytoskeletal filaments in the liver, which can lead to a hemorrhagic shock (Falconer and Yeung, 1992). Microcystins were implicated in animal poisonings and even adverse effects on human

health were reported. In Brazil, unsufficient treatment of water contaminated with microcystin-LR caused the death of 40 dialysis patients (Carmichael *et al.*, 2001; Jochimsen *et al.*, 1998).

1.1.2.1 MICROCYSTIN BIOSYNTHESIS

Microcystin is synthesised by a large multienzyme complex (Dittmann *et al.*, 1997; Tillett *et al.*, 2000) comprising nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS) and additional modifying enzymes (**Fig. 4**). This enables the incorporation of non-proteinogenous amino acids and the introduction of modification like epimerisations and methylations.

NRPS incorporate each single amino acid by the "multiple-carrier-thio-template"-mechanism into the growing peptide chain. The NRPS comprise a linear order of modules and each module performs the recognition and the activation of a single amino acid and the subsequent linkage to the peptide chain. The sequence of the peptide is directly determined by the arrangement of the modules (Finking and Marahiel, 2004; Schwarzer *et al.*, 2003).

The Adda moiety in microcystin is synthesised by PKS. PKS catalyse the sequential condensation of short chain carbonic acids. Similar to NRPS, PKS are modular and the structure of the product can be deduced from the number and the arrangement of the modules.
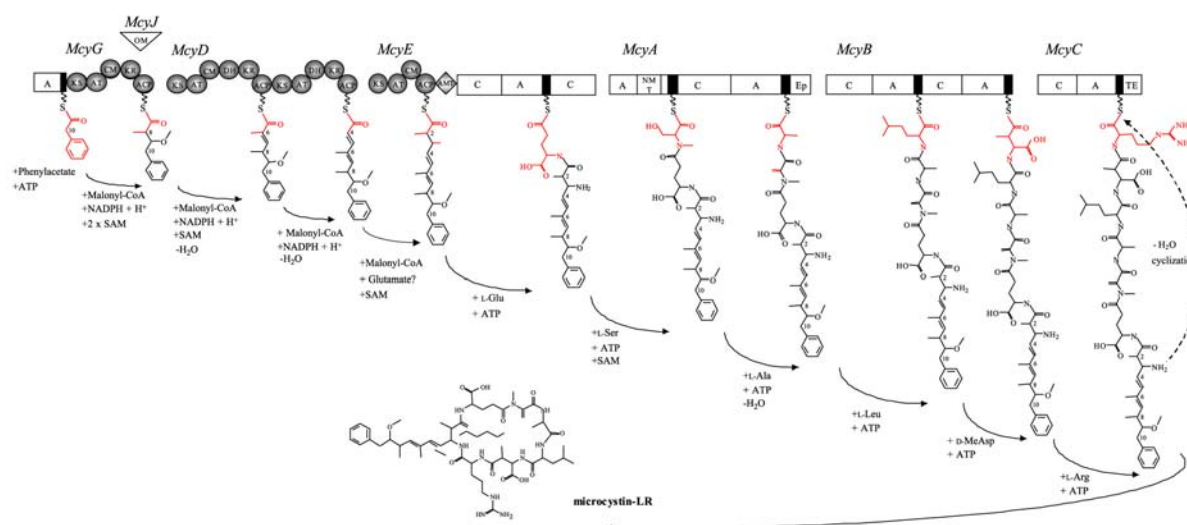
Fig. 4: Model for the formation of microcystin and predicted domain structures of the six multienzymes (McyA-E, G) involved in microcystin biosynthesis. White rectangles represent peptide synthetase domains (NRPS). The NRPS thiolation motif is shown as a black bar. Grey circles represent polyketide synthase domains (PKS). The stepwise incorporation of the individual microcystin moieties is indicated in red. KS, h-ketoacyl synthase; AT, acyltransferase; ACP, acyl carrier protein; KR, ketoacyl reductase; DH, dehydratase; CM, C-methyltransferase; OM; O-methyltransferase, A, aminoacyl adenylation; C, condensation; AMT, aminotransferase. Precursors are indicated below the arrows. From Dittmann & Börner, 2005.

The genes encoding the microcystin biosynthesis are organised in a bidirectional operon with *mcy*ABC transcribed in one direction and *mcy*D-J in the opposite direction. In addition to the biosynthesis genes a putative ABC-transporter McyH is encoded, which may facilitate the proposed export of microcystin (Pearson *et al.*, 2004).

1.1.2.2 FUNCTION OF MICROCYSTINS

The synthesis of microcystin involves the expression of a large enzyme complex and 48 single reaction steps (Tillett *et al.*, 2000), which implies that it is of significant benefit to the orgnanism justifying this great effort. Several studies tried to answer the question of microcystin function, but no primary function could be assigned beyond doubt so far. A variety of studies have analysed the factors that stimulate the production of microcystin. Multiple impact like nutrients in particular phosphorus, nitrogen and inorganic carbon and environmental factors like light and pH were shown to influence the microcystin production (Downing *et al.*, 2005; Downing *et al.*,

2005; Hesse and Kohl, 2001; Kaebernick *et al.*, 2000; Lee *et al.*, 2000; Long *et al.*, 2001; Lyck *et al.*, 1996; Song *et al.*, 1998; Watanabe and Oishi, 1985; Wiedner *et al.*, 2003). However, none of these parameters could be identified as the dominating criterion. Nevertheless, most studies found an influence of the growth phase and microcystin production was highest during exponential growth. Thus the function could not be inferred from the knowledge of parameters driving microcystin production.

In the following the most discussed hypotheses shall be introduced. Different modes of microcystin action were suggested which can be classified as interspecies, intraspecies or intracellular function.

1.1.2.2.1 INTERSPECIES FUNCTION

Due to its toxicity microcystin was considered a protection against ingestion by *Daphnia galeata* (Christoffersen, 1996). Nevertheless, other studies contradict by pointing out that *Daphnia* cannot distinguish between toxic and nontoxic strains (Rohrlack *et al.*, 2001) and beyond this they are not able to ingest the large colonies usually formed by microcystin producers. Additionally, the feeding inhibition observed on *Daphnia* fed with toxic and non-toxic *Microcystis* strains could not be attributed to microcystin (Kaebernick *et al.*, 2001). It was inferred that reduced growth of *Daphnia* fed with non-toxic strains compared to a *Scenedesmus* diet can be attributed to general avoidance of *Microcystis* (Lurling, 2003).

Furthermore, allelopathic interactions among cyanobacteria and other lake species such as *Daphnia*, green algae or dinoflagellates are discussed. However, the significance of these laboratory experiments is unclear, because the microcystin concentrations used exceeded those that can be found in the natural environment. Allelopathic interactions between toxic and non-toxic strains were disproven by competition experiments (Kardinaal *et al.*, 2007; Takeya *et al.*, 2004)

1.1.2.2.2 INTRASPECIES FUNCTION

Some reports propose a role of microcystin as an infochemical, similar to that of homoserine lactones in quorum sensing. Two proteins, MrpA and MrpB (**m**icrocystin-**r**elated **p**rotein), that exhibit significant similarity to the quorum-sensing regulated proteins RhiA and RhiB from *Rhizobium leguminosarum* were shown to be less expressed in the *mcy*B mutant. Thus

accumulation of microcystin in the surrounding media might trigger a coordinated response of the whole *M. aeruginosa* community (Dittmann *et al.*, 2001). Others report that microcystin released by cell lysis stimulates expression of the microcystin synthesis proteins and propose that the enhanced production of microcystin enhances the fitness of the remaining cells (Schatz *et al.*, 2007).

1.1.2.2.3 INTRACELLULAR FUNCTIONS

The major part of microcystin is usually cell-bound and not released to the medium. Some studies showed that microcystin is located at distinct sites within the cell. Immunogold localisation experiments (Gerbersdorf, 2006; Young *et al.*, 2005) found microcystin to be bound to thylakoids, phosphate bodies and carboxysomes. A thylakoid localisation was further supported by the isolation of these (Jüttner and Luthi, 2008).

Zilliges compared the proteome of *M. aeruginosa* PCC 7806 wild type and microcystin-deficient Δ*mcy*B mutant by 2D gel electrophoresis and could show that differences are often found in proteins related to CO_2 fixation (Zilliges *et al.*, 2008). Most proteins of the Calvin cycle including RubisCO were differentially expressed. Furthermore, the direct binding of microcystin to RubisCO was proposed and confirmed *in vitro*. The author postulated a role of microcystin in redox-regulated processes similar to that of thioredoxins. The influence of carbon availability on microcystin production was previously described (Jähnichen *et al.*, 2001). As mentioned above, carbon fixation and accumulation of intracellular carbohydrates are key factors of buoyancy regulation, as is the colony size. Two extraccellular proteins were identified that might contribute to colony formation, which both seemed to be affected by microcystin. These proteins – microvirin (Mvn) and MrpC – will be introduced in the following.

1.2 Microvirin

Microvirin (Mvn) was identified during the analysis of the partial genome sequence of *M. aeruginosa* PCC 7806 available at that time (Kehr, 2003). The aim was to identify genes that might be related to microcystin. The microvirin gene (*mvn*) was chosen because it is located downstream of a 84 bp sequence (*mcy*-Box, **Fig. 5**), which displayed 84% identity to a nucleotide region that overlaps one of the transcriptional start points of the *mcyA* gene (Kaebernick *et al.*, 2002). The deduced protein sequence was used as query in a BLAST search

(http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the only protein exhibiting significant similarity (above 20%) in 2003 was cyanovirin-N (see chapter 1.4.8). Thus the protein was named microvirin. Cyanovirin-N (CV-N) is a oligomannose-binding lectin. Lectins bind oligosaccharides and are usually involved in cell-cell recognition and attachment (see chapter 1.4).

In order to analyse the expression of Mvn in *M. aerugiosa* PCC 7806 and its microcystin-deficient mutants, the protein was expressed heterologously and an antibody was raised against the purified recombinant protein. It was shown that the expression increased at higher culture densities. Additionally, the expression was delayed in the Δ*mcy*B mutant providing first evidence of an influence of microcystin on Mvn.
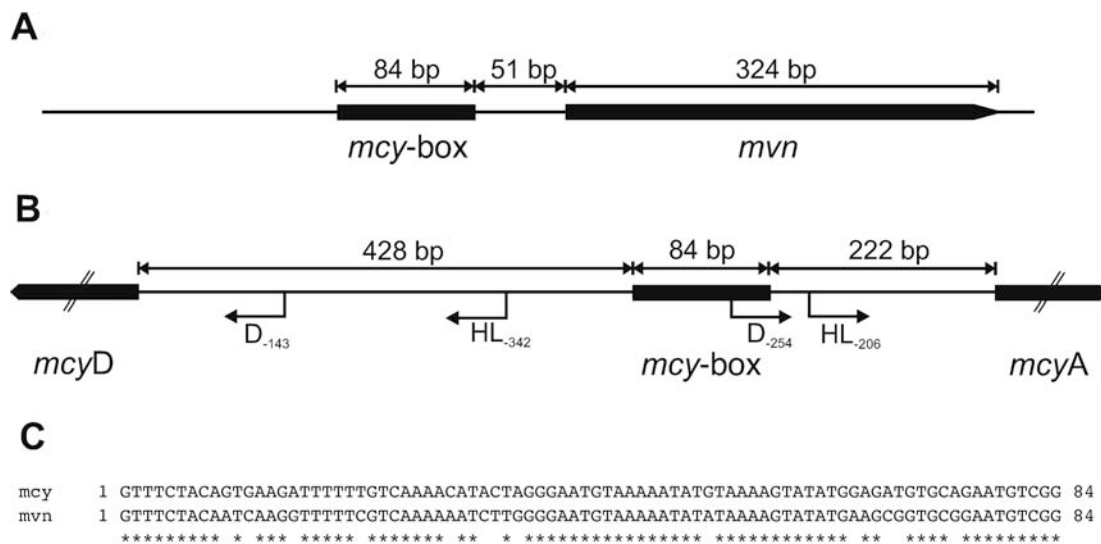
Fig. 5: Schematic representation of the conserved nucleotide box (*mcy-box*) upstream of A) the microvirin (*mvn*) gene and of B) microcystin biosynthesis genes (*mcy*). Arrows indicate distances from translational and transcriptional start sites. C) Sequence comparision of the nucleotide regions adjacent to the *mvn* and *mcy* genes, respectively.

It was proposed that Mvn might play a role in microcystin modulated colony formation in *M. aeruginosa*. A mutant was generated by insertional mutagenesis, but initial investigations did not reveal a peculiar phenotype. However, the laboratory cultured strain *M. aerugniosa* PCC 7806 is unable to form colonies anymore and therefore the hypothesis could not be proven by a knockout phenotype.

1.3 MrpC

In addition to Mvn a second extracellular protein called MrpC that is strongly affected by microcystin was found. The MrpC protein was identified by Zilliges *et al*. and characterised as the most abundant extracellular protein from the supernatant of a *M. aeruginosa* PCC 7806 culture (Zilliges *et al.*, 2008). The expression of MrpC was strongly increased in the microcystin-deficient Δ*mcy*B mutant compared to the wild type under all conditions tested. The distribution of the MrpC encoding gene showed a correlation with a microcystin genotype and together with the expression studies the results implied a strong relation to microcystin. Further biochemical characterisation proved MrpC to be the target of *O*-glycosylation, presumably by a glycosyltransferase encoded downstream of the *mrp*C. Presence of a transit peptide indicated a transmembrane localisation via the Sec pathway. Immunofluorescence microscopy confirmed the increased abundance of the protein in the Δ*mcy*B mutant and showed that MrpC covers the whole cell surface of *M. aeruginosa* (**Fig. 6**). Additionally, MrpC was shown to form connections between individual cells of the Δ*mcy*B mutant. Therefore, the protein was implicated in microcystin dependent colony formation in *M. aeruginosa* PCC 7806.
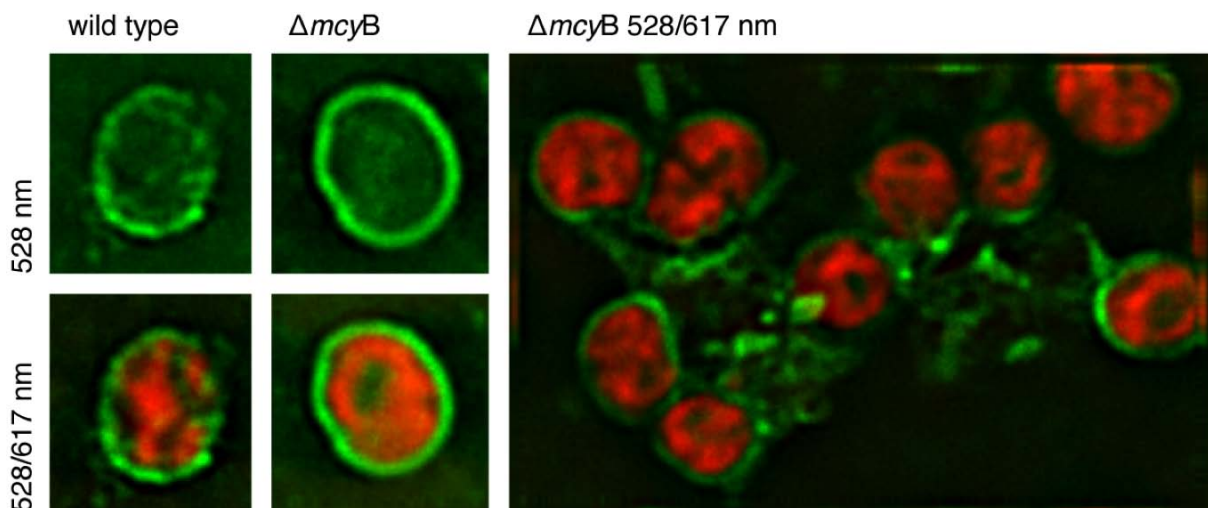
Fig. 6: Immunofluorescence micrographs of the Δ*mcy*B mutant and WT strains of *M. aeruginosa* PCC 7806 obtained by using the antibody against MrpC and a FITC-coupled secondary antibody. The green fluorescence represents the MrpC protein, which is distributed over the cell surface of *M. aerugiosa* in a sphere-like manner. The protein is much more abundant in the mutant and seems to establish connection between individual cells. Images were recorded showing only the antibody fluorescence (528 nm) or additionally the chlorophyll autofluorescence (528/617 nm). From Zilliges *et al*., 2008.

1.4 Lectins

Lectins are a heterogeneous group of proteins that reversibly and with high specificity bind to carbohydrates. Lectins are found in virtually all organisms ranging from bacteria to fungi and to plants and animals. The first description of a lectin refers to Stillmark (1888) who observed the erythrocyte-agglutinating effect of a protein isolated from the castor tree (*Ricinus communis*) which he named ricin. Until the second half of the 20th century lectins were called hemagglutinins. Various lectins showed different hemagglutinating activity on red blood cells from different animals, an observation that led Karl Landsteiner to discover the human A, B, 0 blood type system in 1900. In 1954 Boyd and Shapleigh introduced the term "lectin" derived form the Latin word "legere" (to choose) to summarise plant agglutinins. This concept was later extended to embrace all sugar-specific agglutinins of nonimmune origin, irrespective of source and blood type specificity (Sharon and Lis, 1972).

Although lectins from different organisms display no or only little similarity on the sequence level, they share some important structural features. A striking feature shared by many lectins is the proportion of β-sheets in the tertiary structure (Sharon and Lis, 1990).

Lectins mediate biological recognition by detecting specific carbohydrate moieties inside cells, on cell surfaces and physiological fluids (Sharon and Lis, 1989). High specificity is achieved by storing information in carbohydrates. In contrast to amino acids or nucleotides, carbohydrates bear a high coding capacity. Monosaccharides contain several approximately chemically equivalent sites for chain elongation. Chemically distinct compounds can be generated by linking one unit at the reducing end to different hydroxy groups of the second unit, also allowing branched structures. Furthermore, the occurrence of different anomeric variants at each linkage has to be taken into account. With a set of 20 different monosaccharide building blocks as many as 1.44×10^{15} hexasaccharides are possible regarding the considerations mentioned above. The number of possible permutations for a hexapeptide from a set of 20 amino acids is only 6.4×10^7 (Gabius *et al.*, 2004). The level of diversity can be further extended by the introduction of substituents like sulfation and epimerisation of D- to L-forms.

It was estimated that over the half of all proteins occuring in nature is glycosylated (Apweiler *et al.*, 1999) and additionally cell membranes are decorated with glycolipids. This emphasises the importance of glycoproteins and lectins that are involved in numerous and diverse biological processes (Gabius *et al.*, 2002). Indeed glycosylation is very common in bacteria (Hitchen and Dell, 2006) and defects in glycosylation led to impaired pathogenicity and attachment of

mucosal pathogens to host cells (Szymanski and Wren, 2005). The majority of glycosylated bacterial proteins identified in pathogens are implicated in interactions with the host (Benz and Schmidt, 2002).

1.4.1 MECHANISM OF CARBOHYDRATE BINDING

The carbohydrate binding sites of lectins exhibit a relatively low affinity to monosaccharides with dissociation constants in the millimolar range, whereas the oligosaccharides are bound with nanomolar affinity. This arises from the structure of the binding pocket which is a rather shallow depression on the protein surface in contrast to e.g. the sugar-binding bacterial periplasmic receptors for glucose or galactose that bind the ligand in the interior of the protein (Lis and Sharon, 1998). Lectins interact with carbohydrates primarily through hydrogen bonds and hydrophobic interactions. Charge-charge interactions are not relevant because most saccharides are uncharged. The hydrogen bonds are formed between hydroxyl groups of the carbohydrate which interact with amino and hydroxyl groups and oxygen atoms of the protein. The hydroxyl groups of sugars enable cooperative hydrogen bonding where an OH group can act as a donor of one hydrogen bond and an acceptor of two hydrogen bonds simultaneously (Weis and Drickamer, 1996).

Although carbohydrates are highly polar, they have significant nonpolar patches formed by aliphatic protons and carbons at the epimeric centers. These patches interact with aromatic residues like tryptophan or phenylalanine in the protein. In addition, several classes of lectins require divalent cations like Ca^{2+} or Mn^{2+} to be functional. Two modes of interaction, direct and indirect, were described. Direct interactions occur in C-type lectins where a Ca^{2+} is required to form direct coordination bonds with the sugar ligand. However, indirect interactions are given if a cation interacts only with amino acid side chains to stabilise the interaction with the ligand.

Most lectins contain two or more carbohydrate binding sites, which is the reason for their hemagglutinating activity. One lectin can bind to at least two carbohydrate moieties on different cells, which results in the precipitation of the cells. Additionally, many lectins form multimers, which further increases the number of binding sites compared to the monomer. The resulting multivalency employed by many if not all lectins is considered to substantially enhance the selectivity and affinity of lectin interactions (Rini, 1995).

1.4.2 FUNCTIONS OF LECTINS

Lectins are involved in many processes in and between organisms and although they play diverse roles the recognition of specific carbohydrates is a common prerequisite to accomplish their function. Biological processes that require a specific targeting are e.g. defense, innate immunity, symbiosis or glycoprotein trafficking (**Tab. 1**, (Sharon and Lis, 2004)). In addition to the examples listed in **Tab. 1** some well-characterised lectins from plants, animals and bacteria are introduced in the following and known cyanobacterial lectins are described.

Tab. 1: Function of lectins. Adapted from Sharon and Lis, 2004.

	Lectin	Role in
Microorganisms	Amoeba	Infection
	Bacteria	Infection
	Infuenza virus	Infection
Plants	Various	Defence
	Legumes	Symbiosis
Animals	Calnexin, calreticulin, ERGIC-53	Control of glycoprotein biosynthesis
	Collectins	Innate immunity
	Dectin-1	Innate immunity
	Galectins	Regulation of cell growth and apoptosis; regulation of the cell cycle; modulation of cell-cell and cell-substratum interactions
	Macrophage mannose receptor	Innate immunity; clearance of sulfated glycoprotein hormones
	Man-6-P receptors	Targeting of lysosomal enzymes
	L-selectin	Lymphocyte homing
	E- and P-selectin	Leukocyte trafficking to sites of inflammation
	Siglecs	Cell-cell interactions in the immune and neural system
	Spermadhesin	Sperm-egg interaction

1.4.3 ANIMAL LECTINS

The examples listed in **Tab. 1** show that lectins have manifold functions in mammals, but the majority are innate immune molecules. Many lectin-like innate immune proteins and receptors that interact with bacteria are known today (Palaniyar *et al.*, 2002). The pulmanory surfactant proteins SP-A and SP-D are C-type (Ca^{2+}-dependent) lectins that maintain surfactant

homeostasis were additionally shown to be important host defense components (Holmskov *et al.*, 2003; Wright, 2005). Both proteins bind to bacteria causing agglutination and thus hinder their entrance into the host cell and dissemination. In addition they enhance the phagocytosis by macrophages. Several pathogens, among them *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Haemophilus influenzae* and yeast and fungi are recognised (Kishore *et al.*, 2006). Another important lectin in the innate immunity is the mannose-binding lectin (MBL), which binds to a large number of pathogens. MBL was shown to activate a novel pathway of complements (lectin pathway) that is independent from antibodies or C1 protease (Dommett *et al.*, 2006).

The lectins calnexin and calreticulin localised in the endoplasmic reticulum (ER) are involved in glycoprotein folding. They serve as molecular chaperones, recognising newly synthesised and glycosylated polypeptide chains and preventing aggregation and export of incompletely folded proteins from the ER (Helenius and Aebi, 2004).

Lectins can also play a role in symbioses between animals and bacteria. The marine nematode *Laxus oneistus* secretes a mannose-specific lectin onto the posterior region of its cuticle to facilitate attachment of its sulfur-oxidising bacterial symbiont (Bulgheresi *et al.*, 2006).

1.4.4 PLANT LECTINS

Legume lectins that are involved in *Rhizobia*-legume symbiosis are the best-investigated plant lectins. According to the lectin recognition hypothesis proposed by Hamblin and Kent (1973), Bohlool and Schmidt (1974), Dazzo and Hubbell (1975) and reviewed by Hirsch (Hirsch, 1999), legume lectins mediate the specificity between the legume host and the *Rhizobium* symbiont. The bacteria expose carbohydrates on their cell surface, e.g. lipopolysaccharides, capsular polysaccharides or acidic exopolysaccharides that can be bound by plant lectins. Several studies showed that mutants in the production of these polysaccharides are often unable to establish symbiosis.

Another function frequently described for plant lectins is the defense against pathogens like fungi, insects, and bacteria. Several chitin-binding lectins (chitin is a long-chain polymer of *N*-acetylglucosamine) and chimeric proteins comprising a chitin-binding domain as well as a chitinase domain exhibit a protective activity against fungi (Peumans and Van Damme, 1995).

Many plant lectins are toxic to insects and mammals and are stored in vacuoles in plant seeds or adult plants. Upon ingestion of the plant by herbivores they are released and deploy their toxicity. The wheat germ agglutinin (WGA) from wheat (*Triticum aestivum*) e.g. is toxic to weevils, and the common bean's phytohemagglutinin (PHA) is toxic to mammals (Chrispeels and Raikhel, 1991). It was shown that PHA causes lesions in the intestinal mucosa.

1.4.5 BACTERIAL LECTINS

Bacterial lectins are often involved in infection and mediate the initial step of host-cell-recognition and attachment. The human pathogen *Helicobacter pylori* attaches to human salvary glycoproteins (Prakobphol *et al.*, 2005) and expresses several lectins and lectin-like proteins (Hynes *et al.*, 2003).

A group of chimeric proteins carrying a lectin domain includes the AB–toxins of Enterobacteria. These toxins comprise two domains, the A and B domain, which are made of two polypeptide chains usually linked by two disulphide bonds. The B domain (binding domain) is a lectin and responsible for receptor recognition, whereas the A domain (activity domain) causes the cytotoxic effects. One group of AB-toxins are the Shiga and Shiga-like toxins from *Shigella sp.* and *E. coli*. They bind to the glycolipid Gb3 on the host-cell-surface and are then internalised by endocytosis (Sandvig, 2001). Glycolipids are also the attachment site for a *Porphyromonas gingivalis* adhesin (Hellstrom *et al.*, 2004).

Bacterial lectins also play an important role in communal behaviour. The dental pathogen *Streptococcus mutans* expresses several secreted glucan-binding proteins (Gbp) during biofilm formation. Deletion of the respective genes significantly altered the physical properties of the biofilm produced. Especially a reduction in thickness of the biofilm and an impaired ability of cell-aggregation was observed (Banas *et al.*, 2007; Lynch *et al.*, 2007).

The involvement of lectins in biofilm formation was also described for the biofilm model organism *Pseudomonas aeruginosa*. The quorum-sensing regulated LecA lectin was detected and the mutants showed a reduced surface coverage and no evidence of microcolony formation. Furthermore, the biofilm development was impaired by the preincubation with hydrophobic galactosides, which are the type of sugars specifically recognised by LecA (Diggle *et al.*, 2006).

1.4.6 CYANOBACTERIAL LECTINS

Very little is known about lectins and their role in cyanobacteria. In recent years few lectins from cyanobacteria were isolated starting with the anti-HIV protein cyanovirin-N from *Nostoc ellipsosporum* (Gustafson *et al.*, 1997) (see chapter 1.4.8). The developmental potential of cyanovirin-N as anti-HIV drug led to screenings for substances with similar activity in cyanobacteria. Hence, lectins have been isolated from different cyanobacterial genera that all exhibit a more or less strong affinity to oligomannose, the same oligosaccharide that is recognised by cyanovirin-N (Ziółkowska and Wlodawer, 2006). Namely these are the *Oscillatoria agardhii* agglutinin (OAA) and scytovirin from *Scytonema varium* (Bokesch *et al.*, 2003; McFeeters *et al.*, 2007; Sato *et al.*, 2007). Both proteins comprise two domains that show sequence homology to each other and contain internal disulphide bonds. Nevertheless, information about their *in vivo* function is lacking so far.

1.4.7 MICROCYSTIS LECTINS

Two lectins, MAL and MVL, were isolated from *Microcystis* strains. The *Microcystis viridis* lectin (MVL) agglutinates rabbit erythrocytes and hemagglutinating activity could be inhibited by yeast mannan. It consists of 113 aa and is composed of two tandemly repeated homologous domains of 54 aa. There are currently no homologous sequences to be found in the databases. Expression studies revealed that the MVL is expressed in cultures cultivated without aeration at the stationary phase. Therefore the authors consider MVL a stress protein that may be involved in adaptation to an unfavourable growing environment (Yamaguchi *et al.*, 1999). MAL has been isolated from *M. aeruginosa* M228. The 55.2 kDa protein comprises three tandemly repeated homologous domains of 61 aa which show partial similarity to the α-amylase from *Clostridium beijerinckii*. The genes adjacent to the *mal* gene display homology to a cytochrome P-450 and a polyketide synthase. The hemagglutinating activity of MAL could be inhibited by *N*-acetyl-D-galactoseamine and lactose. The expression of the protein was highest at low temperature (15°C) and low light (12 μEm^{-2}) (Jimbo *et al.*, 2000; Yamaguchi *et al.*, 1998; Yamaguchi *et al.*, 2000).

1.4.8 CYANOVIRIN-N

Cyanovirin-N (CV-N) is a lectin isolated from the filamtenous cyanobacterium *Nostoc ellipsosporum* during a screening for compounds with anti-HIV activity. It is a small 11 kDa lectin with specifity for α1,2-mannan. The protein was found to bind to such carbohydrate epitopes present in the gp120 envelope protein of the HI-virus. Through binding to gp120 CV-N disturbs the interaction with the cell surface receptor CD4 and thus prevents the virus from infecting the host cell (Bewley and Otero-Quintero, 2001; Boyd *et al.*, 1997; Chang and Bewley, 2002; Dey *et al.*, 2000; Esser *et al.*, 1999; Mori and Boyd, 2001; O'Keefe *et al.*, 2000; Shenoy *et al.*, 2001). Due to its potential use as an HIV therapeutic detailed analysis focussed on the biochemical and structural properties of CV-N are available in the literature, but yet no information on the role of this lectin in *Nostoc ellipsosporum* exists. The most important features will be outlined in the following.

CV-N comprises two domains that share 32% sequence identity to each other and that may result from a duplication event during evolution. Several structures were obtained from cyanovirin-N (**Fig. 7**) by NMR as well as by X-ray crystallography (Bewley, 2001; Bewley *et al.*, 2002; Bewley and Otero-Quintero, 2001; Botos *et al.*, 2002; Botos *et al.*, 2002; Fromme *et al.*, 2007; Sandstrom *et al.*, 2004; Shenoy *et al.*, 2002; Yang *et al.*). The protein exists as a monomer in solution and is made of two distinct symmetric domains A and B that are formed by strand exchange of the sequence repeats. This means that domain A is made of residues 1-39 and 90-101 and domain B is formed by residues 39-90. The protein contains four cysteine residues and an intra-domain disulphide bond is present in each domain. The whole structure is dominated by anti-parallel β-sheets (Bewley *et al.*, 1998). The crystallised protein was shown to form domain-swapped dimers (**Fig. 7**A-C), in which domain A of one monomer interacts with domain B of the second monomer and vice versa (Yang *et al.*, 1999). Mutants were created that formed stable dimers in solution (Han *et al.*, 2002).
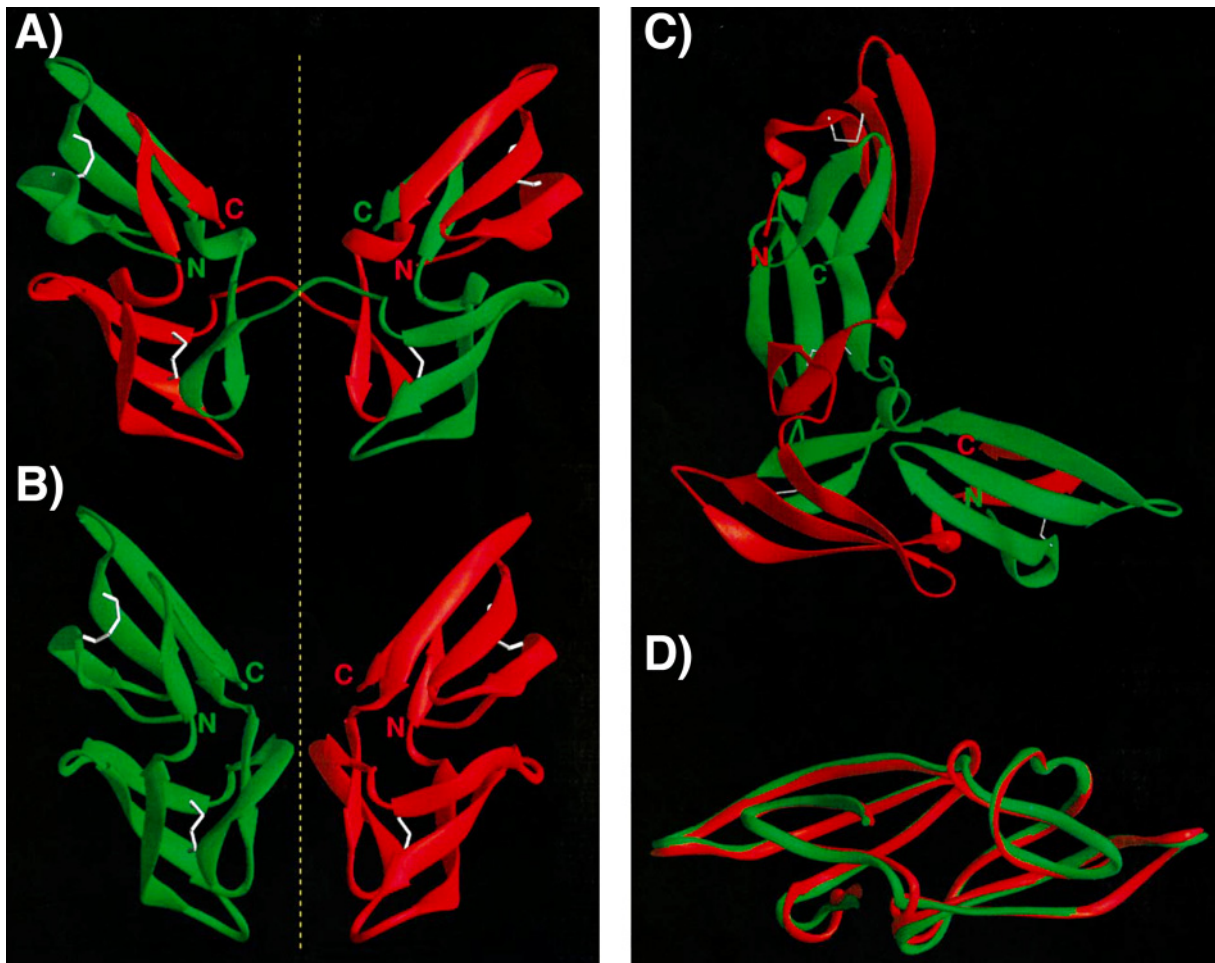
Fig. 7: Crystal structure on CV-N: A-C) Different views of the domain swapped dimer of CV-N. Individual monomers are shown in green and red. The yellow dashed line indicates the symmetry. D) Structure of a CV-N monomer. The high proportion of anti-parallel β-sheets and the pronounced symmetry of multimers are frequently found in lectins. From Yang *et al.*, 1999.

1.4.8.1 CYANOVIRIN-N DOMAINS

In recent years several cyanovirin-N-like proteins have been identified, mostly in filamentous ascomycetes. The European Bioinformatics Instiute (EBI) contains an entry in its InterPro database under the accesion number IPR011058 (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR011058) that lists all proteins containing a CV-N domain. Apparently some of these proteins contain two CV-N domains, while very few only contain half a domain that represents just one of the internal repeats of cyanovirin-N. Percudani and coworkers analysed the CV-N homologues concerning their secondary structure and their evolutionary relationship. The major findings will be summarised in the following (Percudani *et al.*, 2005). Several representatives of the CV-N domain family proteins contain additional conserved domains. Some members of this group possess a peptidogycan and chitin-binding

LysM domain that is inserted into the hinge region between the two CV-N repeats. Furthermore, signal-peptides for membrane translocation were identified in the CV-N homologues of the fern *Ceratopteris richardii* indicating an extracellular function. The cysteine residues involved in disulphide bond formation in cyanovirin-N are conserved among the homologues that posses a signal peptide while they are replaced in most of the intracellular proteins.

The overall tertiary structure of the CV-N domain family members seems to be conserved across the whole family, because hydrophobic residues crucial for protein folding are conserved, while e.g. the amino acids that form the carbohydrate binding pocket are rather variable.

The rather patchy organism distribution of the CV-N domain implies that the organisms acquirred the protein after the separation of the lineages by horizontal gene transfer (HGT) events. However, the internally repeated structure of cyanovirin-N arose from a unique ancient duplication.

1.5 Aim of this Work

Many reports published recently suggest functions for microcystin that do not focus on its toxicity but rather postulate a physiological relevance of the peptide. Proteomic studies have shown a major impact of the absence of microcystin on the abundance of a number of proteins. Among these, the extracellular glycoprotein MrpC as well as glycosyltransferases were identified, which proposed an influence of microcystin on cell surface composition.

The objective of this study is the characterisation of microvirin (Mvn), which previously was shown to be influenced by the presence of microcystin. Homology based prediction implied the secretion of Mvn and a carbohydrate binding capability. Thus the subcellular localisation should be examined and the carbohydrate binding activity and specificity should be elucidated. In order to confirm the proposed functional relationship of microvirin and microcystin further, the distribution of the mvn gene among laboratory and field strains of *Microcystis* should be examined.

Several publications have shown the ability of microcystin to bind to proteins. Therefore, it has to be tested if the relationship of Mvn and microcytin becomes manifested in an interaction of both. Thus, binding of microcystin to the heterologously expressed Mvn should be tested. If a

binding of microcystin is observed the conditions that promote the binding as well as the mechanism should be investigated.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 BACTERIAL STRAINS

2.1.1.1 MICROCYSTIS AERUGINOSA

The strain *Microcystis aeruginosa* PCC 7806 used in this study was obtained from the "Pasteur Culture Collection" (Paris, France). The mutants used in this study were derived from this strain. *M. aeruginosa* Δ*mcy*B (Dittmann *et al.*, 1997) and Δ*mcy*H mutants (Pearson *et al.*, 2004) generated by insertional mutagenses are deficient in microcystin production. The Δ*mvn* mutant was produced by disruption of the *mvn* gene (Kehr, 2003). All mutants were selected on chloramphenicol.

Additionally, a set of *M. aeruginosa* strains was used to investigate the distribution of the mvn gene within the species (**Tab. 2**).

Tab. 2: List of *M. aeruginosa* strains used to investigate the distribution of the mvn gene.

M. aeruginosa strain	Source
PCC 7005 PCC 7820 PCC 7941 PCC 9354 PCC 9355 PCC 9432 PCC 9622 PCC 9624 PCC 9701 PCC 9717 PCC 9804 PCC 9805 PCC 9806 PCC 9807 PCC 9808 PCC 9809 PCC 9812 PCC 9905 PCC 100-24 PCC 100-25	"Pasteur Culture Collection", Paris, France
HUB 5.3	Humboldt University, Berlin, Germany
NIES 44 NIES 89 NIES 100 NIES 104 NIES 299	National Institute for Envrionmental Studies, Tsukuba, Japan
UWOCC CBS UWOCC MRC UWOCC MRD	University of Wisconsin at OshKosh Culture Collection, Madison, USA

2.1.1.2 OTHER CYANOBACTERIA

Nostoc punctiforme ATCC 29133 from the "American Tissue and Culture Collection" and *Synechocystis* PCC 6803 from the "Pasteur Culture Collection" were used as controls in immunofluorescence microscopy.

2.1.1.3 ESCHERICHIA COLI

For cloning purposes the strain *E. coli* XL-1 blue (Stratagene) was used, heterologous expression was performed in the strain *E. coli* BL21 (DE3) (Novagen).

2.1.2 KITS

Bio-Rad Protein Assay	Biorad
FluoroTag™FITC Conjugation Kit	Sigma-Aldrich
Jetsorb „Gel Extraction Kit"	Genomed
Nickel-NTA-Superflow	Qiagen
PCR Purification Kit	Qiagen
Plasmid Mini Prep	Qiagen
Taq DNA-Polymerase PCR Kit	Qiagen
SuperSignal West Pico	Pierce

2.1.3 CHEMICALS

2-mercaptoethanol	C. Roth
Acetone	C. Roth
Acrylamide/Bisacrylamide (37.5:1)	C. Roth
Acetonitril "HPLC Gradient Grade"	C. Roth
Agar, washed	Difco
Agarose	Biozym Diagnostik
Ampicillin	Roche Diagnostics
APS	C. Roth
Bacto-Agar	Difco

Bacto-Trypton	Difco
Boric acid	C. Roth
Bovine serum albumin, fraction V	C. Roth
Bromphenol blue	SERVA Feinbiochemika
Chloramphenicol	Roche Diagnostics
Coomassie staining "Roti-Blue"	C. Roth
Dithiothreitol	C. Roth
dNTP	MBI Fermentas
DTNB	C. Roth
EDTA	C. Roth
Ethanol	C. Roth
Ethidium bromide	C. Roth
Formaldehyde 37%	C. Roth
GelCode Blue Stain Reagent	Pierce
Glycerol	C. Roth
Glycine	C. Roth
HEPES	Amersham Pharmacia
Hydrochloric acid	C. Roth
IPTG	C. Roth
Isopropanol	C. Roth
Magnesium chloride	C. Roth
Methanol	C. Roth
PMSF	Serva
Potassium chloride	C. Roth
N-Propylgallate	C. Roth
Sodium acetate	C. Roth
Sodium chloride	C. Roth
Sodium dihydrogenphosphate	C. Roth
Sodium dodecylsulfate (SDS)	SERVA Feinbiochemika
Sodium hydrogenphosphate	C. Roth
Sodium hydroxide	C. Roth
TEMED	C. Roth

Tris	C. Roth
Tween 20	Sigma
Urea	ICN Biochemicals
X-Gal	C. Roth
Yeast extract	Difco

2.1.4 ENZYMES

Lysozyme	GERMED
Restriction Endonucleases	MBI Fermentas
	New England Biolabs
T4-DNA-Ligase	MBI Fermentas
Taq-Polymerase	Qiagen

2.1.5 ANTIBODIES

The following antibodies were used in this study (The used titer is given for all antibodies. In some cases antibodies were applied in a different titer for immunoblotting and immunofluorescence microscopy (IFM).

Tab. 3: Antibodies used in this study.

Antibody	Antigen	Source	Titer	Reference
Primary antibodies				
Anti-Microcystin	Adda moiety of microcystin from *M. aeruginosa*	Mouse, polyclonal	1:10,000	Alexis
Anti-MrpC	MrpC from *M.aeruginosa*	Guinea pig, polyclonal	1:5000 or 1:500	Zilliges *et al.*, 2008
Anti-Mvn	Microvirin from *M.aeruginosa*	Rabbit, polyclonal	1:10,000 or 1:500 (IFM)	Kehr, 2003
Anti-Poly-Histidin	Poly-Histidin	Mouse, monoclonal	1:10,000	Sigma
Secondary antibodies				
Anti-Guinea Pig IgG FITC conjugate	Mouse IgG	Goat	1:100 (IFM)	Sigma
Anti-Mouse IgG Horseradish Peroxidase conjugate	Mouse IgG	Sheep	1:10,000	Amersham Pharmacia
Anti-Rabbit IgG FITC conjugate	Rabbit IgG	Goat	1:100 (IFM)	Sigma
Anti-Rabbit IgG Horseradish Peroxidase conjugate	Rabbit IgG	Goat	1:10,000	Sigma

2.1.6 NUCLEIC ACIDS

2.1.6.1 PLASMIDS

pDrive   Qiagen

pet15b   Novagen

2.1.6.2 PRIMER

Name	Sequence	Comment
mvn_fw	ATGCCTAATTTTTCGCACACTTGTAG	Screening, amplification and sequencing of mvn genes from various *Microcystis* strains
mvn_rv	TCCAATTTCCAGTTGGCTGTCGTT	
mcyE_fw	TTCCCCTTAACTCGACATGG	Detection of *mcy* genotypes among *Microcystis* strains. The primers were deduced from conserved regions in the *mcy*E gene.
mcyE_rv	TAAAGTCGCCAATCCAGCAA	

2.1.7 FILTERS AND MEMBRANES

3MM Filter-Paper	Whatman
Hyperfilm MP X-ray detection film	Amersham Pharmacia
PVDF-Blotting-Membrane Hybond C	Amersham Pharmacia

2.1.8 LIST OF MANUFACTURERS

Company	Based in
Agilent Technologies	Darmstadt, Germany
Ambion, Inc.	Austin, USA
Amersham Biosciences Europe GmbH	Freiburg, Germany
Applied Biosystems	Weiterstadt, Germany
Applied Precision	Issaquah, USA
Biolabs	Frankfurt am Main, Germany
Bio-Rad Laboratories	Richmond, USA
Boehringer GmbH	Mannheim, Germany
C. Roth GmbH & Co. KG	Karlsruhe, Germany
Difco	Detroit, USA
DuPont de Nemours GmbH	Bad Homburg, Germany
Eppendorf	Hamburg, Germany
Eurogentech	Seraing, Belgien
Fluka-Biochemika	Steinheim, Germany
GERMED	Berlin, Germany

Gibco/ BRL Life Technologies	New York, USA
Heraeus	Hanau, Germany
ICN Biochemicals/ MP Biomedicals	Irvine, USA
Invitrogen GmbH	Karlsruhe, Germany
MBI Fermentas GmbH	St. Leon-Rot, Germany
Merck	Darmstadt, Germany
Millipore Cooperation	Bedford, USA
New England Biolabs	Schwalbach, Germany
Novagen	Nottingham, UK
PerkinElmer Instruments	Shelton, USA
Philips Instruments	Eindhoven, Netherlands
Promega Cooperation	Madison, USA
Qiagen	Hilden, Germany
Roche Diagnostics GmbH	Mannheim, Germany
Schleicher & Schüll	Dassel, Germany
Serva Feinbiochemika & Co. KG	Heidelberg, Germany
Shimadzu	Kyoto, Japan
Sigma Chemical Company	St. Louis, USA
Waters	Eschborn, Germany
Whatman Paper Ldt.	Maidstone, UK
Zeiss	Jena, Germany

2.2 Methods

2.2.1 MICROBIOLOGICAL METHODS

2.2.1.1 CULTIVATION OF CYANOBACTERIA

Cyanobacteria were cultivated in BG11-medium in Erlenmeyer flasks or on plates containing BG11-medium and 0.7% agar (Rippka *et al.*, 1979). Stock cultures were grown in liquid at 23°C and continuous illumination (~20-30 $\mu Em^{-2}s^{-1}$). Exposure experiments were conducted using aliquots of a preculture grown at 16 $\mu Em^{-2}s^{-1}$ and constant aeration in a batch vessel. The culture aliquots were transferred to sterile batch vessels and subjected to the desired light conditions under constant aeration for two hours. The following light conditions were applied: High light (70 $\mu Em^{-2}s^{-1}$), low light (16 $\mu Em^{-2}s^{-1}$) and darkness. External microcystin was added in some case (for details refer to the results section).

2.2.1.2 CULTIVATION OF ESCHERICHIA COLI

Escherichia coli cells were cultivated under standard conditions either in liquid LB medium or on LB agar in petri dishes (Sambrook and Russel, 2001). Cultures for preparation of plasmid vector DNA were incubated in 3-4 ml liquid LB medium at 37°C and shaking at 220 rpm. Proper antibiotics were added in the appropiate concentrations.

2.2.2 MOLECULAR BIOLOGICAL METHODS

2.2.2.1 PREPARATION OF GENOMIC DNA FROM MICROCYSTIS AERUGINOSA

The isolation of genomic DNA from *Microcystis aeruginosa* was performed as described previously (Hisbergues *et al.*, 2003). Cyanobacterial cells were harvested by centrifugation (4000 x g, 10 min) and the pellet was washed twice in TE-buffer. The pellet was resuspended in 0.5 ml TES-buffer and incubated on ice for 1 h. Afterwards lysozyme was added to a final concentration of 2 mg/ml and the cells were incubated at 37°C in a waterbath for 1 h. Then SDS (2% final concentration) and ProteinaseK (50 μg/ml final concentration) were added and the mixture was incubated at 50°C for 2-3 h. Following one volume of phenole/chloroform (1:1) was added, the tubes were shaken and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a fresh tube and the extraction step was repeated. After centrifugation the

supernatant was mixed with chloroform/isoamylalcohol (24:1) and centrifuged as before. The upper aqueous phase was mixed with 0.7 vol isopropanol to precipitate the DNA and centrifuged at 13,000 rpm for 30 min. The resulting pellet was washed with 70% ethanol and air-dried after centrifugation. The DNA was resuspended in water or TE-buffer. Finally, 1 µl of an RNase-Mix was added and the tubes were incubated at 37°C for 1h.

TE buffer   10 mM Tris-HCl, 1 mM EDTA

TES buffer   25% w/v Saccharose, 50 mM Tris-HCl, 100 mM EDTA, pH 8.0

2.2.2.2 PREPARATION OF PLASMID DNA FROM ESCHERICHIA COLI

Plasmid DNA from *E. coli* was isolated according to the method of alkaline lysis (Sambrook and Russel, 2001). Cells from a 3 ml culture grown overnight in LB medium supplemented with the appropriate antibiotic were harvested by centrifugation and resuspended in 300 µl buffer P1. An equal volume of buffer P2 was added after incubation at RT for 5 min and the tubes were flipped 4-6 times. The cells were incubated at RT for 5 min before 300 µl of buffer P3 were added. The tubes were flipped again and kept on ice for at least 5 min. Cellular debris were pelleted by centrifugation in a table centrifuge (13,000 rpm, 4°C, 30 min). The supernatant was transferred to a fresh tube and the DNA was precipitated with 0.7 vol 2-propanol. After centrifugation (13,000 rpm, 4°C, 30 min) the pellet was washed with 70% ethanol and centrifuged again. The pellet was air-dried and resuspended in water or TE-buffer.

P1   50 mM Tris-HCl pH 8.0; 10 mM EDTA

P2   200 mM NaOH; 1% SDS

P3   3 M Potassium acetate, pH 5.0

2.2.2.3 QUANTIFICATION OF DNA

DNA concentrations were determined by measuring the absorption at 260 nm in a photometer. The absorption of one arbitrary unit corresponds to 50 µg/µl double-stranded DNA.

2.2.2.4 *AGAROSE GEL ELECTROPHORESIS OF* DNA

DNA fragments were separated on 0.5%-1.2% (w/v) agarose gels by electrophoresis (Sambrook and Russel, 2001). Ethidium bromide was added to the gels in final concentration of 0.05 µg/ml to visualise the DNA under UV illumination. The gels were run in TBE running buffer at a constant voltage of 100 V. DNA samples were mixed with DNA loading dye prior to application onto the gel. An aliquot from a *Pst*I digest of phage λ DNA was applied as size marker on each gel.

TBE running buffer	45 mM Tris-Borate, pH 8.5; 1.8 mM EDTA
5 x DNA loading dye	50 % Ficoll; 1 mM EDTA, pH 8.0; 0.05 % (w/v) Bromophenole blue; 0.05 % (w/v) Xylene cyanol

2.2.2.5 *ELUTION OF* DNA *FRAGMENTS FROM AGAROSE GELS*

DNA fragments were eluted from agarose gels using the Jetsorb "Gel Extraction Kit" (Genomed) according to the manufacturer's manual.

2.2.2.6 *POLYMERASE CHAIN REACTION*

Amplification of DNA fragments was performed using the Taq DNA-Polymerase PCR Kit (Qiagen). The web-based software "Primer3" (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design primers. Reactions were carried out in a volume of 20 µl containing the Taq-buffer, 2 µl MgCl$_2$, 10 µmol of each primer, 10 µmol dNTPs, 1 U Taq-polymerase and 1 µl template DNA. The reaction was run in a thermocycler using a suitable amplification program. Common steps were the initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, a primer annealing step at the primer specific temperature for 30 s and the sequence elongation at 72°C. The time of elongation was chosen depending on the expected length of the PCR product assuming an amplification speed of 1 kb/min. The PCR reaction was completed with a final elongation step at 72°C for 10 min.

Different template DNA was used depending on the particular analysis. Purified genomic DNA was applied in a final concentration of 50-200 ng while plasmid DNA was used in a concentration of ~10 ng. Colony PCR was performed for quick analysis of a large set of

bacteria or clones. A bacterial suspension was diluted 20-100fold and boiled for 10 min for this before 1µl was applied to the PCR reaction mix.

2.2.2.7 PURIFICATION OF DNA

Primers, nucleotides and other impurities were removed from PCR reactions by purification with the Qiaquick PCR Purification Kit (Qiagen) following the manufacturer's instructions.

2.2.2.8 DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

DNA was cleaved with the restriction endonuclease of choice in a reaction volume of 20-50 µl according to manufacturer's instructions.

2.2.2.9 LIGATION OF DNA FRAGMENT IN PLASMID VECTORS

Purified PCR products were directly ligated into the pDrive vector (Qiagen) following the company's protocol.

Directed cloning of DNA fragments into the pet15b expression vector was achieved by digesting the vector with *NdeI* and *BamHI* restriction endonucleases. Afterwards, the DNA fragment of choice was cut out from the pDrive cloning vector using the same enzymes, purified by gel extraction (see 2.2.2.5) and subsequently ligated into the linearised pet15 vector using the T4 DNA ligase (Fermentas) in the recommended buffer. The ligation mix was incubated at 4°C overnight.

2.2.2.10 SEQUENCING OF DNA

DNA was cloned into the pDrive vector and the column purified (Qiagen) vector DNA was delivered for automatic sequencing to "SMB Service in Molecular Biology" (Berlin). Raw sequence files were viewed and edited with "4Peaks" software (http://mekentosj.com/4peaks) on a Macintosh computer running MacOS X.

2.2.2.11 TRANSFORMATION OF ESCHERICHIA COLI

Cells of *E. coli* were transformed utilising the CaCl$_2$-chemically induced competence (Sambrook and Russel, 2001) of the XL-1 strain (Stratagene). A volume of 200 µl of XL-1 cell culture was mixed with the respective ligated plasmid vector and kept on ice for 20 min. The mixture was then subjected to a heat shock in a water bath at 42°C for 1 min. Afterwards, 500 µl of SOC medium were added and cells were incubated for 1 hour at 37°C and shaking at 220 rpm. Cells were spread on LB agar plates containing the appropriate antibiotic. In case of cloning into the pDrive vector (Qiagen) 40 µl X-Gal solution (20 mg/ml in DMF) and 40 µl of IPTG solution (0.1 M) were added to the agar to facilitate blue-white selection of positive clones. The plates were incubated at 37°C overnight.

Positive clones were picked and used to inoculate 3-4 ml LB medium containing the proper antibiotic. The cells were grown shaking at 220 rpm and 37°C overnight and plasmids were isolated (see chapter 2.2.2.2).

2.2.3 PROTEIN BIOCHEMICAL METHODS

2.2.3.1 HETEROLOGOUS EXPRESSION

For the expression of recombinant His-Mvn 25 ml of LB-Medium supplemented with 100 µg/ml Ampicillin were inoculated with *E. coli* cells containing the expression construct and grown overnight at 37°C on a shaker (220 rpm). The following day, the overnight culture was used to inoculate the large-scale expression culture in a dilution of 1/50. The culture was grown under conditions mentioned above until an OD$_{600}$=0.6 was reached. The expression was induced by addition of IPTG to a final concentration of 1 mM and the cells were grown for 3 h. Finally the cells were harvested by centrifugation at 4000 x g for 20 min. The pellets were either frozen or stored at -20°C or subsequently subjected to protein extraction.

2.2.3.2 PURIFICATION OF HIS-MVN FROM E. COLI

The recombinant His-Mvn was purified from the periplasmic fraction. The pellet from an expression culture was resuspended in 40 ml osmotic shock buffer (30 mM Tris-HCl, 20% sucrose, pH 8.0) supplemented with EDTA to a final concentration of 1 mM and incubated on ice for 10 min with gentle shaking. The cells were centrifuged (6000 x g, 20 min, 4°C), the

supernatant was removed and the pellet was resuspended in the same volume of an ice-cold 5 mM $MgSO_4$ solution and shaken for 10 min on ice. The cells were centrifuged again as described above and the supernatant was retained and extensively dialysed against lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) with three exchanges of the buffer at 4°C. Afterwards, the lysate was transferred to a fresh 50 ml tube and imidazol was added to a final concentration of 10 mM followed by the addition of 4 ml Ni-NTA slurry (Qiagen). Then the tube was mounted on a rotary shaker and incubated for 1 h at 4°C. Subsequently the mixture was applied to the column with the bottom outlet capped and the Ni-NTA was allowed to settle before the bottom cap was removed. The column flow-through was discarded and the column was washed with 40 ml wash buffer (lysis buffer containing 30 mM imidazol). Finally the column was eluted with 8 x 0.5 ml elution buffer (lysis buffer containing 250 mM imidazol). The purity was estimated from SDS-PAGE gel and highly concentrated fractions were pooled.

2.2.3.3 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentrations were determined using the Bio-Rad Protein Assay according to the manufacturer's instructions. Briefly, 1-5 µl of protein solution was added to a mixture of 0.2 ml dye reagent and 0.8 ml H_2O, mixed and incubated for 10 min at RT. Absorbance was measured at 595 nm and the concentrations were calculated using standard curve generated by dilutions of BSA.

2.2.3.4 SDS-POLYACRYAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins were separated by the method of discontinuous gel electrophoresis (Laemmli, 1970). The gels consisted of a separating gel containing 10-15% acrylamide depending on the protein size range that was to be examined and a stacking gel of 4% acrylamide. Samples were mixed with 5x loading buffer and heated at 95°C for 10 min. Depending on the application a reductant was added to the loading buffer or not (see results). The gels were run at a constant current of 25 mA per gel in the Mini Protean II system (Bio-Rad). Afterwards the gels were washed in distilled water for 15 min and stained with GelCode Blue Stain Reagent (Pierce).

Separating gel   10-15% (v/v) acrylamide/bisacrylamide 37.5:1 (v/v); 375 mM Tris-HCl, pH
                        8.8; 0.1% (w/v) SDS

Stacking gel	4% (v/v) acrylamide/bisacrylamide 37.5:1 (v/v); 125 mM Tris-HCl, pH 6.8; 0.1% (w/v) SDS
Running buffer	192 mM Glycin; 25 mM Tris; 0.1% (w/v) SDS
5x Loading buffer	250 mM Tris, pH 6.8; 0.5% bromophenole blue; 10% (w/v) SDS; 50% (v/v) Glycerol; (500 mM 2-mercaptoethanol)

2.2.3.5 IMMUNOBLOTS

Protein samples were diluted in 5x loading buffer containing 5 mM β-mercaptoethanol, heated for 10 min at 95°C and subsequently separated by SDS-PAGE on gels (Laemmli, 1970) and immobilised on nylon membranes (Amersham). The membranes were blocked with 5% w/v milk powder in PBS-T (phosphate buffered saline containing 0.3% v/v Tween20) for 1 h at 4°C. An antibody was applied in PBS-T in the appropriate dilution. After incubation for 1 h, the membranes were washed three times with 25 ml of PBS-T. Subsequently, a second antibody (anti-mouse or -goat horseradish peroxidase conjugate, Sigma) was added (1:10,000) for 1 h followed by three further washing steps with 25 ml of PBS-T. Immunoblots were developed with the SuperSignal West Pico Chemiluminescent Kit (Pierce) and exposed to X-ray film (Amersham).

2.2.3.6 STRIPPING OF WESTERNBLOTS

Antibodies were removed from westernblot membranes using the Restore Western Blot Stripping Buffer (Pierce). The membranes were covered with the stripping buffer and incubated for 15 min at 37°C. Afterwards the membranes were washed in PBS three times.

2.2.3.7 GEL FILTRATION FPLC

2.2.3.7.1 GEL FILTRATION OF M. AERUGINOSA CYTOSOLIC EXTRACTS

Aliquots (1.0 ml) of whole cell extracts of PCC 7806 (WT) and the ΔmcyB mutant were applied to a Superdex 75 prep grade HiLoad 16/60 column (GE Healthcare). Isocratic elution was performed at 10°C with a flow rate of 1 ml min^{-1}, and 1.0 ml fractions were collected. Fractions (0.5 ml per sample) were analysed in immunoblots (dot blot unit; Schleicher and Schüll) with the

antibody against Mvn. Sizes of multimeric Mvn isoforms were determined using the retention times of standard proteins (thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, vitamin B_{12}; BioRad).

2.2.3.7.2 GEL FILTRATION OF RECOMBINANT HIS-MVN

Aliquots (100 μl of a 1 μg/μl solution) of Mvn samples from the microcystin binding assay (see 2.2.3.11) were applied to a Superdex 75 10/300 GL column (GE Healthcare). Isocratic elution was performed at 10°C with a flow rate of 0.5 ml min^{-1}, and 0.5 ml fractions were collected. Sizes were determined using the retention times of standard proteins (thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, vitamin B_{12}; BioRad).

2.2.3.8 MASS SPECTROMETRY

The FTICR MS used was a Finnigan LTQ FTMS (Thermo Electron). To assign the sequence, a tryptic digest of Mvn was reduced by DTT (DL-Dithiothreitol, 10 μmol/l in solution) for two hours at 50°C. For separation, the same mass spectrometer was coupled to an Agilent 1100 Nano LC system (Agilent Technologies). To obtain information about the occurrence of S-S bonds, a non-reduced tryptic digest was analysed as well. Sequence coverage of 100% was reached. The position of one of the two occurring S-S bonds could be assigned. Mass accuracy of the protein and the tryptic peptides was far below instrument specifications (all below 2 ppm, specifications for external calibration: 4 ppm).

2.2.3.9 OLIGOSACCHARIDE MICROARRAY

The oligosaccharide microarray was done in cooperation with Matthew D. Disney and Peter H. Seeberger at the ETH (Zurich, Switzerland). The carbohydrate array containing a series of sugars that resemble the high mannose structure displayed by gp120 was constructed as described (Adams *et al.*, 2004). Carbohydrate structures with a thiol-terminated polyethylene glycol chain on their reducing ends were synthesised as described (Ratner *et al.*, 2004; Ratner *et al.*, 2004; Ratner *et al.*, 2003).

The FITC-labeled Mvn (see 2.2.3.12) was placed into a solution containing 50 mM HEPES buffer (pH 7.5), 0.1 M NaCl, 1% w/v BSA and 1 mM $CaCl_2$. Each slide was incubated with

about 5 µg Mvn in a solution of 20 µl that was distributed over the surface of the slide using a small sheet of Parafilm. After incubating at RT for 1 h, the Parafilm was removed and the slide was washed twice in a 50 ml solution 50 mM HEPES (pH 7.5), 1% v/v Tween 20, 0.1% w/v BSA and then a final rinse with 50 ml of distilled water was used. Slides were then centrifuged to dry and scanned using a Scan Array 500 scanner from GSI Lumonics.

2.2.3.10   DIALYSIS OF PROTEINS

Proteins were dialysed to change the buffer or to remove unbound microcystin during the microcystin binding assay (see 2.2.3.11). Dialysis tubings (Serva) with an exclusion size of 12 kDa were pretreated according to the manufacturer's recommendations and loaded with the samples. Dialysis was performed in 1 L of the buffer of choice at 4°C under constant stirring over night. The buffer was changed three times.

2.2.3.11   MICROCYSTIN BINIDING ASSAYS

The binding of microcystin to microvirin was assayed as follows: The binding was tested under reducing and non-reducing conditions in PBS buffer pH 7.4. Reducing conditions were established by adding 10 mM DTT (final concentration) to the Mvn samples. These were incubated 30 min at 37°C before microcystin was added to a final concentration of 10 ng/µl and incubation was continued for 30 min. A second assay was performed almost identically, but after the reduction step dithio-*bis*(2-nitrobenzoic acid) (DTNB) was added to the samples to block the thiol groups of the reduced protein. The samples were incubated for 30 min at 37°C and microcystin was added as described above. To remove the unbound microcystin the samples were dialysed against PBS buffer. Finally the samples were mixed with SDS-PAGE loading buffer without a reducing agent and SDS-PAGE was conducted as described (see chapter 2.2.3.4). Following SDS-PAGE the samples were immobilised by westernblotting and microcystin bound to Mvn was detected using an anti-microcystin antibody (Alexis). The membranes were stripped after detection (see chapter 2.2.3.6) and reprobed with an anti-Mvn antibody to confirm the identity of the protein.

The same assay was performed using a microcystin variant that was modified at its *N*-methyl-dehydroalanine residue. The methyl-group of said moiety was linked with a cysteamin molecule via a thioether bond. Dr. K. Ishida (Jena) provided the modified microcystin.

2.2.3.12 FLUORESCENCE LABELLING OF HIS-MICROVIRIN

Purified recombinant His-Mvn was labelled with the FluoroTag FITC Conjugation Kit (Sigma) according to the manufacturer's instructions. The labelled protein was purified by gel filtration on Sephadex G-25M columns and eluted with PBS. For prolonged storage 0.1% w/v sodium azide and 1% w/v BSA were added to the labelled protein and the samples were kept at 4°C in the dark.

2.2.3.13 LECTIN BINDING ANALYSIS

The lectin binding analysis (LBA) was performed using the His-Mvn labelled with fluorescence. Aliquots of a cyanobacterial culture (20-30 µl) were applied onto glass slides and fixed by drying at RT. The cells were covered with 50 µl of a FITC-microvirin solution (0.1 µg/µl) and incubated at RT for 60 min. The solution was removed with filter paper. The samples were washed three times with PBS. The remaining PBS buffer was drawn off with filter paper. The cells were viewed under a fluorescence microscope (Zeiss Axioscope) using the filter set 13 (Zeiss) and pictures were taken with a digital microscope camera (Zeiss).

2.2.3.14 ISOLATION OF LPS

Lipopolysaccharides were isolated from *M. aeruginosa* PCC 7806 using a hot-phenol method as described previously (Westphal and Jann, 1965). Harvested cells were washed three times with distilled water, freeze dried and 1 g of cell powder was subjected to hot-phenol-water extraction. The crude LPS extract was dialysed against water overnight, freeze dried and dissolved in 100 µl distilled water.

2.2.3.15 IMMUNOFLUORESCENCE MICROSCOPY

Cells of mid-logarithmic growth phase (OD$_{750}$ 0.5) were harvested by centrifugation at 4000xg, at RT for 5 min, washed once in PBS and resuspended in PBS. Cells were fixed in 3.7 % (v/v) formaldehyde in PBS on ice for 1 h. After three PBS washing steps, cells were resuspended in GTE buffer and spread-to-dry on poly-L-lysine coated glass slides. Glass slides with immobilised cells were dipped into methanol for 5 min at -20°C and subsequently into -20°C acetone for 30 s. Preparations were blocked in 2 % BSA (w/v in PBS) and incubated with the

primary antibody at a dilution of 1:500 in 2 % BSA (w/v in PBS) at RT for 1 h. After two PBS washes preparations were incubated with a FITC-labelled secondary antibody (at a dilution of 1:100 in 2% BSA [w/v in PBS]; Sigma-Aldrich) for 1 h. Cells were mounted in a drop of 4% (v/v) n-propylgallate dissolved in 87% (v/v) glycerol and stored at -20°C for up to 4 weeks.

The preparation of field samples for immunofluorescence microscopy was identical to the preparations of laboratory batch cultures, except for the fixation step. Field samples were already fixed with formaldehyde directly after the sampling.

Sample observation, image acquisition and processing were carried out using the DeltaVision spectris system (Applied Precision) with the pre-installed default softWorx software package. Two sets of excitation and emission filters were used for visualisation: the "RD-TR-PE" filter pair (555 nm/617 nm) to visualise red/orange autofluorescence of cyanobacteria and the "FITC" filter pair (490 nm/528 nm) to visualise FITC-coupled green immunostaining. Acquired raw images were deconvolved by iterative constrained deconvolution to enhance image quality and contrast using the algorithms implemented in the softWorx software package.

PBS   140 mM NaCl, 2.7 mM KCl; 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

GTE   50 mM glucose, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0

2.2.3.16   MICROCYSTIN QUANTIFICATION

Quantification of cell-bound and supernatant microcystin was done by HPLC. 10 ml samples were taken from cultures of *M. aeruginosa* wild type and mutant at culture densities of OD_{750} 0.5 and 1.0. The cells were harvested by centrifugation; the supernatant was filtered (0.2 micron filters), transferred to a fresh tube and evaporated (Savant SC210A Vacuum Concentrator). The cell pellet was dissolved in 1 ml methanol (HPLC grade) and cells were broken by sonication (12 x 15 s, highest power settings). After centrifugation (13000 x g, 10 min, 4°C) the supernatant was filtered and evaporated as described above. The successive treatment was identical for both cell pellet and supernatant samples. The extracts were dissolved in 50% methanol and subjected to HPLC analysis. A calibration curve was made using a microcystin-LR standard (Calbiochem).

2.2.3.17 HPLC ANALYSIS

The HPLC separation of cell-extract and supernatant samples was conducted on a Shimadzu HPLC unit comprising the system controller SCL-10AVP, the pump LC-10Ai, the autosampler SIL-10A, the fraction collector FRC-10A and the Photodiode-array-detector (PDA-detector) SPD-M-10AVP. Separation was carried out on a "SymmetryShield RP18" column (Waters) with a particle size of 3.5 μm, 4.6 mm inner diameter and 100 mm length and a precolumn (3.9 mm x 20 mm) with an identical sorbent. The column was eluted with gradient of water and acetonitrile with a flow rate of 1 ml/min. Both solutions contained 0.05 % TFA.

Gradient program:

Retention time [min]	0	10	40	42
% Water	70	65	30	0
% acetonitrile	30	35	70	100

2.2.3.18 PHYLOGENETIC ANALYSIS

2.2.3.18.1 SEQUENCE RETRIEVAL

Sequence data was obtained from the Interpro database hosted at the EBI which contains an entry (http://www.ebi.ac.uk/interpro/lEntry?ac=IPR011058) listing all known proteins possessing cyanovirin-N domains. Screening various *M. aeruginosa* strains for microvirin homologues via PCR and sequencing the respective PCR products obtained additional sequences. Amino acid sequences were aligned using the multiple alignment algorithms implemented in the ClustalX program version 1.83 (Thompson *et al.*, 1997). While BLOSUM 62 (Henikoff and Henikoff, 1993) was chosen as the protein weight matrix, all other parameters were left at default settings. The alignment was manually edited to remove gaps with the Bioedit v7.0.5.3 software (Hall, 1999). The alignment file was converted into the proper format that served as input for the phylogenetic analysis programs (see below).

2.2.3.18.2 RECONSTRUCTION OF PHYLOGENETIC TREES

The phylogenetic trees topologies were calculated by using the methods of Bayesian inference of phylogeny and the distance matrix method.

2.2.3.18.2.1 BAYESIAN INFERENCE OF PHYLOGENY USING MRBAYES

For the Bayesian method (Larget and Simon, 1999) the software MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) was utilised. After the aligned sequence files stored in nexus file format were provided as input for the program, the following parameters were applied: To determine the required likelihood function it was assumed that a portion of the sites in a given sequence was practically invariable while substitution rates for others were drawn from a Γ distribution. MrBayes then required the setting of a prior probability distribution for the parameters of the likelihood function set before. Here, the Jones-Taylor-Thornton matrix (Jones *et al.*, 1992) was chosen to model substitution rates. The algorithm was started with the instruction to calculate 1,000,000 generations with 4 chains, while sampling every 100th generation to create a tree whose branch length was to be recorded by the program. The analysis was continued until a probability value <0.1 was reached.

At the end of the run, after a reasonable burn-in was chosen (usually 50% of all tree data), trees were summed up in a consensus tree, which was to include all compatible groups.

2.2.3.18.2.2 PHYLOGENY BASED ON THE NEIGHBOUR-JOINING METHODS USING PHYLIP

Phylogenetic analysis according to the neighbour-joining method was carried out using the PHYLIP package in version 3.65 (Felsenstein, 2005). First 1000 pseudoreplicates were generated with the Seqboot module. These were used as input for the PROTDIST program, which calculated the distance matrices using the JTT model. The NEIGHBOR program evaluated these distance matrices and the resulting trees served as input for the CONSENSE module, which produced a majority-rule consensus tree.

2.2.3.19 IN SILICO RESOURCES

2.2.3.19.1 SOFTWARE

Agarose gel documentation	"Quanitity One v4.6.3"
	BioRad
Alignments and Phylogeny	"Bioedit v7.0.5.3"
	http://www.mbio.ncsu.edu/BioEdit/bioedit.html
	"ClustalX v1.83"

	http://www.embl.de/~chenna/clustal/darwin/
	"MEGA 3.1"
	http://www.megasoftware.net
	"MrBayes 3.1.2"
	http://mrbayes.csit.fsu.edu/download.php
	"PHYLIP v3.65"
	http://evolution.genetics.washington.edu/phylip/getme.html
	"TreeView 1.6.6"
	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
Illustrations	"Adobe Illustrator CS2"
Image processing	"Adobe Photoshop CS2"
Sequence editor	"4Peaks"
	http://mekentosj.com/4peaks
Spreadsheet calculations	"Microsoft Excel:Mac 2004"
Word processing	"Microsoft Word:Mac 2004"

2.2.3.19.2 ONLINE RESOURCES

Web applications:

Primer Design	"Primer3"
	(http://frodo.wi.mit.edu/cgi/bin/primer3/primer3_www.cgi
Protein Secrection prediction	http://www.cbs.dtu.dk/services/SignalP/
	http://www.psort.org/psortb/
Databases:	
Genomic resources	http://www.ncbi.nlm.nih.gov
	http://genopole.pasteur.fr/maeru; login required
	http://www.kazusa.or.jp/cyano
Protein families	http://www.ebi.ac.uk/interpro

3 RESULTS

The gene encoding Mvn was initially identified within the scope of elucidating the physiological function of microcystin. Early analyses of the protein were focused on its expression in the *M. aeruginosa* PCC 7806 wild type and the microcystin-deficient Δ*mcy*B mutant. It was shown that the absence of microcystin resulted in a significant alteration in microvirin expression. The protein was detected on westernblots at a molecular weight that was far above the deduced size of 12 kDa (**Fig. 8**). Two bands were visible at 50 kDa and 54 kDa in the wild type. It was concluded that the bands might represent a heteromultimer constituted of Mvn and an unknown binding partner. It was further inferred regarding the homology to the lectin cyanovirin-N that the binding partner of Mvn might carry oligosaccharide moieties and that Mvn exhibits a strong sugar binding capability. Microvirin was considered an extracellular protein although no experiments were conducted to prove this hypothesis, but the literature on bacterial lectins described extracellular roles for this kind of proteins in most instances.
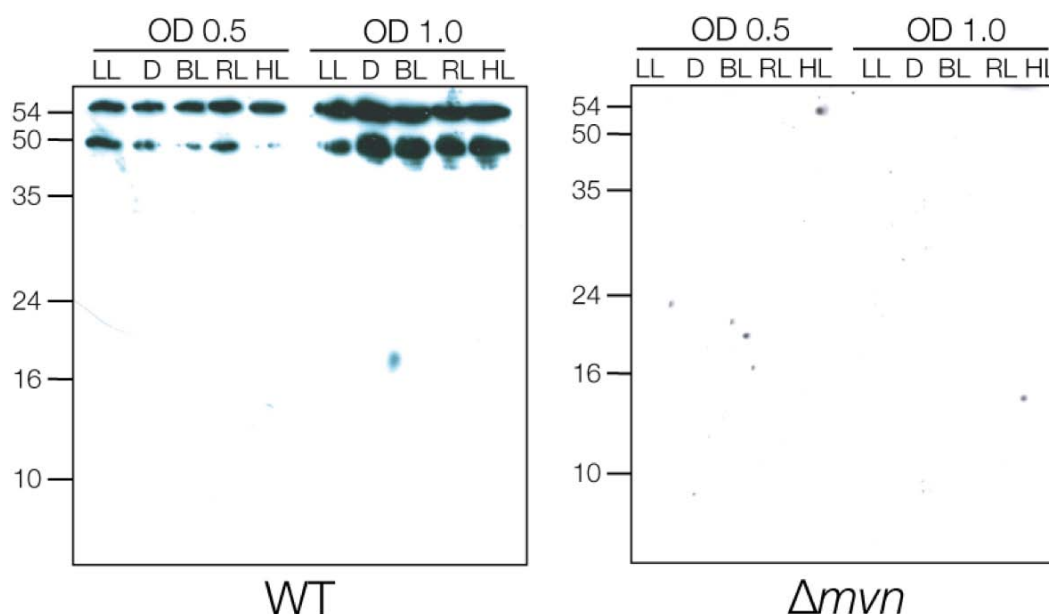
Fig. 8: Immunoblot analyses of Mvn in soluble extracts of *M. aeruginosa* wild type and Δ*mvn* mutant. Cells were grown to culture densities of OD_{750} 0.5 and 1.0 and exposed to low light (LL), darkness (D), blue light (BL), red light (RL) and high light (HL) for 2 h. The right panel shows the Δ*mvn* mutant that confirms the specificity of the antibody. From Kehr *et al.*, 2006.

These initial results implied that microvirin might be involved in extracellular carbohydrate recognition and cell-cell interaction. This process might be affected by the production of microcystin.

3.1 Characterisation of microvirin

3.1.1 CHARACTERISATION OF HETEROLOGOUSLY EXPRESSED HIS-MVN

Microvirin exhibits remarkable overall resemblance to cyanovirin-N. Several features such as the ability to form dimers and the formation of intramolecular disulphides bonds are crucial for the function of the protein and highly affect the carbohydrate binding properties and the extraordinary stability of CV-N.

For further characterisation of MVN the protein was heterologously expressed in *E. coli* BL21 and purified by Ni^{2+}-affinity chromatography. **Fig. 9** shows an SDS-PAGE gel loaded with the purified protein. Depending on the presence of reducing agents in the loading buffer Mvn either ran as dimer (reducing conditions) or monomer (non-reducing conditions).
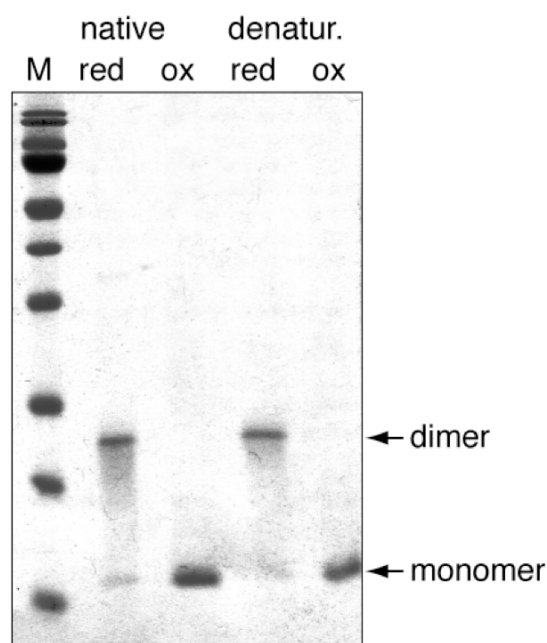
Fig. 9: SDS-PAGE of His-Mvn. The heterologously expressed His-Mvn was purified under native and denaturing conditions and separated under reducing (red) and non-reducing (ox) conditions.

3.1.2 MASS SPECTROMETRIC ANALYSIS OF HIS-MVN

The whole His-Mvn molecule was subjected to NSI FTICR mass spectrometric anaylsis under non-reducing conditions. The determined m/z (**Fig. 10**) complied the simulated value for a monomer of His-Mvn without the N-terminal methionine and and two internal disulphide bonds. The excision of the N-terminal methionine is a common posttranslational modification occuring in bacterial proteins, which is catalysed by the methionyl-aminopeptidase (MAP) and depends on the nature of the second amino acid residue in the peptide chain. The highest MAP activity is described for a glycine residue at this particular position (Hirel *et al.*, 1989), so the loss of the N-terminal methionine of His-MVN may be attributed to MAP activity.

His-MVN
measured $[M+9H]^{9+}$

His-MVN
simulated $[M+9H]^{9+}$
two disulfide bonds

$C_{601}H_{912}N_{177}O_{209}S_8{}^{9+}$

His-MVN
simulated $[M+9H]^{9+}$
reduced

$C_{601}H_{916}N_{177}O_{209}S_8{}^{9+}$
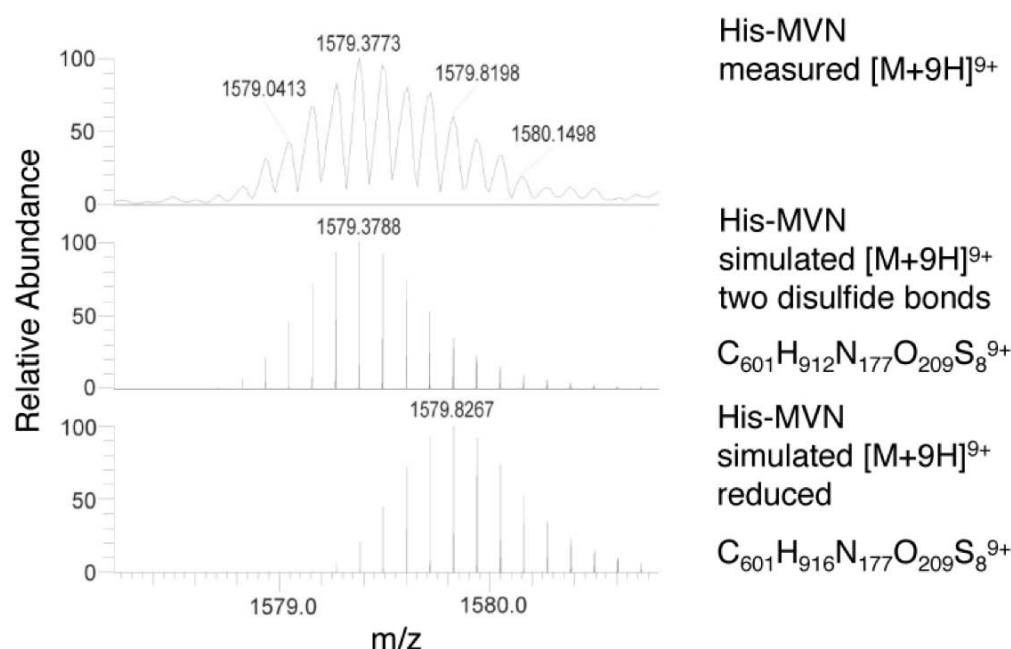
Fig. 10: Mass spectrometric analysis of recombinant His-Mvn using FTICR MS. The first graph shows the measured spectrum, whereas the second and third graph show simulated spectra for His-Mvn containing either no or two disulphide bonds, respectively.

3.1.3 ASSIGMENT OF DISULPHIDE BONDS IN HIS-MVN

To gain further information on the exact localisation of the disulphide bonds a mass spectrometic analysis of trypsin-cleaved His-MVN was performed under reducing (see supplementary data) and non-reducing conditions. Under non-reducing conditions peptides covering 87% percent of the entire Mvn could be identified, while the sequence coverage of the reduced sample was 100%. The N-terminal peptide as well as the fragment aa 84-107 could

not be detected in the non-reducing sample and in addition the predicted peptide covering aa 57-83 gave only a weak signal. Nevertheless, a peptide representing the residues aa 57-107 was identified and contained at least one disulphide bond or a mixture of one and two disulphide bonds. The peptide representing the residues aa 17-48 produced a peak, which complies with the calculated m/z of a disulphide bond between cysteines C28 and C44 (see **Fig. 11** and **Tab. 4**). This was confirmed by data obtained under reducing conditions. The disulphide bond between C28 and C44 could be clearly identified and two disulphide bonds were identified in the whole protein, thus only one disulphide bond must be present in the fragment aa 57-107. This peptide contains four cysteines and the close neighbourhood of the cysteines impeded a further assignment, as no suitable protease cleavage site was present between cysteines 79 and 87.
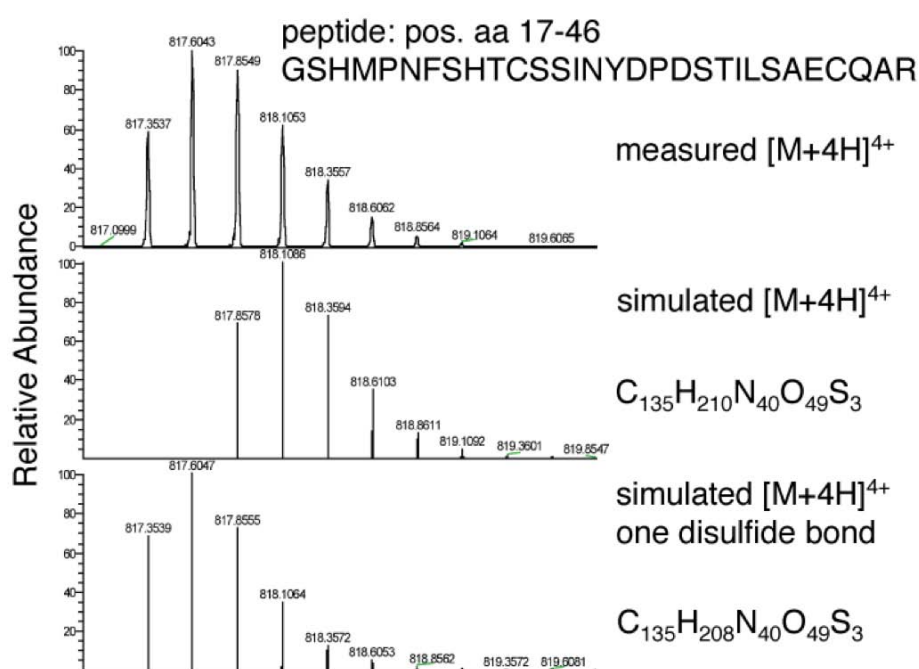
Fig. 11: Mass spectrometric analysis of recombinant His-Mvn using FTICR MS after trypsin digestion. The first graph shows the measured spectrum of the tryptic fragment aa 17-46, whereas the second and third graph show simulated spectra for His-Mvn containing either no or one disulphide bond, respectively.

Tab. 4: Summary of all fragments identified after trypsin digestion using FTICR MS. The sequence of each fragment and its position in the whole molecule as well as the detected corresponding ions are given.

Sequence	Pos. aa in protein	Ion	m/z$_{obs}$	m/z$_{sim}$	mass accuracy
GSSHHHHHHSSGLVPR	1-16	$[M+nH]^{n+}$	n.d.		-
		$[M+O+nH]^{n+}$	n.d.		-
GSHMPNFSHTCSSINYDPDSTILSAECQAR	17-46	$[M(-2H)+2H]^{2+}$	1634.2036	1634.2021	0.9 ppm
		$[M(-2H)+3H]^{3+}$	1089.8032	1089.8038	0.6 ppm
		$[M(-2H)+4H]^{4+}$	817.6043	817.6047	0.5 ppm
		($\rightarrow$ 1 S-S: AA 27(Cys)-43(Cys))			
DGEWLPTELR	47-56	$[M+2H]^{2+}$	608.3031	608.3039	1.3 ppm
		$[M+H]^{+}$	1215.6003	1215.6004	0.1 ppm
LSDHIGNIDGELQFGDQNFQETCQDCR	57-83	$[M(-2H)+3H]^{3+}$	1027.7754	1027.7763	0.9 ppm
		($\rightarrow$ 1 S-S: AA 79(Cys)-82(Cys))			
LEFGDGEQSVWLVCTCQTMDGEWK	84-107	$[M+nH]^{n+}$	n.d.	n.d.	-
		$[M+O+nH]^{n+}$	n.d.	n.d.	-
		$[M(-2H)+nH]^{n+}$	n.d.	n.d.	-
STQILLDSQIDNNDSQLEIG	108-127	$[M+2H]^{2+}$	1102.0412	1102.0399	1.2 ppm
Missed cleavage: LSDHIGNIDGELQFGDQNFQETCQDCRLEFGDGEQSVWLVCTCQTMDGEWK	57-107	$[M(-4H)+4H]^{4+}$ (two S-S bonds)	1456.3772	1456.3750	1.5 ppm
		$[M(-2H)+4H]^{4+}$ (one S-S-bond)	1456.8783	1456.8790	0.5 ppm

3.1.4 SIZE DETERMINATION OF NATIVE MVN FROM *M. AERUGINOSA*

During a previous study (Kehr, 2003), Mvn was detected as two bands of approximately 50 and 54 kDa on immunoblots of *M. aeruginosa* PCC 7806. This is far above the calculated size of a monomer (12 kDa) or dimer and was discussed as the result of multimerisation or the strong interaction with a binding partner of unknown kind. Nevertheless, the proteins were separated by denaturing SDS-PAGE and thus no definite conclusion of the oligomerisation state of Mvn *in vivo* can be drawn. To address the question of oligomerisation *in vivo*, cell extracts of *M.*

aeruginosa wild type and Δ*mcy*B mutant were subjected to gel filtration (see chapter 2.2.3.7.1) and the eluted fractions were probed for Mvn on a dot blot. The protein eluted in both, wild type and Δ*mcy*B mutant, after 55-57 min which corresponds to 55-64 kDa according to the calibration with a molecular weight standard (**Fig. 12**). This agrees with the size observed in previous immunoblots of protein extracts of cytosolic fractions. No signals were obtained for higher molecular weight forms of the protein. Thus it can be concluded that the multimeric forms detected by immunoblotting after denaturing and reducing SDS-PAGE represent the native oligmerisation state of Mvn.
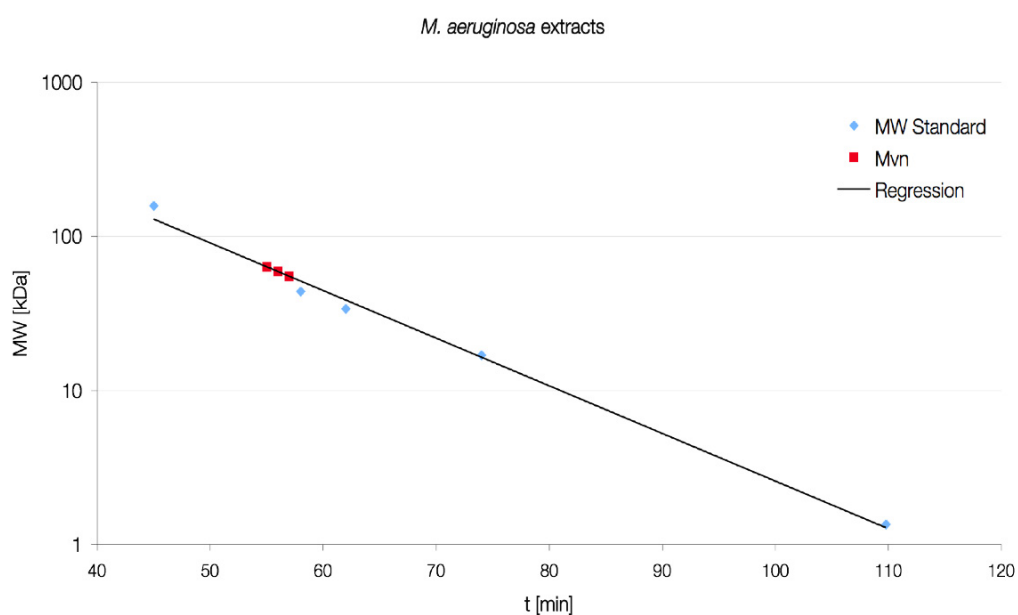
Fig. 12: Size determination of Mvn in *M. aeruginosa* cell extracts. Cell extracts were subjected to gel filtration and Mvn (red squares) eluted after 55-57 min, which corresponds to a molecular weight of 55-64 kDa.

3.1.5 CARBOHYDRATE SPECIFICITY OF MVN

Microvirin shows significant similarity to cyanovirin-N, which specifically recognises $\alpha(1,2)$-linked mannose residues like the ones present in the carbohydrate moieties of the HIV envelope protein gp120 (Boyd *et al.*, 1997). Therefore, similar carbohydrate specificity should be

expected for Mvn. A carbohydrate microarray (Ratner *et al.*, 2004) spotted with different oligomannose derivates was already used to test the carbohydrate specificity of cyanovirin-N (Ratner *et al.*, 2004). The very same array was hybridised with FITC-labelled Mvn (see chapter 2.2.3.9) to verify this assumption.

Fig. 13 shows the carbohydrate microarray probed with FITC-labelled Mvn. Eight different carbohydrates (strucures are displayed in the lower part of **Fig. 13**) were spotted in replicates (horizontal squares) in four different concentrations for each (oligo-)saccharide (vertical squares).
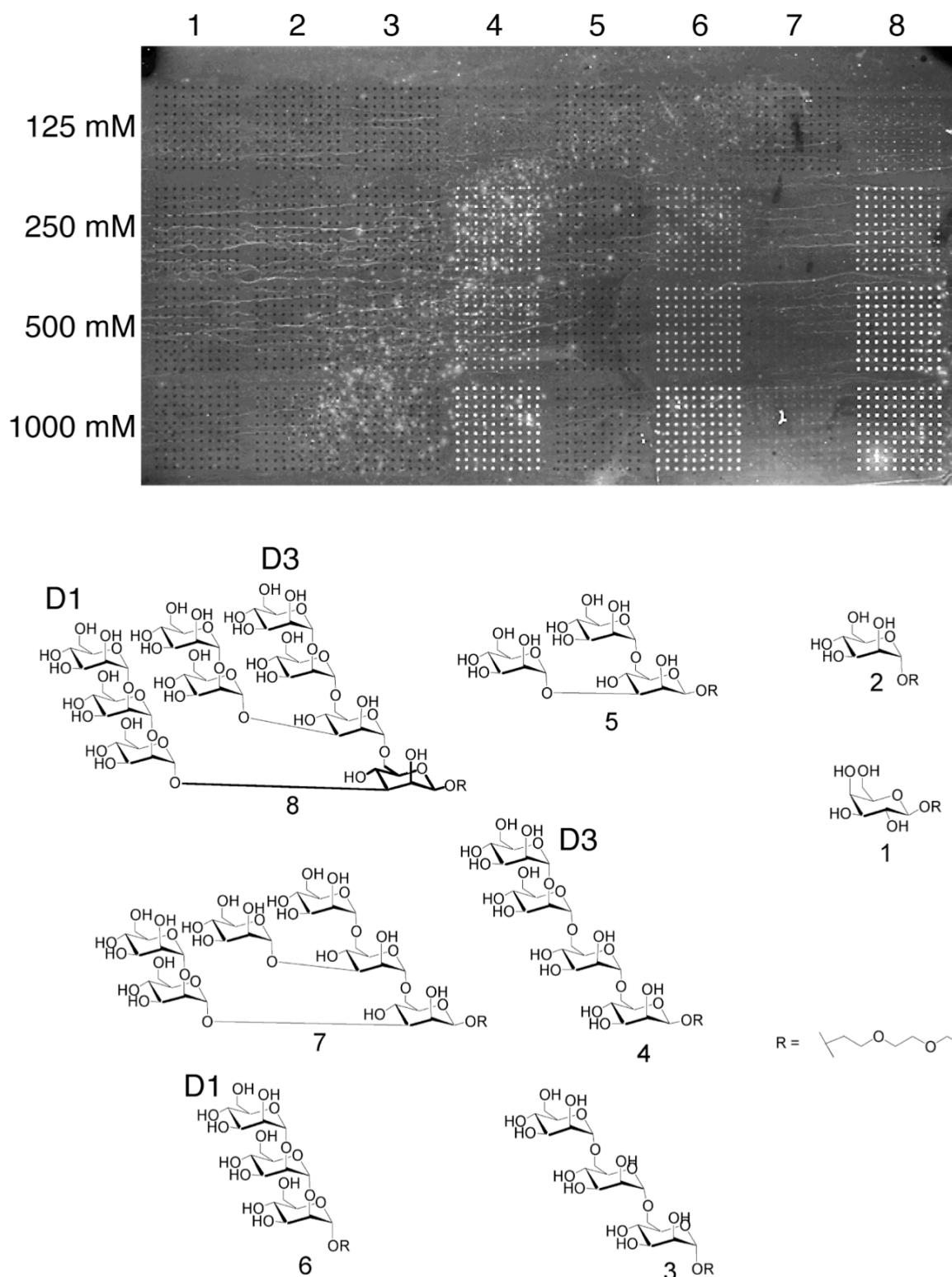
Fig. 13: Carbohydrate mircroarray. Image of the carbohydrate microarray incubated with fluorescently labelled Mvn (upper panel). Synthetic mannans 2-8 and galactose 1 as negative control (lower panel) were spotted on the glass slide at concentrations of 1000, 500, 250 and 125 mM. The D1 and D3 arms of mannoside 8 that are separately represented by mannosides 4 and 6 are indicated. Carbohydrates were synthesized as described in Ratner *et al.* (2002).

The results unambiguously show that Mvn binds to oligomannose that carries a terminal $\alpha(1,2)$-linked mannose unit. The lectin binds best to a mannan9 (structure 8 in **Fig. 13**) that comprises three oligosaccharide arms possessing terminal $\alpha(1,2)$-linked mannose units. Structures 4 and 6 represent the D3 and D1 arms of the mannan9 and each alone is sufficient to achieve a binding of Mvn (**Fig. 13**, lane 4 and 6). The importance of the terminal $\alpha(1,2)$-linked mannose residue is emphasised by the fact that no binding was observed to structure 3, which is the same as structure 4 but without the terminal mannose unit. The same applies for structure 7, which is identical to structure 8, but lacks the terminal mannose units at all three branches of the oligosaccharide. Nevertheless, a faint fluorescence signal could be detected at the highest carbohydrate concentration applied. The D1 arm of structure 7 still contains a terminal $\alpha(1,2)$-linked mannose unit but is only made of two sugar units. This implies that a minimum chain length of three carbohydrate units is required to facilitate optimal binding. This agrees with the fact that lectins usually recognise oligosaccharides but not monosaccharides. The same applies for Mvn, which fails to bind to mannose monosaccharide (structure and lane 2).

3.1.6 ANTI-HIV ACTIVITY OF MVN

Mvn exhibits almost the same sugar binding specificity as CV-N and the anti-HIV activity of CV-N results from the recognition of the respective carbohydrates on the viral cell envelope protein GP120. The binding of CV-N to these glycoepitopes prevents the virus from entering the host cell (Bolmstedt *et al.*, 2001; Botos *et al.*, 2002; Esser *et al.*, 1999; O'Keefe *et al.*, 2000). Therefore, intensive studies were conducted to test eligibility of CV-N as a drug to prevent HIV transmission. The results presented above suggested that Mvn might possess a similar potential. A previous study already found evidence that Mvn can indeed bind to components of HI virus lysates *in vitro* (Kehr, 2003). In order to assess the *in vivo* activity of Mvn the purified His-Mvn was tested in the group of Dominique Schols (Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium).

The assays were perfomed on three different HI virus strains including one strain (IIIB HHA-res) that was resistant to inhibition by the mannose-binding lectin HHA (Balzarini *et al.*, 2005; Balzarini *et al.*, 2004). Apart from Mvn additional mannose-binding lectins were tested as a reference. These included CV-N, HHA (*Hippeastrum hybrid* agglutinin) and GNA (*Galanthus nivalis* agglutinin) and the inhibitory activity of these was demonstrated earlier (Balzarini *et al.*,

2004). In addition to the 50% inhibitory concentration (IC_{50}) the 50% cellular toxicity (CC_{50}) was determined to evaluate possible cytotoxic effects caused by the lectin treatment.

Tab. 5: HIV inhibitory activity and cytotoxic effects of Mvn in comparison to other mannose-specific lectins. The activity against three different HIV strains was tested, including one strain (IIIB HHA-res) resistant to inactivation by HHA.

lectin	IC$_{50}$ (μg/ml)			CC$_{50}$ (μg/ml)
	HIV-1 NL4.3	HIV-1 IIIB	IIIB HHA-res	
HHA	0.27	0.19	>100	>100
GNA	0.056	0.17	>100	>100
CV-N	0.19	0.06	>4	2.1
Mvn	0.07	0.054	>20	>20

The results presented in **Tab. 5** show that Mvn indeed exhibits an anti-HIV activity that is similar to that of CV-N and exceeds those determined for other lectins. Interestingly, the cytotoxicity is an order of magnitude lower compared to CV-N. Furthermore, the results confirm the data obtained by the carbohydrate microarray. The specificity of CV-N was assayed using the same carbohydrate array. In contrast to Mvn the former was less specific recognising a broder spectrum of mannose derivates (Ratner *et al.*, 2004). Thus the higher cytotoxic impact of CV-N might result from addtional interactions of the lectin.

3.1.7 DETECTION OF MANNAN MOIETIES ON THE *M. AERUGINOSA* CELL SURFACE

Complex carbohydrates are presented by bacteria on their cell surface to accomplish a role e.g. in attachment or recognition (Karlyshev *et al.*, 2004; Sherlock *et al.*, 2005; Wu *et al.*, 2007). Such carbohydrates can be part of glycoproteins (Hitchen and Dell, 2006; Schaffer and Messner, 2004) or lipopolysaccharides (Rocchetta *et al.*, 1999). Most bacterial glycoproteins were found to be associated with the cell surface (Upreti *et al.*, 2003). As mentioned in the introduction (see chapter 1.4.2) lectins serve as recognition molecules of sugar signals exposed on cell surfaces. In order to test whether a mannan type oligosaccharide is present on the *M. aeruginosa* PCC 7806 cell surface a lectin binding analysis (LBA) was carried out. The lectin binding analysis is a technique commonly used to determine the composition of carbohydrate structures on cell surfaces. Lectins with known sugar specificity are linked to fluorescence dyes and used as probes (Hedemann *et al.*, 2007; Robitaille *et al.*, 2006). The use of a set of lectins

each coupled with a diffierent fluorescene dye can be employed to study complex samples containing a number of species e.g. pathogenic bacteria from clinical isolates (Greiner *et al.*, 2005) or bacteria from environmental samples (Boeckelmann *et al.*, 2002; Neu *et al.*, 2002; Neu *et al.*, 2004).

In this study this technique was applied to identify *M. aeruginosa* strains, which express the particular sugar that is recognised by Mvn. The expressed His-Mvn was coupled with the green fluorescent dye FITC and used as probe against cells that were immobilised on glass slides. In order to obtain reliable results proper controls were needed. A common way to confirm specific binding of a certain lectin used in LBA is to preincubate the lectin with the corresponding oligosaccharide. The carbohydrate binding sites should be occupied by the sugar rendering the lectin unable to bind further carbohydrate. The mannan oligosaccharide that is specifically recognised by Mvn was not available from any supplier and thus a different set of controls was choosen. At first, strains from different cyanobacterial genera, namely *Synechocystis* PCC 6803 and *Nostoc punctiforme* ATCC 29133 were used. Both strains were available in the laboratory and do not contain genes encoding for Mvn homologues. Furthermore both strains can be easily distinguished from *Microcystis* by size (*Synechocystis*) or morphology (filamentous *Nostoc*). Therefore, the assay could also be performed with cell mixtures and the general possibility to use the Mvn LBA approach on complex samples was evaluated.

Fig. 14 shows fluorescence micrographs from representative samples of the LBA. The two upper images show the same region of *M. aeruginosa* PCC 7806 cells incubated with FITC-labelled His-Mvn. The right image shows the red autofluorescence of the cells, whereas the left image displays the merge of the red autofluorescence and the green FITC fluorescence. The green fluorescence clearly outshines the autofluorescence of the cells indicating an extensive binding of the labelled Mvn to the cell surface of the bacteria. The specificity of the interaction is confirmed by the controls presented in the lower fluorescence micrographs. The first image in the middle row shows the merged channel recording of *M. aeruginosa* PCC 7806 cells without any Mvn applied. Green fluorescence is completely absent from this sample. The same is valid for the following micrographs in the middle row that show samples of *Synechocystis* and *Nostoc* incubated with the FITC-labelled His-Mvn. These results unambigously show that the strong green fluorescence observed in the *M. aeruginosa* PCC 7806 sample (upper left) can only originate from the binding of Mvn. The complete absence of green fluorescence in the controls corroborates the specificity of the binding. Unspecific signals might have occured by

FITC-labelled His-Mvn retained in the mucilage of the cells due to insufficient washing after incubation, but these were not observed. Thus it can be finally concluded that oligomannose type carbohydrates identical or at least very similar to that successfully used in the carbohydrate microarray (see chapter 3.1.5) are present on the cell surface of *M. aeruginosa* PCC 7806.

Obviously the next step was to evaluate whether the FITC-labelled His-Mvn can also be used to discriminate between cells in complex samples that do possess mannan cell surface carbohydrates and those that do not contain this particular sugar. The lower row of images shows the results obtained with mixtures of cells. The lower left image displays the merged channel recording of a sample containing *M. aeruginosa* PCC 7806 and *Synechocystis* PCC 6803. The green fluorescence is only visible on the smaller cells that represent the *Microcystis* cells, while the larger *Synechocystis* cells only exhibit the red autofluorescence. The fluorescence micrograph in the lower right showing a mixture of *Microcystis* and *Nostoc* cells displays a similar and even more obvious pattern since the *Nostoc* filaments can be easily distinguished from the unicellular *Microcystis*.
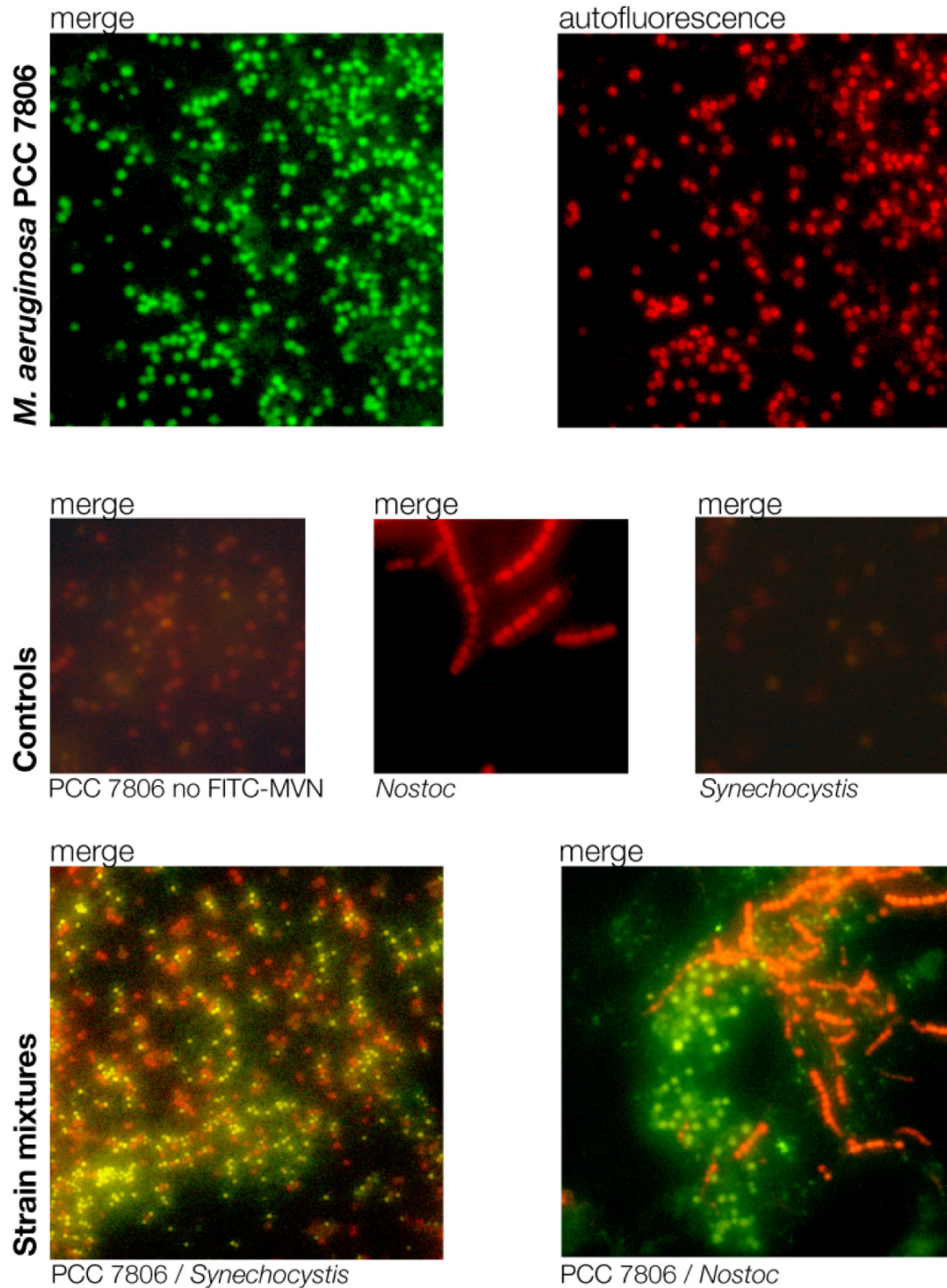
Fig. 14: Lectin binding analysis using FITC-labelled Mvn. The green fluorescence represents the bound FITC-labelled Mvn in contrast to the red fluorescence that originates from the excitation of chlorophyll. Images either show a merge of red and green fluorescence (merge) or just the red fluorescence (autofluorescence). *Synechocystis* PCC 6803 and *Nostoc punctiforme* ATCC 29133 were used as negative controls.

### 3.1.8 IN SITU DETECTION OF MVN IN *M. AERUGINOSA* PCC 7806

Mvn recognises oligosaccharide moieties on the cell surface of *M. aeruginosa*, but this does not necessarily mean that the protein itself is bound to the cell surface *in vivo*. Microvirin is a lectin and in bacteria these types of proteins are ususaly involved in processes of cell-cell recognition. They mediate attachment of cells in either an inter- or intraspecies manner. Therefore, lectins have to be released from the cell to the cell surface or the culture medium. This is indeed the case for most bacterial lectins characterised so far (see chapter 1.4). Apart from this, further evidence points to an extracellular function of Mvn. The ability of the protein to form intracellular disulphide bonds has been demonstrated (chapter 3.1.3) and poses a strong argument for a translocation through the cytoplasmic membrane since disulphide bonds in bacterial proteins are established in the periplasm (Collet and Bardwell, 2002). The presence of disulphide bonds in the heterologously expressed His-Mvn suggests that the protein is translocated into the periplasm in *E. coli*. Disulphide bonds in *E. coli* are established in the periplasm by the DsbA thiol-disulphide oxidoreductase (Sevier and Kaiser, 2002). On the other hand, *in silico* analyses using common prediction tools like signalP (http://protfun.net/services/SignalP/) or PSORT (http://psort.ims.u-tokyo.ac.jp/) support a cytosolic localisation of Microvirin.

In order to address the question of *in vivo* localisation of microvirin immunofluorescence microscopy (IFM) studies were conducted on fixed cells of *M. aeruginosa* PCC 7806. The IFM technique was already used successfully in the PCC 7806 strain (Guljamow *et al.*, 2007). An Mvn mutant that could serve as control was available and therefore, this method was chosen. The cells were incubated with an anti-Mvn antibody and afterwards hybridised with a FITC-labelled secondary antibody. After each antibody hybridisation step the cells were washed in PBS buffer and finally the samples were observed under a fluorescence microscope. In contrast to the protocol used by Guljamow *et al*. the lysozyme incubation step that resulted in the permeabilisation of the cells was omitted because an extracellular localisation of microvirin was expected.

Fig. 15 shows a set of representative fluorescence micrographs summarising the results. The upper part of **Fig. 15** displays images taken of control experiments. Micrographs were recorded at 617 nm (autofluorescence), 528 nm (FITC fluorescence) and a merged image shows an overlay of both wavelengths. Several controls were used. The upper row of images shows wild type cells hybridised only with the secondary fluorescently labelled antibody and only the fixed cells without any antibody applied. The images acquired from both experiments virtually look the same indicating no unspecific reaction of the secondary antibody. Also the

experimental procedure was sophisticated and particularly the washing steps after antibody treatment were sufficient to remove unbound antibodies. Apparently there is little green fluorescence visible in both samples on the images acquired with the FITC filter set (528 nm), but since no fluorescent antibody was applied in one of the experiments, the weak green fluorescence must be endogenous of the bacteria. The second row of **Fig. 15** displays images of control experiments performed with primary and secondary antibodies using the Δ*mvn* mutant and *Synechocystis* PCC 6803. Apart from the endogenous green fluorescent of the cells that was also visible in the topmost images there is no strong green fluorescence associated with the cells itself, but a rather bright green fluorescence signal could be observed surrounding the Δ*mvn* mutant cells. It turned out that this fluorescence does not correspond to bound antibodies, but results from the excitation of the mucilage or compounds embedded in the mucilage. The amount of mucilage increased with prolonged culturing of the laboratory strains and thus the phenomenon was regularly observed at higher culture densities. This effect was even stronger in field samples that usually produce considerably higher amounts of mucilage (see chapter 3.5).

However, the wild type cells display a bright green fluorescence tightly associated with the cell surface (**Fig. 15**, lower left images) that was not observed in any control experiment. In contrast to the endogenous green fluorescence the specific signal was also visible on the merge of both wavelengths. Obviously the green fluorescence signal had a greater diameter compared to the red autofluorescence, which is visible where two cells are in close proximity. Since the antibodies cannot enter the cells the results clearly show that microvirin is indeed secreted and located at the cell surface in the wild type. This is also valid for the Δ*mcy*B mutant (**Fig. 15**, lower right images).
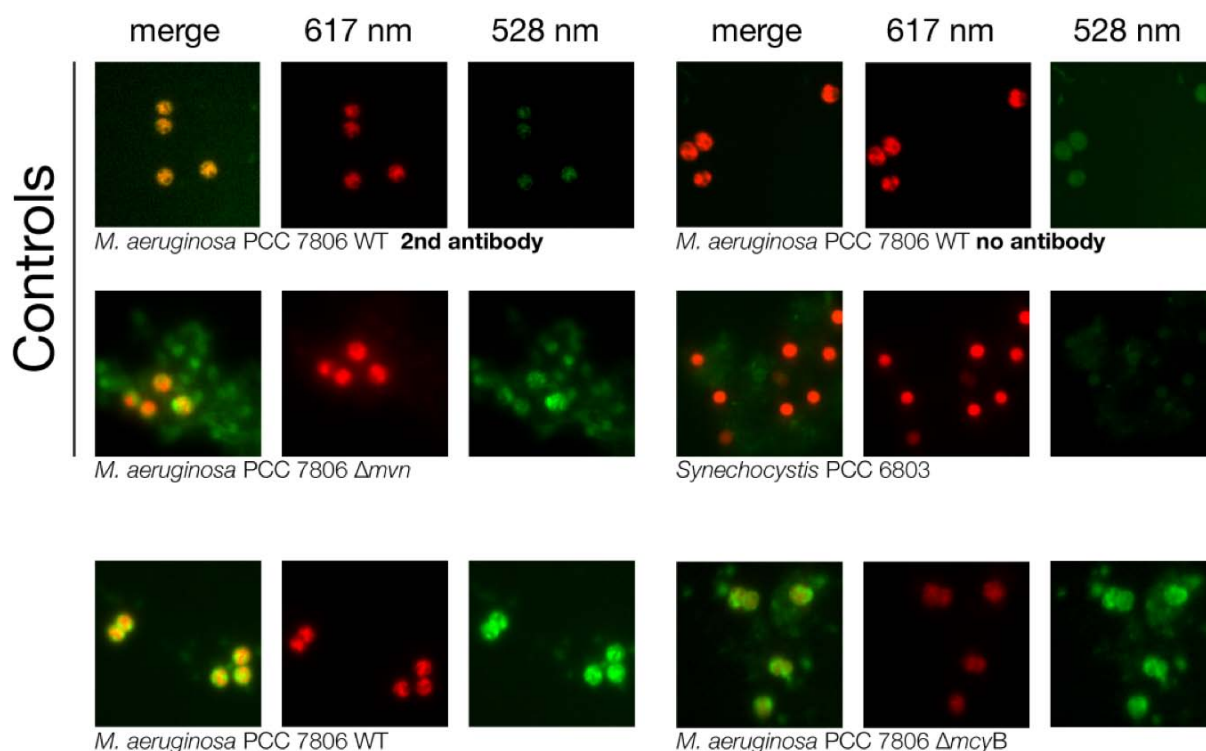
Fig. 15: Immunofluorescence microscopy using an anti-Mvn antibody. Cells were fixed, hybridised with an anti-Mvn antibody and a FITC-labelled secondary antibody. Images show both the green or red fluorescence and a merge of both channels. The Δ*mvn* mutant and *Synechocystis* PCC 6803 as well as the wild type hybridised with only the secondary antibody or without any antibody served as controls.

3.1.9 MICROVIRIN BINDS TO LPS

In the previous chapter it was unambigously shown that oligosaccharides recognised by Mvn are present on the cell surface of *M. aeruginosa* PCC 7806. Bacterial surface-exposed carbohydrates are linked to membrane-associated glycoproteins or glycosylated extracellular proteins like e.g. S-layer proteins (Schaffer and Messner, 2004; Smarda *et al.*, 2002). Lipopolysaccharides of the outer membrane contain carbohydrate moieties that reach out into the surrounding medium and make a major contribution to the extracellular glycome (Papageorgiou *et al.*, 2004; Weckesser *et al.*, 1979). Thus extracellular glycosylated compounds might be potential targets of Mvn binding.

To test whether LPS are binding partners of Mvn a gel shift assay was performed using purified LPS isolated from *M. aeruginosa* PCC 7806 according to the hot phenol method (Westphal and Jann, 1965). The protein was incubated with LPS and separated by SDS-PAGE. The following immunoblot (**Fig. 16**A) showed that the amount of monomeric Mvn decreased in the presence of LPS and an additional band appeared running slightly above the dimer of Mvn at 28 kDa

(**Fig. 16**A, lane 3). However, neither of the two bands of 50 kDa or 54 kDa that were detected in whole cell protein extracts was observed. Given the homology to CV-N, Mvn might harbour to carbohydrate binding and thus a second binding partner absent from the LPS extract might be needed to achieve a shift to 50 kDa or 54 kDa (see **Fig. 8**).
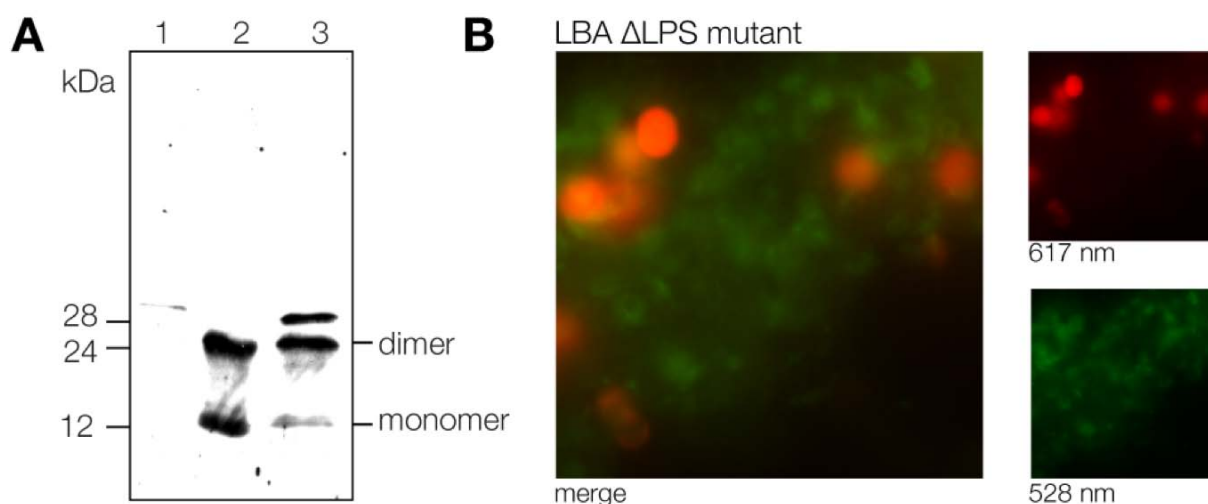
Fig. 16: Microvirin binds to LPS. A) Immunoblot of a gel-shift experiment with His-Mvn and purified LPS isolated from *M. aeruginosa* PCC 7806 wild type. The gel was loaded with purified LPS (lane 1), purified His-Mvn (lane 2) and His-Mvn incubated with the isolated LPS (lane3). B) Lectin binding analysis of an ΔLPS mutant of *M. aeruginosa* PCC 7806. No Mvn binding to the mutant cells was observed. Mvn was detected using the anti-Mvn antibody in both experiments.

An *M. aeruginosa* PCC 7806 knockout mutant of a gene encoding a glycosyltransferase that is involved in the synthesis of lipopolysaccharides was available (kindly provided by K. Goeldner) and could be used for further analysis. The said gene showed similarity to the *rfa*Q/*waa*Q gene (both gene names refer to the same gene, where *rfa*Q is used in older literature). The RfaQ protein is involved in the synthesis of the inner LPS core in *E. coli* (Klena *et al.*, 1992). Typically, LPS consist of a lipid, the inner core oligosaccharide and the variable O-antigen. The knockout of the WaaQ protein in *Pasteurella multocida* led to severely truncated LPS missing the O-antigen (Harper *et al.*, 2004). The *M. aeruginosa* PCC 7806 ΔLPS mutant generated by insertional mutagenesis (**Fig. 17**A) of the *rfa*Q homologue also showed (**Fig. 17**B, arrow 1) a drastic reduction of O-antigen attached to LPS (Göldner, 2007). In addition, the LPS containing only the core oligosaccharide migrated faster on SDS-PAGE gels (**Fig. 17**B, arrow 2), which was also described for other RfaQ mutants. This results from the absence of a heptose unit that is transferred to the LPS core by RfaQ (Klena *et al.*, 1992).
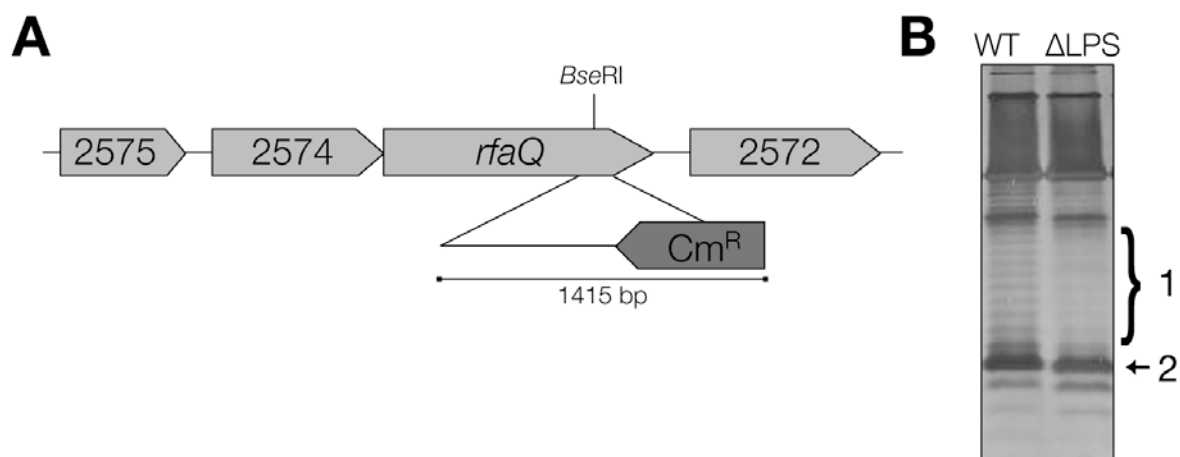
Fig. 17: *M. aeruginosa* **ΔLPS mutant.** A) Schematic display of the mutagenesis of the *rfa*Q homologue of *M. aeruginosa* PCC 7806 by insertion of a chloramphenicol resistance gene. The numbers of the adjacent genes refer to the IPF numbers of the *M. aeruginosa* PCC 7806 genome annotation. B) SDS-PAGE of isolated LPS from *M. aeruginosa* PCC 7806 wild type and ΔLPS mutant. The LPS isolated from the mutant show a strong reduction of the O-antigen portion (1) and a slightly smaller core LPS (2). Adapted from Göldner, 2007.

The ΔLPS mutant was subjected to a lectin binding analysis and no binding of the lectin was observed on any micrograph (**Fig. 16**B). Obviously the mutation led to a complete loss of the Mvn-specific oligosaccharide exposed at the cell surface. However, the glycosyltransferase might be involved in other pathways and therefore it cannot be excluded that other putative binding partners are also affected by the mutation. Furthermore, the ΔLPS mutant seemed to produce more extracellular mucilage, which can be seen on the images as green background fluorescence. However, no effort was spend to quantify the mucilage, but an enhanced mucilage production was already reported for LPS mutants of *E. coli* (Parker *et al.*, 1992).

3.1.10 THE LOSS OF MVN LEADS TO A REDUCED PRODUCTION OF MICROCYSTIN

The altered expression of Mvn (**Fig. 8**) in the Δ*mcy*B mutant implies a functional correlation of microcystin and microvirin. The absence of microcystin results in a delayed onset of Mvn expression. To further investigate the relationship of microcystin biosynthesis and Mvn, the microcystin content of the Δ*mvn* mutant was compared to that of the wild type. Methanolic extracts were prepared from 10 ml aliquots of triplicate cultures at an OD_{750} of 0.5 and 1.0, respectively. HPLC analysis allowed the identification of microcystins based on specific retention times and the spectrum of the characteristic Adda moiety. Wild type extracts

contained the previously described major peaks for microcystin-LR and D-Asp-microcystin-LR and several undescribed peaks.
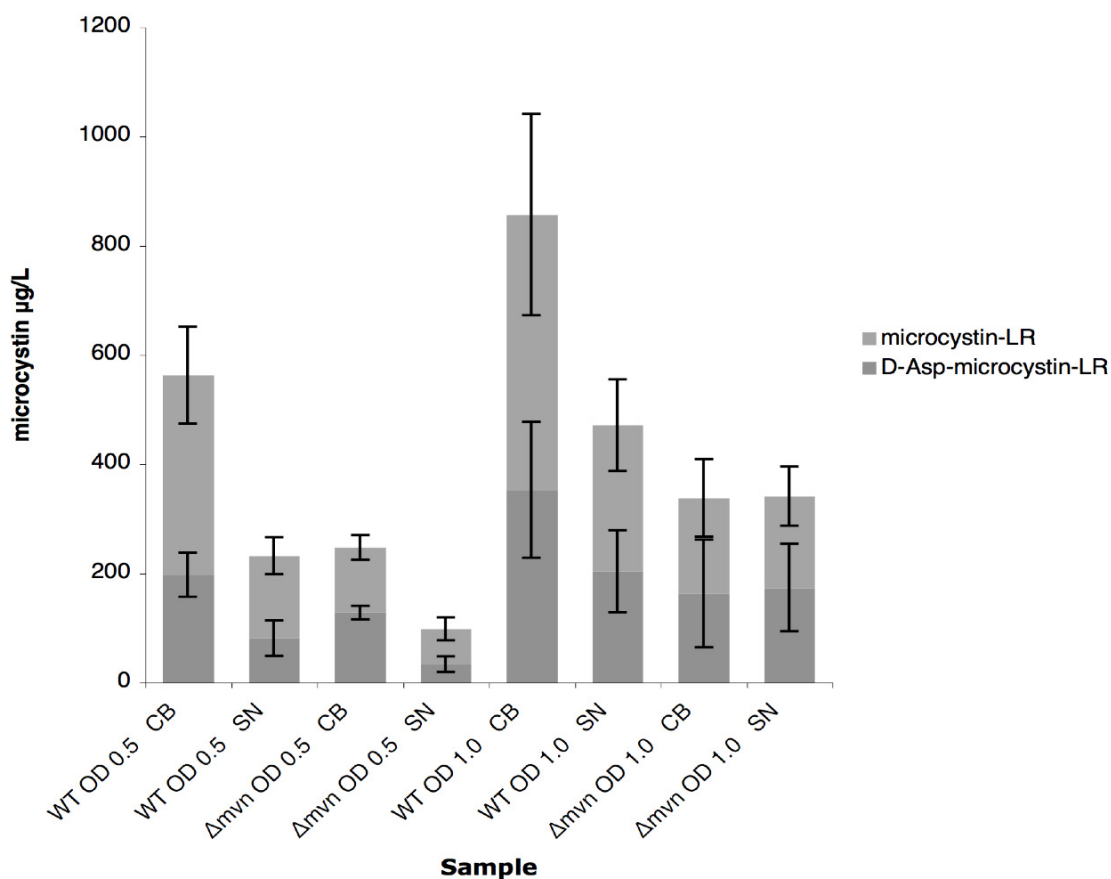
Fig. 18: Microcystin content of *M. aeruginosa* PCC 7806 wild type and Δ*mvn* mutant.
The cultures were grown in triplicates and samples were taken at culture densities of OD_{750} 0.5 and 1.0. Microcystin was quantified from the cell-bound (CB) and culture supernatant (SN) fractions.

The results are summarised in **Fig. 18** and broken down into fractions of cell-bound (CB) and culture supernatant microcystin fractions. In both wild type and mutant the amount of microcystin increases with the culture density but the Δ*mvn* mutant produces less than 50% of the wild type microcystin level. Furthermore, the ratio of cell-bound to released microcystin differs between wild type and mutant at higher cell densities. At OD_{750} 0.5 approximately 70% of total microcystin is cell-bound in the wild type and mutant, while at OD_{750} 0.5 this ratio is shifted towards the released microcystin in the Δ*mvn* mutant which accounts up to 50% of total microcystin. However, in the wild type the ratio remained unaffected during growth.

3.2 Direct interaction of microcystin and microvirin

3.2.1 BINDING OF MICROCYSTIN TO MVN

Several reports describe the interaction of microcystin with cellular components - in particular with proteins. Immunogold staining of *Microcystis* cross sections showed microcystin to be associated with thylakoids and phosphate bodies (Young *et al.*, 2005). Others who found microcystins to be associated with phycobilisomes (Jüttner and Luthi, 2008) also support thylakoid localisation of microcystin. Binding of microcystin to proteins was shown to be very tight even enduring denaturing separation on SDS-PAGE (Zilliges, 2007). Zilliges also showed that microcystin binds to the large subunit (RbcL) of RubisCO and that binding can be suppressed by pre-treatment of the enzyme with DTNB. DTNB reacts with free sulfhydryl groups and hence microcystin must bind covalently to reduced thiol moieties of cysteines since these are rendered inaccessible due to DTNB modification. Besides RbcL where microcystin binding was investigated *in vitro*, several other proteins were identified as putative mc binding partners. These include the phycobilisome subunits CpcA and CpcB, phosphoribulokinase (Prk) and gluthathione reductase. Additionally, most of them (CpcA, CpcB, Prk, RbcL) showed a differential expression in the Δ*mcy*B mutant. A role of mc in the regulation of enzyme activity and stability similar to thioredoxin was proposed in the same study.

Like the proteins listed above Mvn shows an altered expression pattern in the Δ*mcy*B mutant. Furthermore, Mvn contains six cysteine residues that may be a potential target of microcystin binding and the oxidation state of the cysteine residues seems to be crucial for the oligomerisation of Mvn.

Binding assays were accomplished with His-Mvn under reducing (10 mM DTT) and non-reducing conditions. The protein solutions were incubated with microcystin and subsequently dialysed against PBS buffer to remove unbound microcystin. The samples were separated by non-reducing denaturing SDS-PAGE, immobilised by western blotting and hybridised with an anti-mc antibody. The same blot was stripped and then re-hybridised with an anti-Mvn antibody to confirm the identity of the protein.
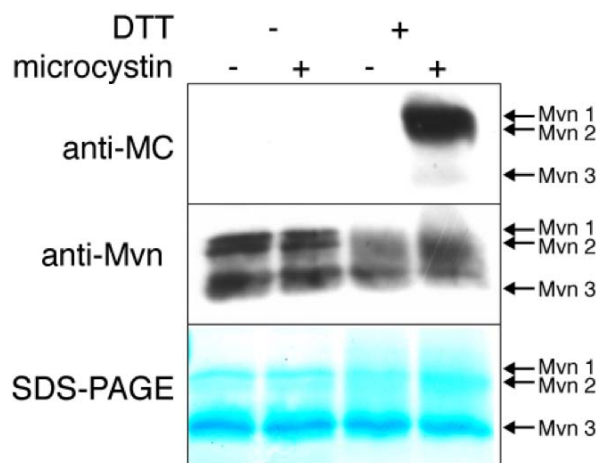
Fig. 19: Binding of microcystin to microvirin. Reduced (10 mM DTT) and non-reduced Mvn was incubated with microcystin and bound microcystin was detected by immunoblotting (anti-MC).

The first two lanes in **Fig. 19** contain the Mvn that was not reduced prior the assay, and three distinct bands that react with the anti-Mvn antibody are visible (Mvn1 and Mvn2 refers to the two upper bands running very close, Mvn3 refers to the lower band). No microcystin signal was obtained for the first two lanes. The third and forth lane contain the reduced protein. Upon reduction the two upper bands (Mvn1 + 2) vanished and were replaced by a more diffuse band in both lanes of the reduced Mvn. Obviously the absence of disulphide bonds resulted in a looser and flexible protein structure. The diffuse band in the lane with microcystin addition gave a stronger signal with the anti-Mvn antibody and a very strong signal with the anti-mc antibody. The results show that microcystin is capable of binding to Mvn under reducing conditions.

3.2.2 MICROCYSTIN BINDING CAN BE SUPPRESSED BY BLOCKING OF THIOL GROUPS

The experiment described in the previous section shows that a reducing environment is a prerequisite for the binding of microcystin to microvirin. This supports the hypothesis that microcystin reacts with the reduced thiol moieties of cysteine residues. However, other modes of binding that depend on e.g. structural changes occurring after the reduction of disulphide bonds might be possible. To exclude this possibility and to prove the initial hypothesis further the following assay was developed. Microvirin was reduced as described before and then dithio-*bis*(2-nitrobenzoic acid) (DTNB) was added to the protein mix. DTNB reacts with free thiol groups releasing one molecule of 2-nitro-5-thiobenzoate (TNB), while the second TNB forms a thioether with the free thiol group (**Fig. 20**). By this treatment the reformation of disulphide bonds was prevented and the protein remained in a state that mimics complete reduction. On

the other hand, no free thiols that could react with microcystin were accessible. Afterwards, the Mvn treated with DTNB was used to test microcystin as described in the previous section.
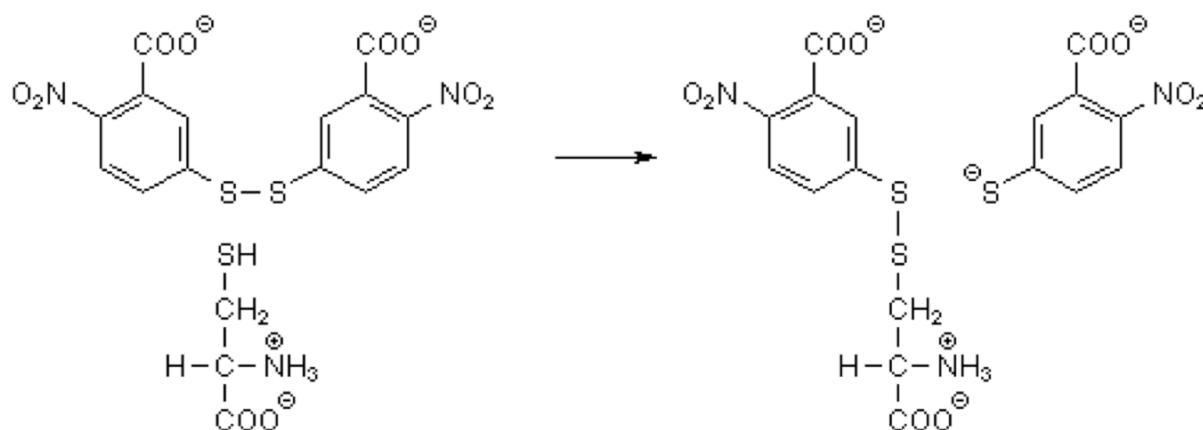
Fig. 20: 5-5″-Dithio-bis(2-nitrobenzoic acid) (DTNB or Ellman's reagent) reacts with the free sulfhydryl side chain of cysteine to form an S-S bond between the protein and a thionitrobenzoic acid (TNB) residue.

Fig. 21 shows the results of the assay. Binding of microcystin was observed in one single lane loaded with the reduced Mvn that was not treated with DTNB. In the following lane containing the Mvn, which was treated with DTNB after reduction, binding of microcystin did not occur. Again the binding was detected only at the upper bands (Mvn 1) like it was shown in the previous chapter. DTNB selectively reacts with the SH groups of cysteines and thus the inhibition of microcystin-binding after DTNB treatment proved that microcystin binds to cysteine residues.

DTT	+	+	+	+	-	-	-	-
DTNB	-	+	-	+	-	+	-	+
microcystin	-	-	+	+	-	-	+	+
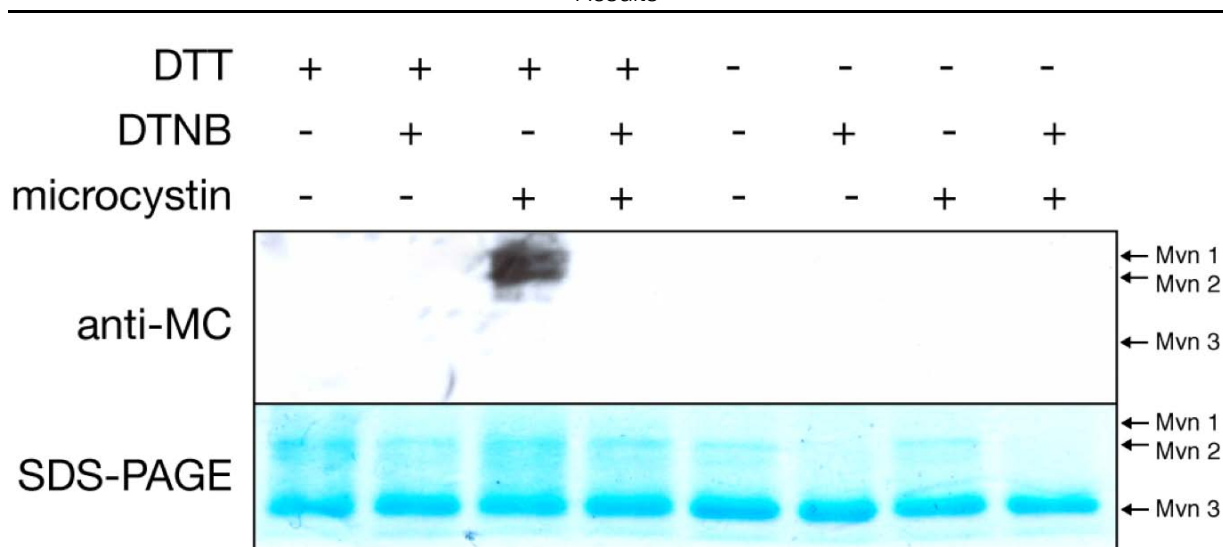
Fig. 21: Binding of microcystin requires reduced cysteine residues. Mvn was reduced with DTT following blocking of the free thiol groups with DTNB, before microcystin was added. Bound microcystin was detected by immunoblotting (anti-MC).

Apparently, microcystin was not able to replace the TNB from the cysteine residues. The binding of TNB is stable under oxidative conditions, but the TNB group can be released from the protein by reducing agents that are routinely used to reduce disulphide bonds (Tawfik, 2002). Thus microcystin cannot act as a reductant itself, but it is dependent on reducing conditions to facilitate binding to cysteine residues.

3.2.3 MICROCYSTIN BINDS TO CYSTEINES VIA *N*-METHYL-DEHYDROALANINE

The results described above unambiguously show that microcystin binds to free thiol groups of Mvn. Nevertheless the question remains, which part of the mc molecule interacts with these moieties. It was demonstrated (MacKintosh *et al.*, 1995) that microcystin binds to Cys273 of human protein phosphatase 1 (PP1) via the vinyl group of the *N*-methyl-dehydroalanine moiety of microcystin (**Fig. 22**A). The mechanism of microcystin binding to Mvn might be similar. To verify this hypothesis microcystin was covalently modified at the particular vinyl group by linking it to the thiol group of cysteamin (**Fig. 22**B). The cysteamin-modified microcystin was kindly provided by Dr. K. Ishida (Hans-Knöll-Institute, Jena).
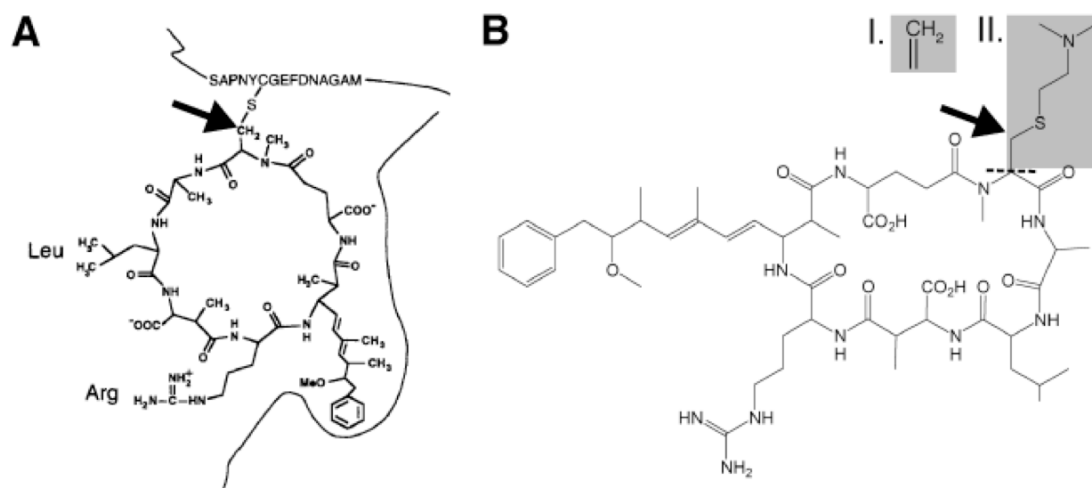
Fig. 22: Which part of microcystin is responsible for protein binding? A) Scheme of microcystin bound to a cysteine residue of a protein phophatase via the *N*-methyl-dehydroalanine moiety (from MacKintosh, 1995). B) Microcystin-LR (I.) and a modified variant with an cysteamin moiety attached to the vinyl group of the *N*-methyl-dehydroalanine residue.

The assay of microcystin binding was performed as described in the previous chapter, but only reduced Mvn was tested this time. Equal amounts of reduced microvirin were mixed with microcystin and the modified microcystin, separated by SDS-PAGE and an immunoblot was developed using the anti-mc antibody. The only signals could be observed in the lane containing the mixture of microcystin and Mvn, while the negative control without microcystin and the lane containing the modified microcystin did not show a reaction (**Fig. 23**). The identity of Microvirin was confirmed after stripping the blot and rehybridising it with the anti-Mvn antibody (data not shown). The anti-mc antibody is directed against the Adda-moiety of the molecule, so the recognition of microcystin should be unaffected by the cysteamin modification. The reactivtiy of the antibody against the modified microcystin was tested in an additional assay (data not shown).
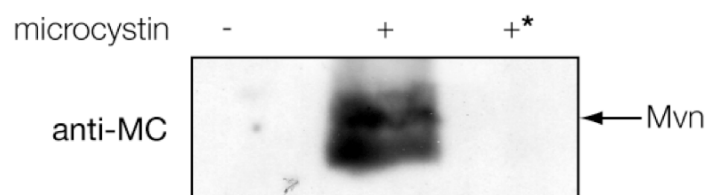
Fig. 23: Microcystin binds to cysteines via *N*-methyl-dehydroalanine. Binding of microcystin (+) and cysteamin-modified microcystin (+*) to microvirin.

3.2.4 INFLUENCE OF MICROCYSTIN BINDING ON THE OLIGOMERISATION OF MVN

The purified His-Mvn showed a different migration behaviour on SDS-PAGE depending on the presence or absence of reductive agents in the loading buffer and dimers or monomers were observed, respectively. The tendency to form dimers was also described for the homologous cyanovirin-N (Barrientos *et al.*, 2002) and little sequence alteration resulted in stable dimeric variants of the protein (Botos *et al.*, 2002; Han *et al.*, 2002; Kelley *et al.*, 2002). It seemed reasonable to analyse the state of oligomerisation of Mvn with microcystin bound to the protein. Usually, internal disulphide bonds enhance the stability of proteins, but on the other hand flexibility of the molecule is reduced. The binding of microcystin to the cysteine residues of Mvn might prevent or reduce disulphide bond formation of the protein and thus change the structural properties of the protein.

Gel filtration was conducted using aliquots of the samples from the microcystin binding assay (see chapter 3.2). **Fig. 24** shows the results of the experiment. The flow rate was set to 0.5 ml per min and the run was monitored measuring the absorption at 220 nm and 280 nm. The retention profile of each sample is shown in the graphs. Additionally, the collected fractions covering the size range above 2 kDa were dotblotted and probed with anti-Mvn and anti-microcystin antibodies. Images of the developed blots are aligned to the graphs showing the signals of the respective fractions. In all cases identical amounts of protein were applied to the FPLC column.

A major peak was detected after 24.5 min in all samples corresponding to a molecular weight of ~30 kDa, which agrees with a His-Mvn dimer (**Fig. 24**). Interestingly, the peak intensity as well as the Mvn signal observed in the westernblots of the particular fractions of each sinlge run varied and higher molecular weight forms were detected by western blots although no significant absorption was measured during gel filtration. The column used for the gel filtration was chosen with regard to the size of Mvn to provide a good resolution in the lower molecular weight range. Therefore, the retention profile for higher molecular weight compounds is rather steep. Thus the higher molecular weight forms of Mvn that were detected by westernblot (**Fig. 24**A+D) might be explained by these fractions being mixtures of multiple oligomeric forms of the protein consisting of varying numbers of monomers. The high molecular weight forms detected by the immunoblot covered the size range of ~90 kDa to ~240 kDa (**Fig. 24**A). These would have passed the detector one by another resulting in a low constant rather than a strong distinct signal. However, all forms were collected in a single fraction giving a strong signal on the immunoblot. A different affinity of the Mvn antibody to conformational variants of Mvn would be

a second explanation for the discrepancies between photodetection and immunoblot. Similar ambigous observations were made in aforementioned experiments (**Fig. 19**) where the antibody reacted in a stronger way with protein bands hardly visible on the stained SDS-PAGE gel, while bands highly abundant on the same gel displayed weak antibody reaction.

The appearance of higher molecular weight forms of Mvn was influenced by multiple factors. A direct comparison of the non-reduced protein with and without microcystin (**Fig. 24**A+B) showed that the presence of microcystin influenced the multimerisation of the protein. Nevertheless, microcystin was not bound to the protein as confirmed by immunoblot. With the presence of microcystin the protein was biased towards the dimer. Contrary observations were made using the reduced protein. Here the addition of microcystin led to the appearance of multimeric forms of the protein of approximately 240 kDa besides the dimer. The high molecular weight forms as well as the dimer could be shown to contain microcystin (**Fig. 24**D). Although the peaks in the FPLC chromatogram showed similar intensities compared to the non-reduced samples, the reduced protein showed a reduced reactivity with the anti-Mvn antibody.
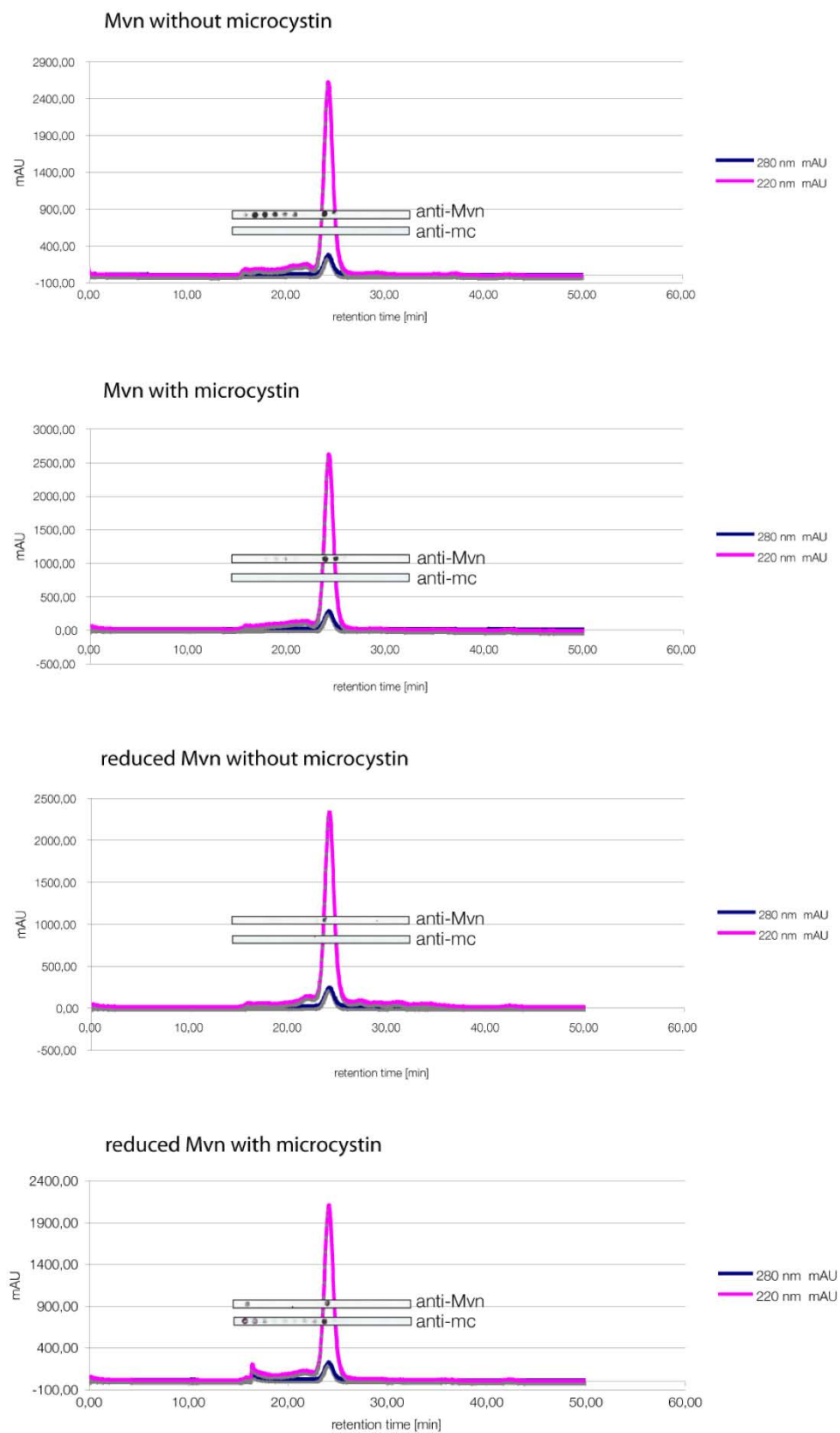
Fig. 24: Influence of microcystin binding and oxidation state on the oligomerisation of Mvn. Reduced or oxidised Mvn incubated with and without microcystin was subjected to gel filtration. The collected fractions were dotblotted and probed with anti-Mvn and anti-mc antibodies. The respective dotblots are projected on the coressponding elution profile.

3.3 Influence of oxidative stress conditions on microcystin-protein interactions

The binding of microcystin to cytosolic proteins of *M. aeruginosa* PCC 7806 *in vivo* was shown previously (Zilliges, 2007). The binding of microcystin to the large subunit of RubisCO (RbcL) was confirmed *in vitro* in the same study. In addition, the differential abundance of known and putative microcystin-binding proteins between wild type and microcystin-deficient mutant was reported. It was proposed that microcystin might influence protein stability. The putative function of microcystin was discussed to be similar to that of thioredoxins and other redox controlling molecules. However, the mechanistic details and the conditions that promote the binding of microcystin to its targets have not been addressed in the respective study. An involvement of microcystin in the response to redox stress conditions should alter the degree of microcystin binding to proteins compared to ambient conditions. To test the microcystin binding under oxidative stress conditions *M. aeruginosa* PCC 7806 was grown under low light conditions (16 $\mu Em^{-2}s^{-1}$) and was exposed to very high light (500 $\mu Em^{-2}s^{-1}$) at different culture densities for two hours. The same experiment was performed with *M. aeruginosa* PCC 7806 cultures, which were grown under iron starvation. Iron limitation also results in oxidative stress. Iron depletion was confirmed by monitoring the blue shift of the chlorophyll absorption peak at ~680 nm that is typical for cells expressing the iron stress induced protein IsiA (Dühring *et al.*, 2006). After exposure, the cells were harvested and proteins were isloated. Equal amounts of protein from each condition were separated by SDS-PAGE and transferred to PVDF-membranes. The blots were hybridised with antibodies against microcystin and microvirin.
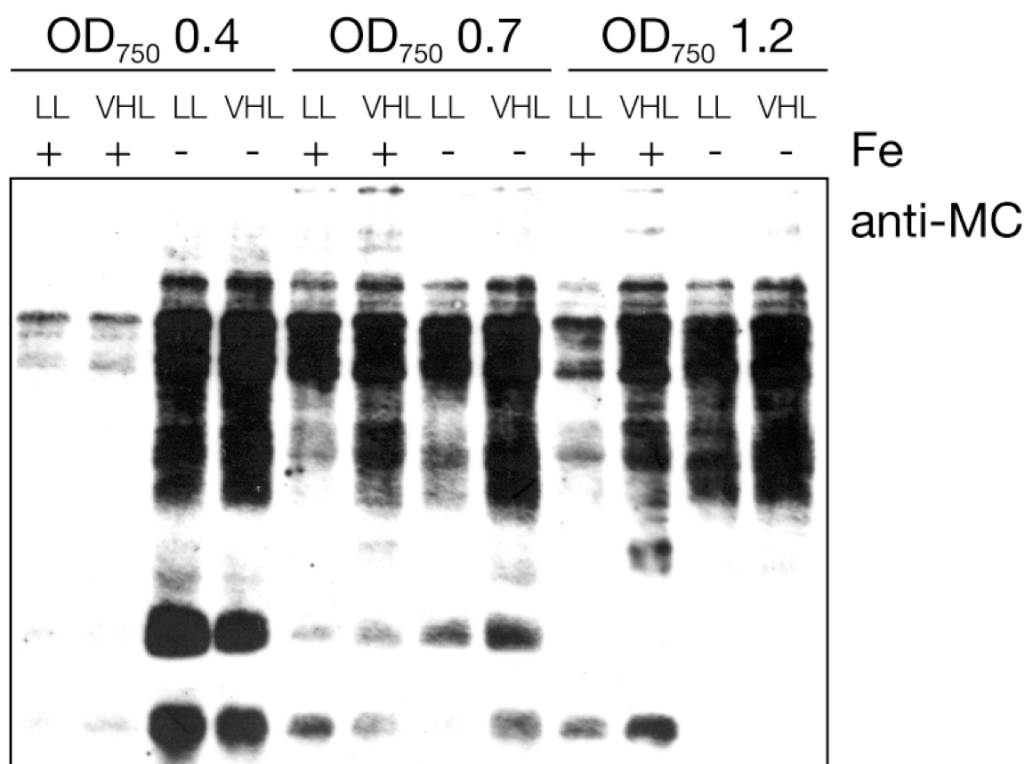
Fig. 25: Detection of protein-associated microcystin under oxidative stress conditions. *M. aeruginosa* cells were subjected to low (LL) and very high light (VHL) conditions for 2 h in culture medium with (+) and without (-) iron and sampled at different culture densities. The soluble protein fraction was probed with an anti-microcystin antibody.

The results summarised in **Fig. 25** show that the binding of microcystin to proteins is enhanced under oxidative stress conditions. Generally the cells grown under iron limited conditions exhibit much more microcystin binding compared to the cells grown in standard BG11 medium. The binding of microcystin was further enhanced when the cells were subjected to light stress by applying very high light. Microcystin binding was significantly lower in cells grown in standard BG11 medium, but an increased binding of microcystin to proteins was observed when the cells were transferred to very high light. This phenomen became more apparent with increasing culture densities and was hardly visible at the lowest culture density tested.

Microvirin was also affected by oxidative stress, but the effects were less pronounced. Under iron depletion two bands were deteced for microvirin (**Fig. 26**). It was shown in Chapter 3.2.4 that the presence as well as the binding of microcystin could alter the oligomerisation of Mvn. Furthermore, the gel filtrations of *M. aeruginosa* cell extracts (see chapter 3.1.4) confirmed that the high molecular weight bands observed on immunoblots represent the native state of the protein.
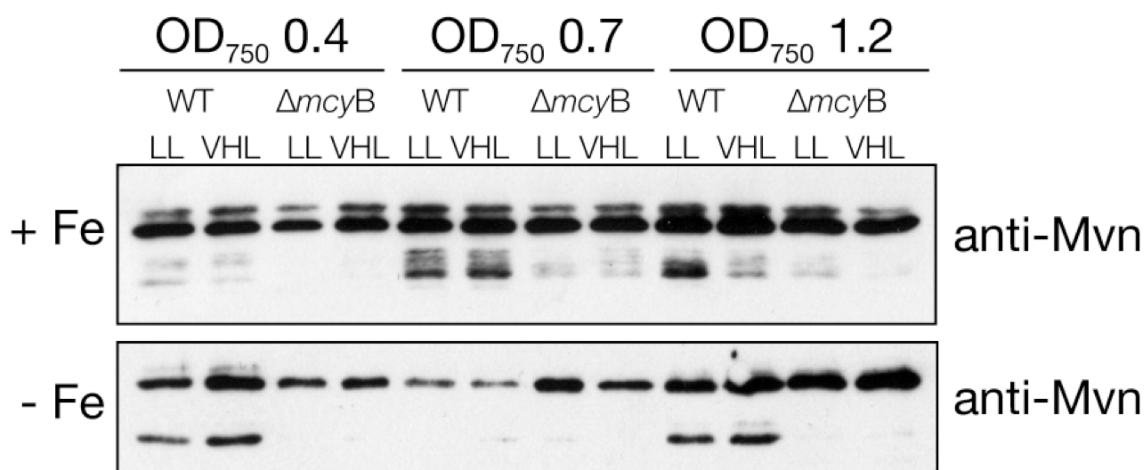
Fig. 26: Detection of Mvn under oxidative stress conditions in *M. aeruginosa* PCC 7806 wild type and Δ*mcy*B mutant. *M. aeruginosa* cells were subjected to low and very high light conditions for 2 h in culture medium with and without iron and sampled at different culture densities. The soluble protein fraction was probed with anti-Mvn antibody.

Fig. 26 shows that under iron rich as well as iron limited conditions no or only little alterations in the appearance of alternative forms of Mvn in the microcystin-deficient mutant could be detected. While significant differences can be observed between presence and absence of iron in the wild type, the pattern of Mvn expression is almost identical between these two conditions in the Δ*mcy*B mutant.

The first study on the expression of Mvn comparing wild type and Δ*mcy*B mutant (Kehr, 2003) not only revealed differences in the abundance of Mvn, but also it was also inferred that the putative binding partner must be differentially expressed. A conditionally changing expression of surface carbohydrates could be easily verified experimentally by a lectin binding analysis. An increased or decreased exposition of surface carbohydrates which represent binding sites for Mvn should result in a significantly altered green fluorescence signal.

A lectin binding analysis (see 2.2.3.13) was performed using culture aliquots sampled at OD_{750} 1.2 from the aforementioned experiment. A strong increase of green fluorescence could be observed in the wild type after the culture was subjected to either light or iron stress (**Fig. 27**). Interestingly, the cells were embedded in higher amounts of mucilage. As mentioned previously the green fluorescence coming from the mucilage does not correspond to bound FITC-Mvn, because it also appears in the controls (data not shown). In contrast to the wild type the Δ*mcy*B mutant did not show such a distinct response to the applied stress conditions. This held true for light stress as well as iron stress. Nevertheless, an enhanced binding of FITC-labelled Mvn to

the cell surface was observed in the wild type under VHL conditions only resulting in a bright green signal of the cell. The Δ*mcy*B mutant did not show such a response.
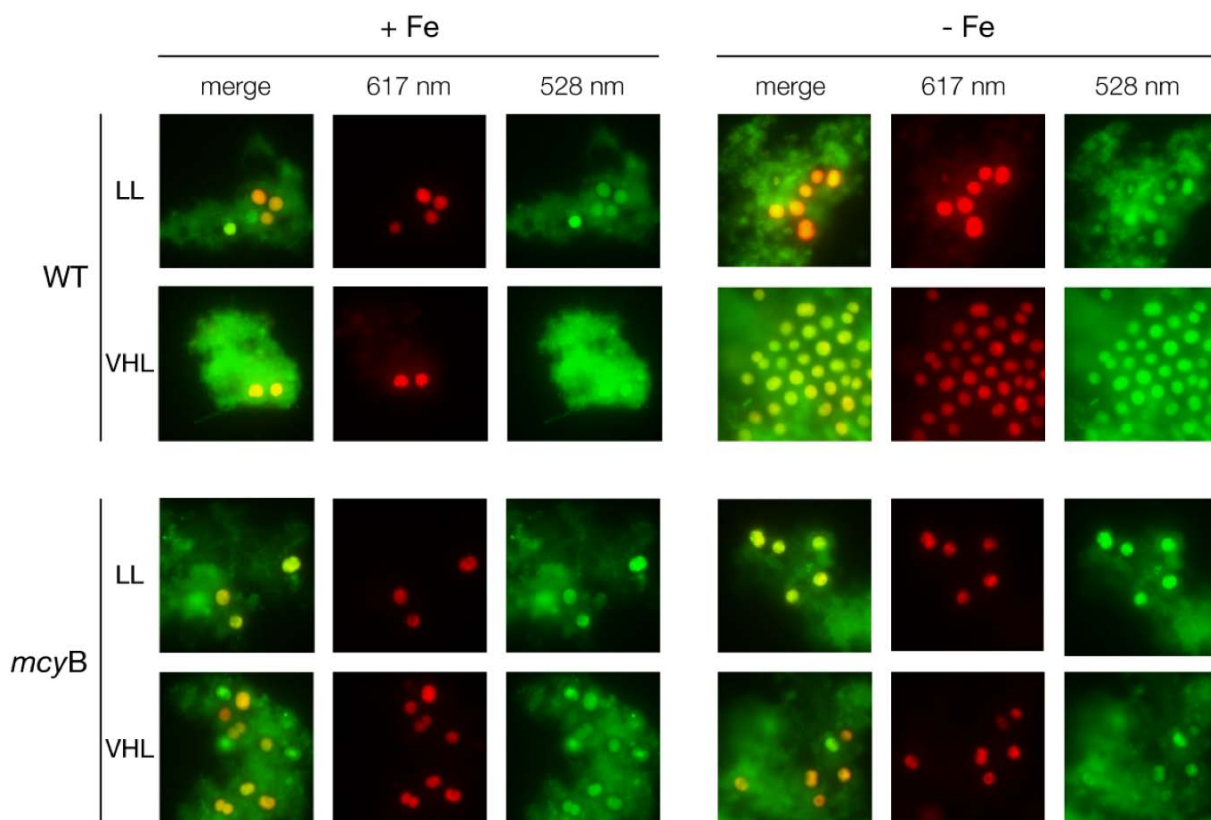
Fig. 27: Lectin binding analysis of *M. aeruginosa* **PCC 7806 wild type and Δ*mcy*B mutant under oxidative stress conditions.** *M. aeruginosa* cells were subjected to low and very high light conditions in culture medium with or without iron for 2 h and sampled at different culture densities.

These data suggest that microcystin affects the expression or activtiy of enzymes responsible for the synthesis of surface and extracellular carbohydrates. Since the binding of Mvn to LPS was shown, it must be assumed that the PCC 7806 wild type reacts to the stress conditions applied here by altering its LPS composition, while the microcystin-deficient mutant is unable to do so.

3.4 Occurence and Evolution of Mvn

3.4.1 DISTRIBUTION OF *MVN* AMONG DIFFERENT *MICROCYSTIS* SPECIES

The above results, especially the binding of microcystin to Mvn raised the question whether the occurence or certain sequence features of the protein correlated with microcystin production. It was already mentioned (see chapter 1.4.8.1) that proteins possessing the cyanovirin-N domains were also found in fungi and in a very small number of bacteria. However, the overall sequence identity of *M. aeruginosa* PCC 7806 to the fungal CV-N domain-like proteins is rather low and even the similarity to the cyanobacterial cyanovirin-N at the amino acid level is only 52%. This raised the question whether an adaptation of the protein to *Microcystis* strains producing microcystin occured. This might be represented by a predominat distribution in toxic strains or – in the case of an equal distribution among the genus – by the conservation of special sequence features. At the time this study was conducted genes encoding similar proteins in cyanobacteria were not found in the databases. Therefore, a PCR screening of *Microcystis* DNAs was performed using primers derived from the *mvn* gene sequence of *M. aeruginosa* PCC 7806. The DNAs used were obtained from strains of the Pasteur Culture Collection (PCC, France), the Microbial Culture Collection at the National Institute for Environmental Studies (NIES, Japan) and one strain from the Humboldt-University (HUB, Berlin).

The results of the PCR screening are summarised in **Tab. 6** and additional information on the presence of microcystins is given for the PCC strains (personal communication, M. Welker, Technical University, Berlin). The primers for the detection of the microcystin synthetase were derived from the conserved regions in the *mcy*E gene. The data on the toxicity of the NIES, CBS, MRC and MRD strains were obtained from the literature (Kaebernick *et al.*, 2001; Kaneko *et al.*, 2007; Kondo *et al.*, 2000; Nishizawa *et al.*, 2007; Tillett *et al.*, 2001).

The results do not show a complete correlation of microvirin and microcystin. Microvirin is not generally present in all strains of *Microcystis aeruginosa* and the gene was detected in a number of strains that do not produce microcystin. However, there seems to be a bias of cooccurence of microcystin and microvirin, because in almost all strains - with PCC 9806 being the one exception by giving ambiguous results in MALDI and PCR - *mvn* genes are present if microcystin biosynthesis genes are present. In other words, no microcystin-producing strain was found that does not contain *mvn* genes.

Tab. 6: Distribution of the *mvn* gene and *mcy* genes in *M. aeruginosa* strains as detected by PCR. Additionally the production of microcystin was assessed by MALDI-TOF (personal communication, M. Welker, Technical University Berlin). Some information was obtained from the literature ([1]Kondo *et al*., 2008 and Nishizawa *et al*., 2007; [2]Tillet *et al*., 2001 and Kaebernick *et al*., 2001; [3]Kaneko *et al*., 2007).

strain	MALDI	PCR	
	microcystin	microcystin	mvn
PCC 7005	-	-	-
PCC 7806	+	+	+
PCC 7820	+	+	+
PCC 7941	+	+	+
PCC 9354	+	+	+
PCC 9355	+	+	+
PCC 9432	-	-	+
PCC 9622	-	-	+
PCC 9624	-	-	-
PCC 9701	-	-	+
PCC 9717	-	-	-
PCC 9804	-	-	+
PCC 9805	-	-	-
PCC 9806	+	-	-
PCC 9807	+	+	+
PCC 9808	-	-	-
PCC 9809	+	+	+
PCC 9812	+	+	+
PCC 9905	-	-	-
PCC 100-24	-	-	-
PCC 100-25	-	-	+
HUB 5.3	n.d.	-	+
NIES 44	n.d.	-[1]	+
NIES 89	n.d.	+[1]	+
NIES 100	n.d.	-[1]	+
NIES 104	n.d.	-[1]	+
NIES 299	n.d.	-[1]	+
CBS	-[2]	+[2]	+
MRC	-[2]	+[2]	+
MRD	+[2]	+[2]	+
NIES 843	n.d.	+[3]	-

3.4.2 Evidence for the loss of Mvn in *M. aeruginosa* NIES 843

The whole genome sequences of the two toxic *M. aeruginosa* strains PCC 7806 and NIES 843 were published recently (Frangeul *et al.*, 2008; Kaneko *et al.*, 2007). Both strains are very similar to each other and can be regarded as the same chemotype. Genes for the production of microcystin and cyanopeptolin were identified in both strains. Furthermore, the genes encoding gas vesicle proteins are highly conserved between both. Interestingly, the NIES 843 strain does not contain an *mvn* gene in contrast to all other toxic *Microcystis* strains evaluated in conjunction with this study. The availability of the whole genome sequences of said strains made it possible to compare the genomic region of mvn from PCC7806 to the corresponding region in the NIES 843 genome.

A sequence stretch of ~5600 nt of the PCC 7806 *mvn* region was used as query in a BLAST search on the NIES 843 genome sequence deposited in the Cyanobase database (http://bacteria.kazusa.or.jp/cyanobase/cgi-bin/blastform.cgi?target=Microcystis).

The results of the BLAST search are visualised in **Fig. 28**. The organisation of the genes in the PCC 7806 strain is displayed schematically on the top and it is projected on the graphical representation of the BLASTN results using this very sequence as query. The BLASTN was performed on the whole genome sequence as well as on the annotated ORF database. Apparently, the whole sequence stretch shows some striking difference when compared to the NIES 843 strain. First of all, the entire query sequence is not clustered in one region of the NIES 843 but it is distributed over two distinct loci (blue and green overlay in **Fig. 28**) of its genome. Furthermore, for some positions no homologous sequence was identified in the NIES 843 genome (yellow overlay in **Fig. 28**), including the *mvn* gene sequence. Interestingly, these deletions (as compared to the PCC 7806 genome) are close to highly repetitive sequences (red overlay in **Fig. 28**). One of these repeated sequences was identified previously in PCC 7806 and resides upstream of the *mvn* gene and within the microcystin synthetase promoter region. In total this element occurs nine times in the PCC 7806 strain and was also found nine times in the NIES 843 genome. Rearrangements between the two *Microcystis* genomes were described previously and it was proposed that repeat elements play a role in those processes (Frangeul *et al.*, 2008). As to the comparison shown here it is striking that putative deletions and rearrangements took place close to highly repetitive loci, which is agrees with the proposal of Frangeul *et al.* (2008).
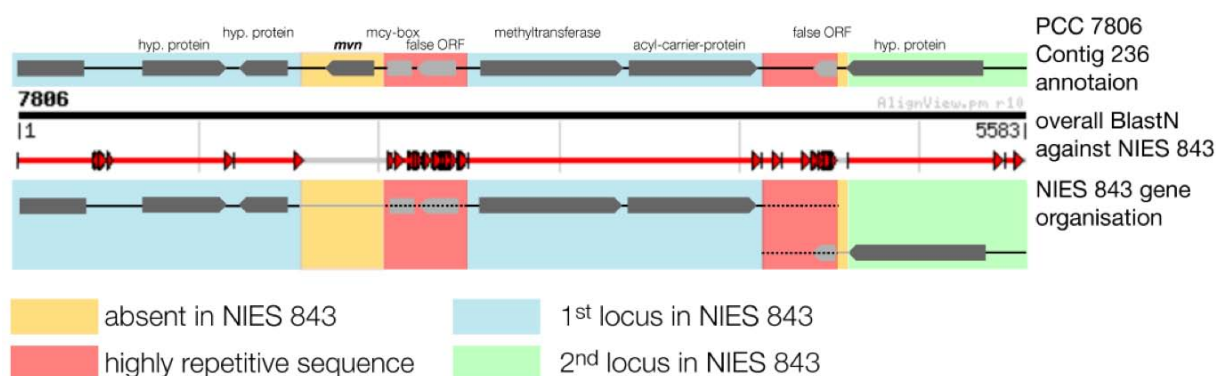
Fig. 28: Projection of the *mvn*-encoding genomic region of *M. aeruginosa* PCC 7806 on the genome of the NIES 843 strain. The homologous sequence is not clustered in the NIES 843 genome, but is scattered at two loci (blue and green). These two sequence stretches are flanked by highly repetetive sequences (red). Sequence stretches absent from NIES 843 (yellow) are in direct proximity to repetetive elements.

3.4.3 COMPARISON OF MVN SEQUENCES FROM VARIOUS *M. AERUGINOSA* STRAINS

The *mvn* gene was present in all microcystin-producing strains investigated. Nevertheless, it was also present in many strains that do not produce microcystin. The observation that microcystin binds to microvirin implies that the microvirin of the microcystin-producing strains might have evolved towards microcystin-binding and thus considerable differences should exist in the primary sequence of microvirins from toxic and non-toxic strains. In contrast to cyanovirin-N, the *M. aeruginosa* PCC 7806 protein for instance contains six instead of four cysteine residues. As shown in chapter 3.2 the thiol groups of the cysteine residues are the targets of microcystin. Therefore, additional cysteine residues may have evolved for this special function and occur only in microvirin from toxic strains. In order to test this hypothesis the PCR products from the strains analysed for the presence of *mvn* genes (previous chapter) were cloned and sequenced.

The alignment in **Fig. 29** shows the deduced amino acid sequences from the sequenced PCR products. The names of the toxic strains are highlighted in yellow and a grey background at the particular positions indicates differences to the sequence of PCC 7806.

Fig. 29: Alignment of *mvn* gene sequences from various *M. aeruginosa* strains. Grey boxes indicate differences in the amino acid sequences. The names of toxic strains are underlaid in yellow, non-toxic strains in orange.

The overall similarity of the Mvn sequences from the different *Microcystis* strains is very high. Only up to 7 amino acids are exchanged among the homologous sequences compared to the PCC 7806 protein and these are mostly conservative exchanges like glutamate to aspartate or aspartate to asparagine. A cysteine to serine exchange was found in four of five NIES strains, but this did not correlate with the presence of *mcy* genes.

3.4.4 PHYLOGENY OF CV-N DOMAINS

The alignment of Mvn protein sequences (**Fig. 29**) shows that the protein is highly conserved in *M. aeruginosa* strains. However, the database entries with highest similarities are hypothetical proteins from ascomycetes, while the only cyanobacterial homologue cyanovirin-N exhibits less overall similarity to Mvn. As noted in the introduction (see chapter 1.4.8.1) the CV-N family proteins have undergone an evolution that involved horizontal gene transfer events, duplications and the acquisition of additional domains. Hence, it seemed worthwhile to elucidate how the *Microcystis* homologues are positioned within the CV-N domain family.

The study of Percudani *et al.* (2005) already showed the CV-N domain to be an evolutionary conserved protein module and it represents a distinct protein familiy listed at the Pfam as well as the Interpro database. The similarity across this family is not sufficient to identify all of its

members by using a BLAST approach, but a hidden Markov model profile was succesfully used in the quoted study to find additional homologues. The following analysis was done using the protein sequences listed in the Pfam database entry PF08881 and the Mvn sequences obtained during this study and introduced in the previous chapter. An aligment was created with clustalX and used as input for the phylogeny programmes. Both methods used (see chapter 2.2.3.18) produced very similar trees, thus only the Bayesian phylogenetic tree of the respective sequences is shown in **Fig. 30**. Some CV-N domain family proteins contain additional conserved domains. Such members of the CV-N family are highlighted in the tree by pictograms showing the domain organisation of these proteins.

All *Microcystis* sequences cluster closely together as it was expected considering the high degree of sequence identity between them. Furthermore, the Mvn proteins from toxic and non-toxic strains do not form separate branches. The only exception is the Mvn from the non-toxic *M. aeruginosa* PCC 9701 that forms a branch relatively distant from the other *Microcystis* proteins. The *Microcystis* sequences are included in a main branch together with ascomycetes sequences. Detailed analyses of this branch revealed features shared by the *Microcystis* proteins and the fungi proteins of this clade. All sequences are rich in cysteine residues (number of cysteine residues are given in yellow squares), which is a common feature of secreted proteins. Thus, sequences were analysed for the presence of signals responsible for membrane translocation using signalP (http://www.cbs.dtu.dk/services/SignalP/). It turned out that all fungal proteins forming a branch with the *Microcystis* proteins were predicted to be secreted proteins. The only exception is the protein Q7S4I2 from *N. crassa*, but that contains an oligopeptide transporter domain with trans-membrane helices and shows cell membrane localisation.

A second branch only contains non-secreted proteins, which possess significantly smaller numbers of cysteine residues. A sub-branch of the non-secreted proteins contains CV-N domain family proteins that carry additional conserved domains. All of them bear a domain that at first was identified in *Rickettsia* as a surface exposed antigen. A further sub-branch contains proteins that additionally carry a peptidoglycan- and chitin-binding LysM domain. Interestingly, these proteins were predicted to be intracellularly localised.

Some sequences do not cluster with either of the main branches and are situated in-between them, which is reflected by the rather low clade probability values at the respective nodes. All other bacterial sequences, in partciular the sequence from the cyanobacterium *Nostoc ellipsosporum* belong to this group. The results for the subcellular localisation were ambiguous,

too. Some proteins clearly showed membrane translocation (Q2KGK0, *M. grisea*; Q0M6G4, *Caulobacter sp.*), while others showed no evidence for secretion (Q0LGR1, *H. aurantiacus*; Q2HHW1, *C. globosum*). The localisation of cyanovirin-N (CVN, *N. ellipsosporum*) could not be predicted with certainty, because there is no information on the gene sequence available. The protein sequence was determined experimentally and hence, a putative signal peptide was not captured (Gustafson *et al.*, 1997). On the other hand, Mvn from *M. aeruginosa* PCC 7806 does not contain a known translocation signal, but it is secreted in a so far unknown manner. Likewise, information on putative translocation signals in the other *Microcystis* homologues is missing, because sequences were obtained by using the *M. aeruginosa* PCC 7806 derived primer set. But obviously in all secreted sequences four cysteine residues are conserved at corresponding positions. These cysteines are also conserved in the *N. ellipsosporum* sequence and in all *M. aeruginosa* sequences (see alignment in the supplement).
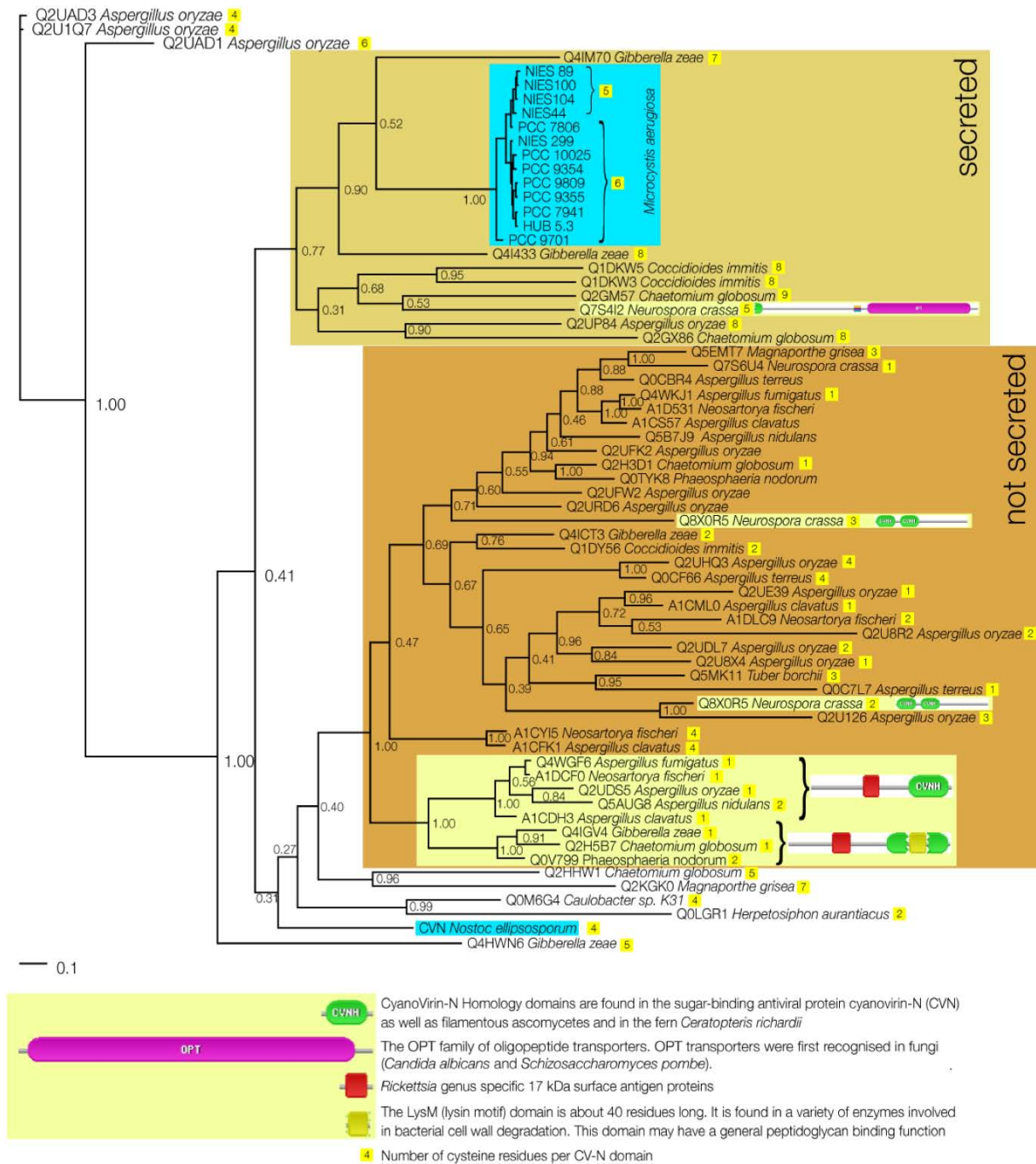
Fig. 30: Bayesian phylogenetic tree of CV-N domain proteins. Clade probability values are given at each node. Blue-green boxes highlight cyanobacterial sequences. Yellow boxes indicate the number of cysteine residues per molecule. Additional domains present in the respective protein are displayed. For two major branches the prediction of the subcellular localisation was consistent with the topology of the tree as indicated (secreted and non-secreted).

The question of the evolutionary origin of Mvn cannot be answered with certainty, but several issues are evident from the phylogenetic tree. First of all, the sequences are highly diverse, which is reflected by the occurence of additional domains, different localisation and the little

overall similarity of distant members of the familiy. For the same reasons, there seem to be diverse functions of the different proteins. Several fungi contain multiple CV-N domain proteins, like e.g. *A. oryzae*, which possesses 14 of these proteins spread over the whole tree occurring in almost all branches. This indicates that duplication and the acquisition of additional domains already took place in an early ascomycetes ancestor. The rare presence of CV-N domain proteins in bacteria suggests that Mvn and CV-N were acquirred by a horizontal gene transfer event. However, it cannot be judged from the data whether this happened independently in *Nostoc* and *Microcystis* or if both proteins share a common cyanobacterial ancestor. Additional homologues were not found in the complete genome sequences of cyanobacteria. During this study the screening for *mvn* sequences was limited to *Microcystis aeruginosa* strains, but a broader screening approach might yield sequences from other cyanobacterial genera.

3.5 Field studies

3.5.1 MORPHOTYPE DIVERSITY AND SAMPLE QUALITY

The immunofluorescence microscopy analyses of laboratory strains revealed the association of microvirin with the cell surface *of M. aeruginosa* PCC 7806. In addition, the results showed that microvirin is a strain specific protein and the lectin binding analyses demonstrated that the associated cell surface carbohydrate was present in a limited number of *Microcystis* strains. All laboratory *Microcystis* strains used in this study have lost their characteristic morphological features throughout prolonged cultivation. This is a commonly observed phenomenon (Reynolds *et al.*, 1981) that is not understood yet. The apparent changes in the colony morphology of laboratory strains - in particular the complete loss of colony formation - imply that especially factors involved in cell-cell attachment and extrallular matrix formation have changed during cultivation. Indeed, a significant loss of total carbohydrate per cell and a rather rough than smooth cell surface were observed in dispersed *M. aeruginosa* compared to colonial field samples (Zhang *et al.*, 2007). Therefore, the results obtained with the laboratory strains have to be interpreted carefully with regard to cell surface structures and cell-cell interactions. To overcome these limitations the immunofluorescence analyses were conducted using field samples that were collected at the Braakman Reservoir (Netherlands; kind gift of Dr. L. Tonk, University of Amsterdam).

Besides Mvn MrpC was included in the analysis. This extracellular glycoprotein was shown to be highly abundant in the Δ*mcy*B mutant and a role in cell-cell attachment was proposed (see

Chapter 1.3). Immunofluorescence detection of MrpC was done with laboratory strains and thus the detection of this protein seems to be reasonable with regard to the same arguments that also apply for Mvn.

The samples from the Braakman Reservoir contained only a small number of different morphotypes, which might be attributed to the time of sampling (mid of September 2007) in late summer. Usually a small number of cyanobacterial species dominates a certain lake later in the year (Znachor *et al.*, 2006). Moreover, some morphotypes of the sampled colonies could be hardly assigned to a certain *Microcystis* species, because the morphological characteristics were ambiguous. **Fig. 31** shows light micrographs of the most abundant morphotypes. Some morphotypes like *M. wesenbergii* (**Fig. 31**A+E) and *M. aeruginosa* (**Fig. 31**B+F) were identified with high certainty. Typical *M. aeruginosa* colonies were lowly abundant in the sample. Others were highly abundant like the types shown in **Fig. 31**C+G and **Fig. 31**D-H, but it was not possible to determine the species. Although both look relatively different, the determination of the species was difficult because several colonies were found that represented smooth transition between those two.

x 50   x 200
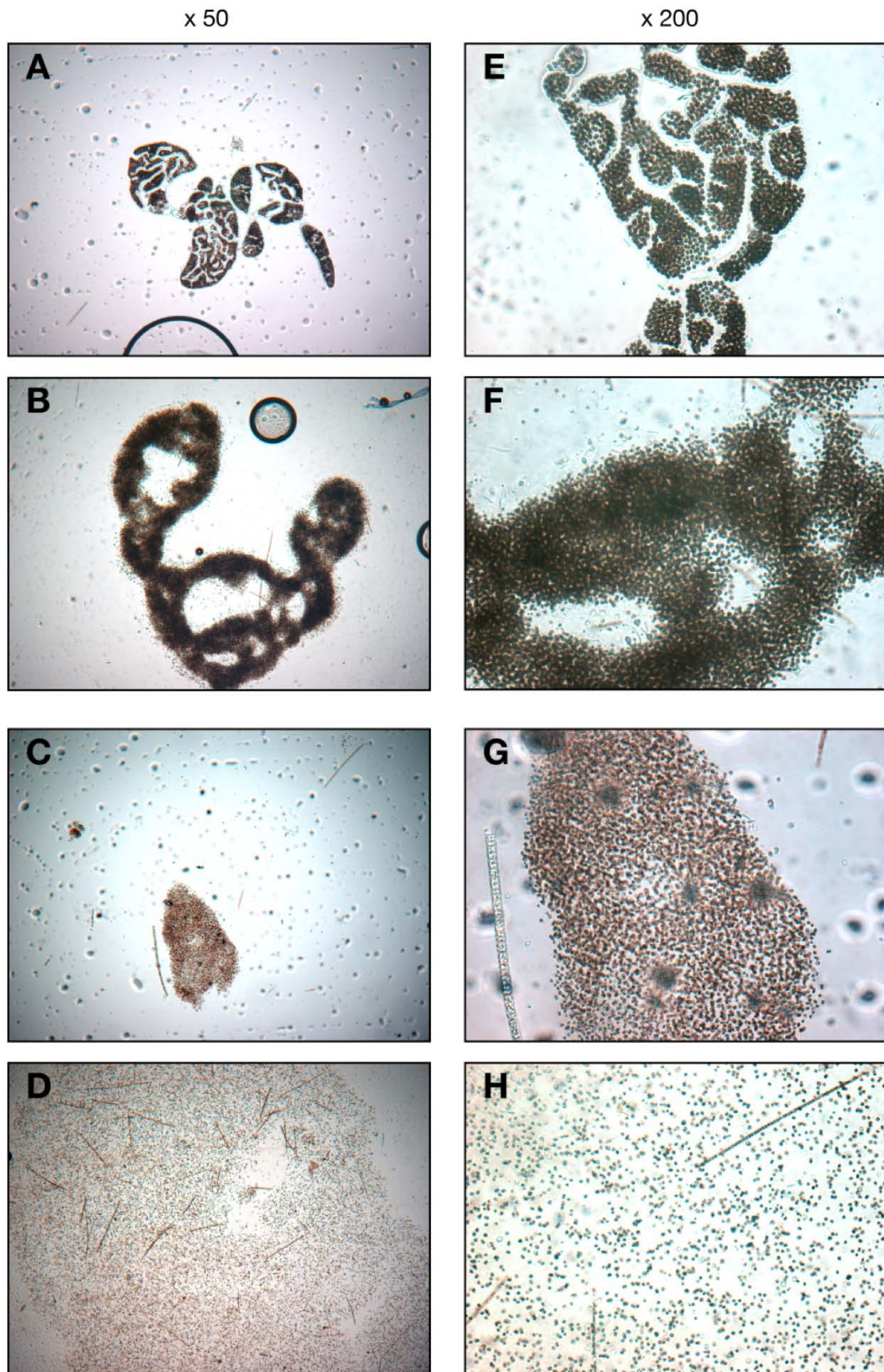
Fig. 31: Dominant morphotypes in samples from the Braakman reservoir. A+E) *M. wesenbergii*, B+F) *M. aeruginosa*, C, D, G + H) *Microcystis* with ambiguous morphology.

The next to be mentioned is the strong green autofluorescence that was observed in almost all colonies evaluated. **Fig. 32** shows a selection of images taken of control experiments. The excitation wavelengths are given for each image column where 617 nm were used for chlorophyll fluorescence and 528 nm excitated the FITC-labelled antibodies. The first columns show a merge of both wavelengths. Since representatives of all colony morphologies of the sample behaved more or less identically in the controls only a small selection is shown here. The two upper rows show colonies that were fixed and mounted on slides without any antibody added. A strong green fluorescence is visible that must result from compounds present in the cells. Such an intensive green autofluorescence was never observed when laboratory strains were examined under the fluorescence microscope.

The lower rows of pictures show cells that were only hybridised with a FITC-labelled secondary antibody against guinea pig IgG, which is used in combination with the anti-MrpC antibody. The green fluorescence is visible again, but does not differ from the green fluorescence of the controls in the upper pictures. Obviously an unspecific binding of the secondary antibody could be excluded. The control pictures obtained with the FITC-labelled secondary anti-rabbit IgG antibody that is used for detection of the anti-Mvn primary antibody looked very similar and are not shown here.

The green fluorescence visible in the control samples made it difficult to judge the obtained signals. The Mvn IFM in laboratary strains showed that positive signals clearly outshined the red autofluorescence in merged images and thus this was also considered as a criterium for a positive signal in the field isolates. The results for MrpC obtained from the laboratory strains (see Chapter 1.3) clearly showed a distinct localisation of the proteins at the cell surface resulting in a strong ring-like green fluorescence. Hence, only ring-like signals were considered to be a real signal in the following.

Initially it was tried to perform PCR on single colonies to confirm the immunofluorescence microscopy results by detecting the genes for the respective genes. Several attempts were undertaken, but the sample qualitiy was not sufficient to amplify DNA from single colonies (data not shown).

merge **617 nm** **528 nm**

no antibody

secondary antibody only
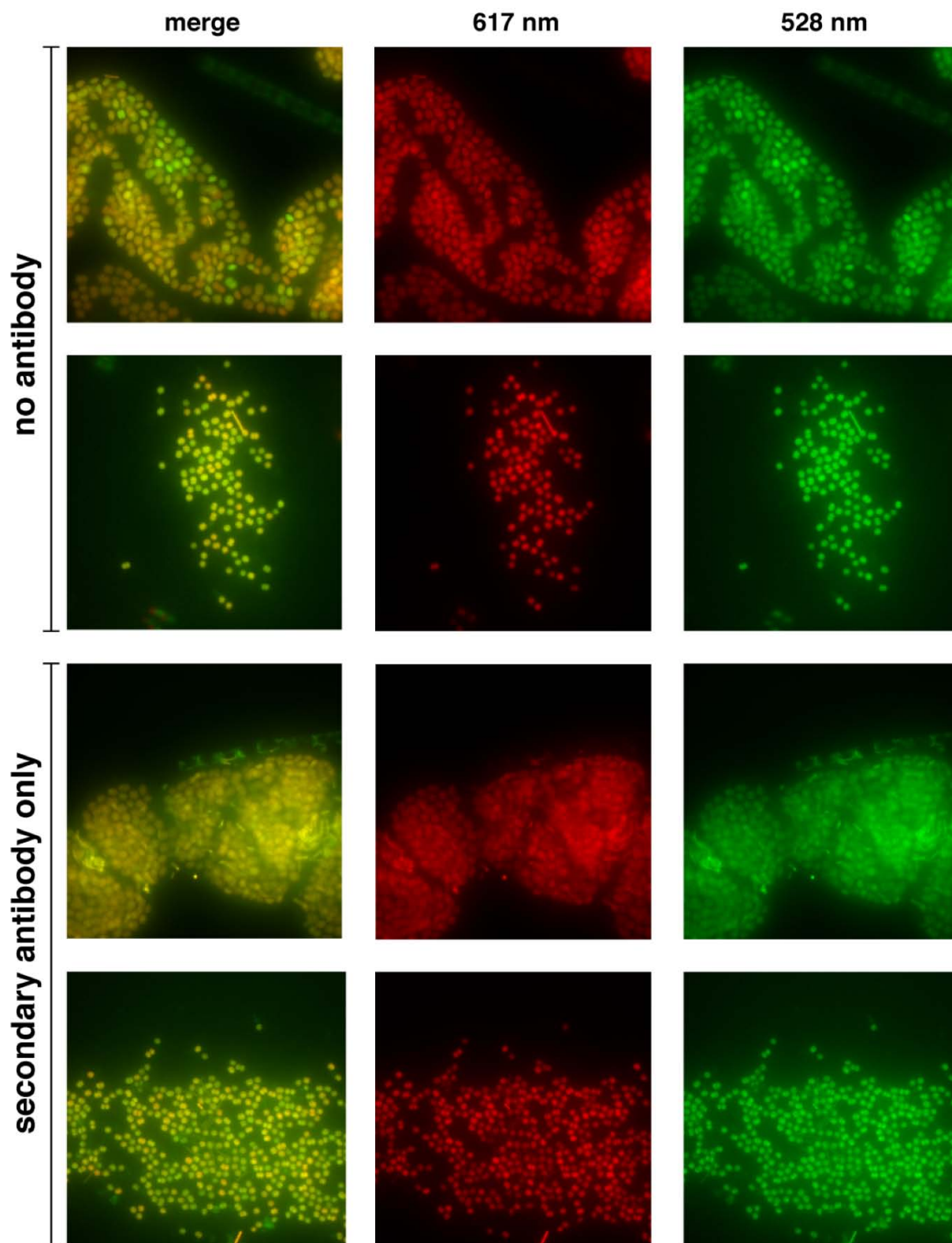
Fig. 32: Immunofluorescence microscopy on samples from the Braakman reservoir. The images show a representative selection of controls where either no antibody or only the secondary antibody was applied. A considerable green endogenous fluorescence is visible that did not originate from the FITC-labelled antibody.

93

3.5.2 Immunofluorescence detection of Mvn

Fig. 33 shows a selection of images of the immunofluorescence detection of Mvn in *Microcystis* colonies from the Braakman Reservoir. The rows A and B show examples of colonies that apparently do not express Mvn. The images displayed in **Fig. 33**B show a *M. wesenbergii* colony, which did never contain any Mvn. **Fig. 33**C+D show examples of cells expressing Mvn on their cell surfaces. In contrast to the laboratory strains analysed previously the green fluorescence is much more pronounced. Interestingly, the fluorescence signal appeared as a ring similar to that observed during the IFM analyses performed with the anti-MrpC antibody (see chapter 1.3). This corroborates a higher abundance of the protein at the cell surface compared to the laboratory strains. On the other hand one could speculate that higher amounts of the specific high mannose oligosaccharide are exposed, which would offer a higher number of attachment sites for Mvn.

Similar to the analyses performed on cultured *Microcystis* strains by PCR and fluorescence techniques (see chapter 3.4.1) the strain specificity of Mvn was ascertained. Furthermore, all cells of a particular colony equally expressed the protein. This indicates that the colonies are constituted of cells from the same or at least closely related *Microcystis* strains. However, it cannot be judged from this data whether a colony is clonal or is constituted from individual cells that belong to the same species. In the latter case the formation of colonies could be the result of cell-cell recognition mediated by surface exposed specificity factors such as carbohydrates and lectins.

merge	617 nm	528 nm

A

B

C

D

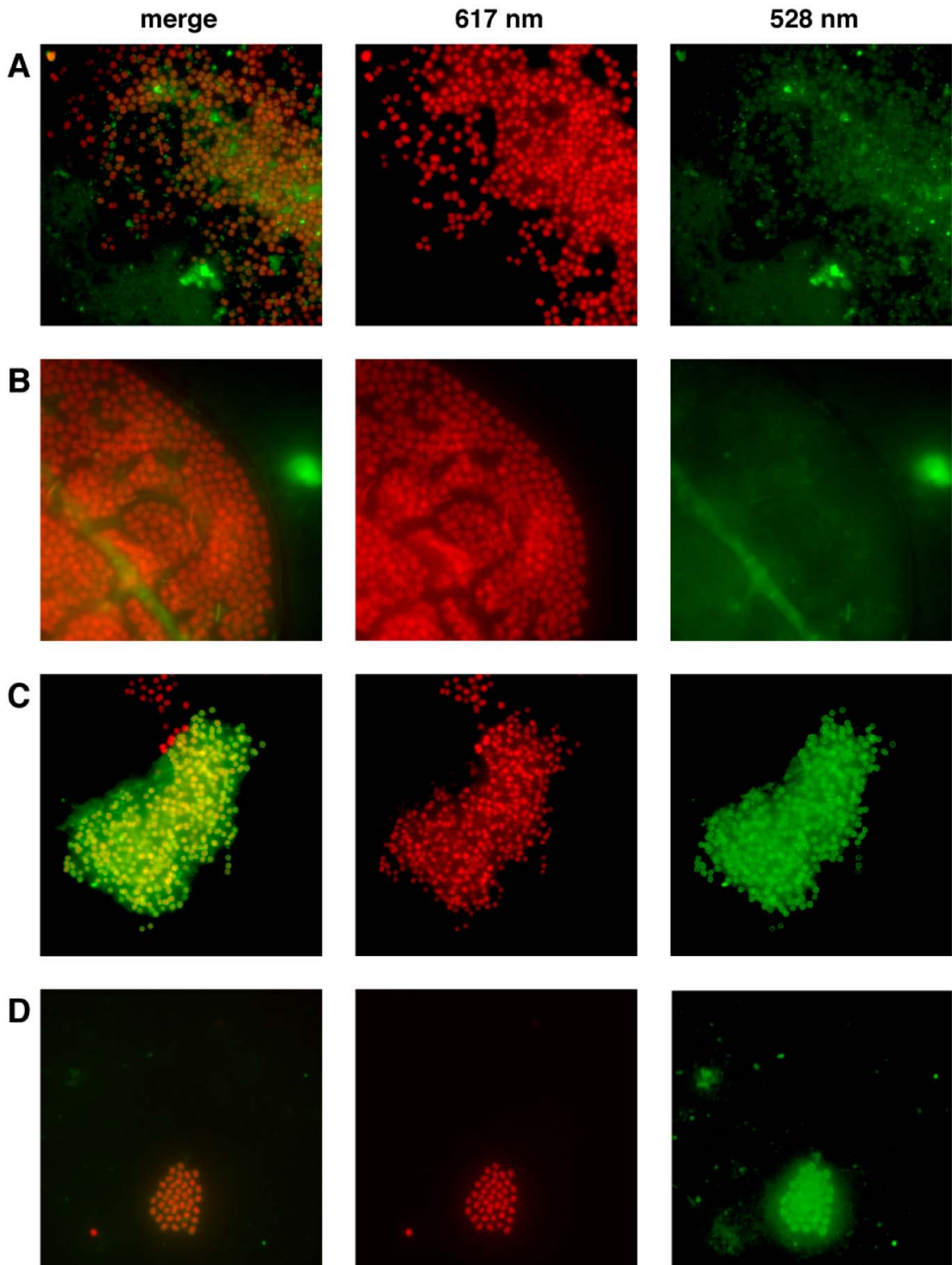Fig. 33: Detection of Mvn by immunofluorescence microscopy on samples from the Braakman reservoir. The images show a representative selection of colonies probed with an anti-Mvn anti-body and a secondary FITC-labelled antibody. A specific signal in addition to the endogenous fluorescence is visible in colonies of panel C and D.

3.5.3 LBA ON FIELD SAMPLES

The LBA show that exposure of oligomannose at the cell surface is a strain specific feature and said carbohydrate was detected in certain colonies (**Fig. 34**). Similar to the detection of Mvn the presence of oligomannose could not be clearly assigned to a particular morphotype. The colonies of *M. wesenbergii* that were easily recognised did never show a specific reaction with the FITC-labelled Mvn. The results strongly support that α1,2-linked mannan occurs in conjunction with microvirin in certain *Microcystis* strains.

merge	617 nm	528 nm

A

B

C

D
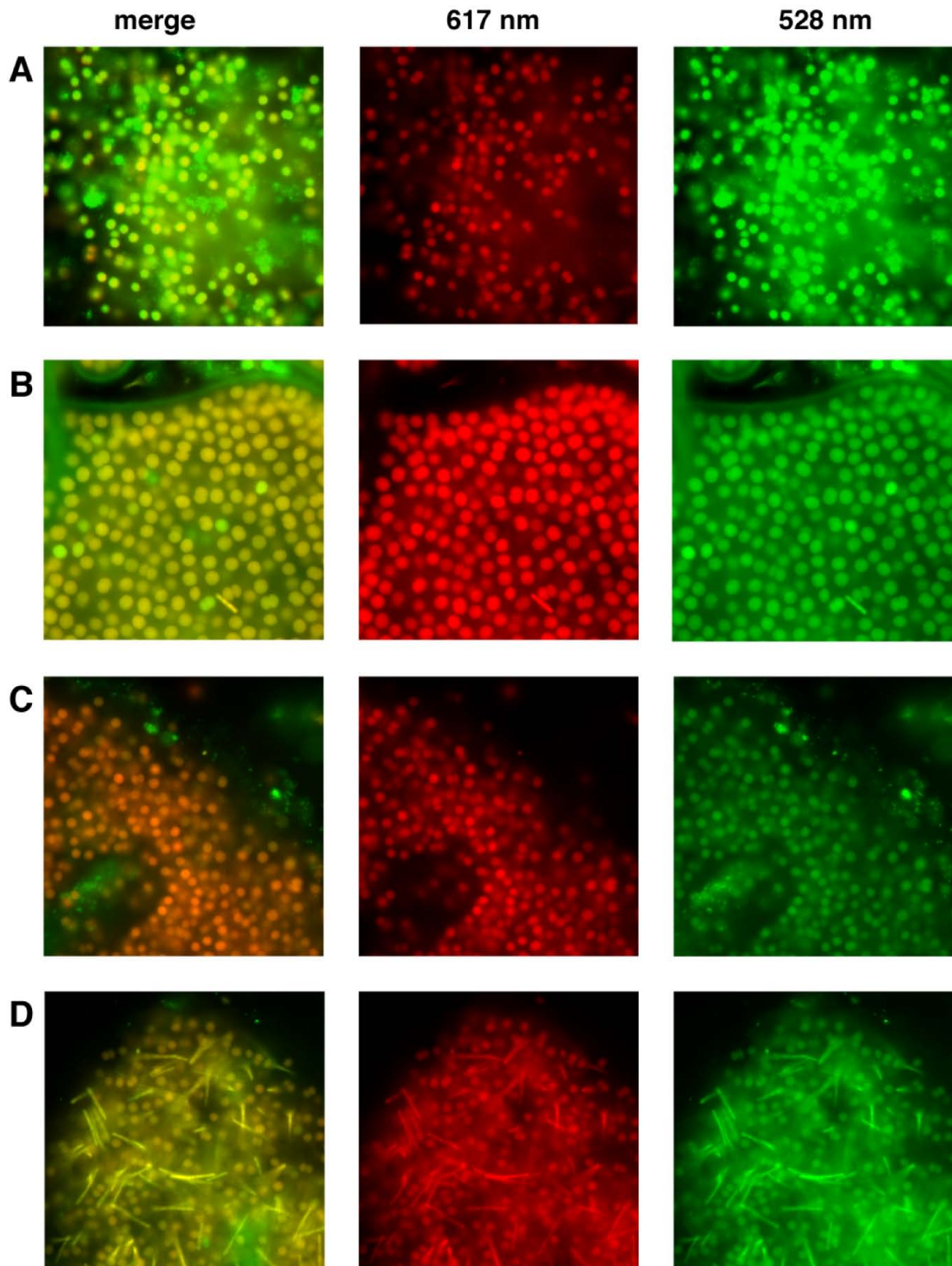
Fig. 34: Lectin binding anaylsis of samples from the Braakman reservoir. A strong signal is visible in the cells of panel A where the green fluorescence of the labelled Mvn by far exceeded the red autofluorescence in the merged image.

3.5.4 IMMUNOFLUORESCENCE DETECTION OF MRPC

The results for the detection of MrpC were rather similar to those of Mvn in terms of distribution among the isolated colonies. *M. wesenbergii* colonies did never contain the protein as shown in **Fig. 35**C where a *M. wesenbergii* colony is shown close to a cell of a different *Microcystis* species that did express MrpC. Cells that were positive for MrpC displayed a strong green ring-shaped fluorescence meaning that the whole cell surface was covered with MrpC proteins. Interestingly, the expression of MrpC showed a gradient through the colonies examined. This means that especially in larger colonies the cells in the center expressed little or even no MrpC, while the protein was highly abundant at the periphery of the colony (**Fig. 35**A+B). It can be excluded that this was an effect of unequal antibody labelling, because with the anti-Mvn antibody no trans-colony gradient was visible using the same sample.
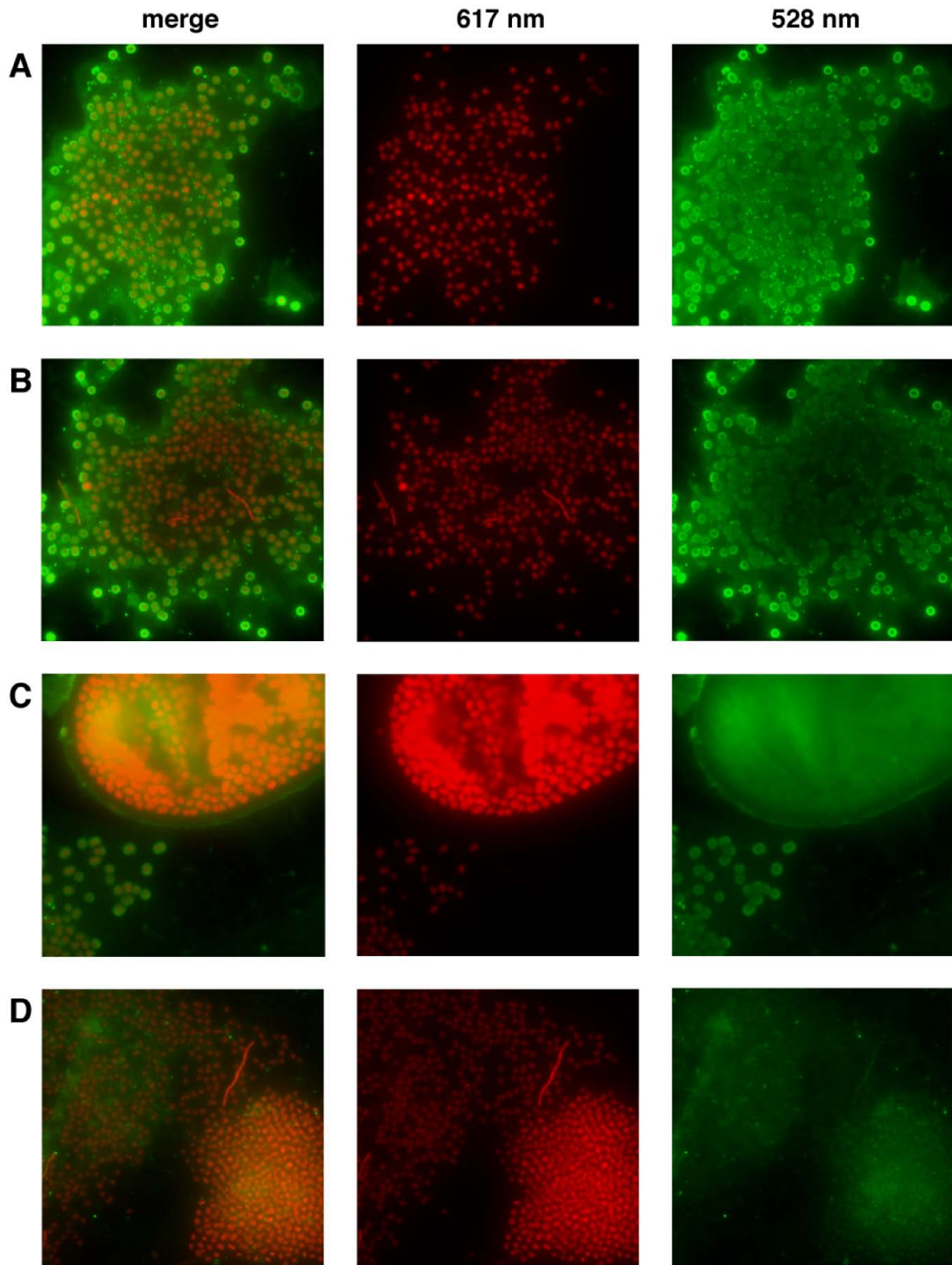
Fig. 35: Detection of MrpC by immunofluorescence microscopy on samples from the Braakman reservoir. The images show a representative selection of colonies probed with an anti-MrpC anti-body and a secondary FITC-labelled antibody. A specific signal in addition to the endogenous fluorescence is visible in colonies of panel A, B and the lower left colony of panel C.

3.5.5 THE MRPC ANTIBODY RECOGNISES ACCOMPANYING BACTERIA

An unexpected observation was made using the MrpC antibody for IFM studies on the field samples. Obviously the antibody cross-reacted with proteins on the cell surface of heterotrophic bacteria embedded in the mucilage of *Microcystis* colonies (**Fig. 36**A+B). Such bacteria were only detected in *Microcystis* colonies that expressed MrpC. The MrpC protein aliquot that was used to raise the antibody in guinea pigs was isolated from the supernatant of a *M. aeruginosa* PCC 7806 culture, because heterologous expression of the protein in *E. coli* was not satisfactory (Zilliges *et al.*, 2008). Thus, the protein was fully glycosylated with specific sugars. The polyclonal antibody might therefore not only recognise protein epitopes but also glyco-epitopes.

Probably the accompanying bacteria express carbohydrates on their cell surfaces that highly resemble those present in *M. aeruginosa*. The association with *Microcystis* colonies might be of substantial benefit for these bacteria, because it can provide a stable environment and the supply of nutritions. It was reported earlier that the axcenisation of cyanobacteria is difficult due to the bacteria present in their mucilage (Rippka, 1988). Here evidence is provided that these associations depend on specific interactions rather than accidental attachment. However, no effort was spent to investigate this in detail, but the observations made here may encourage future studies.

528 nm · 617 nm
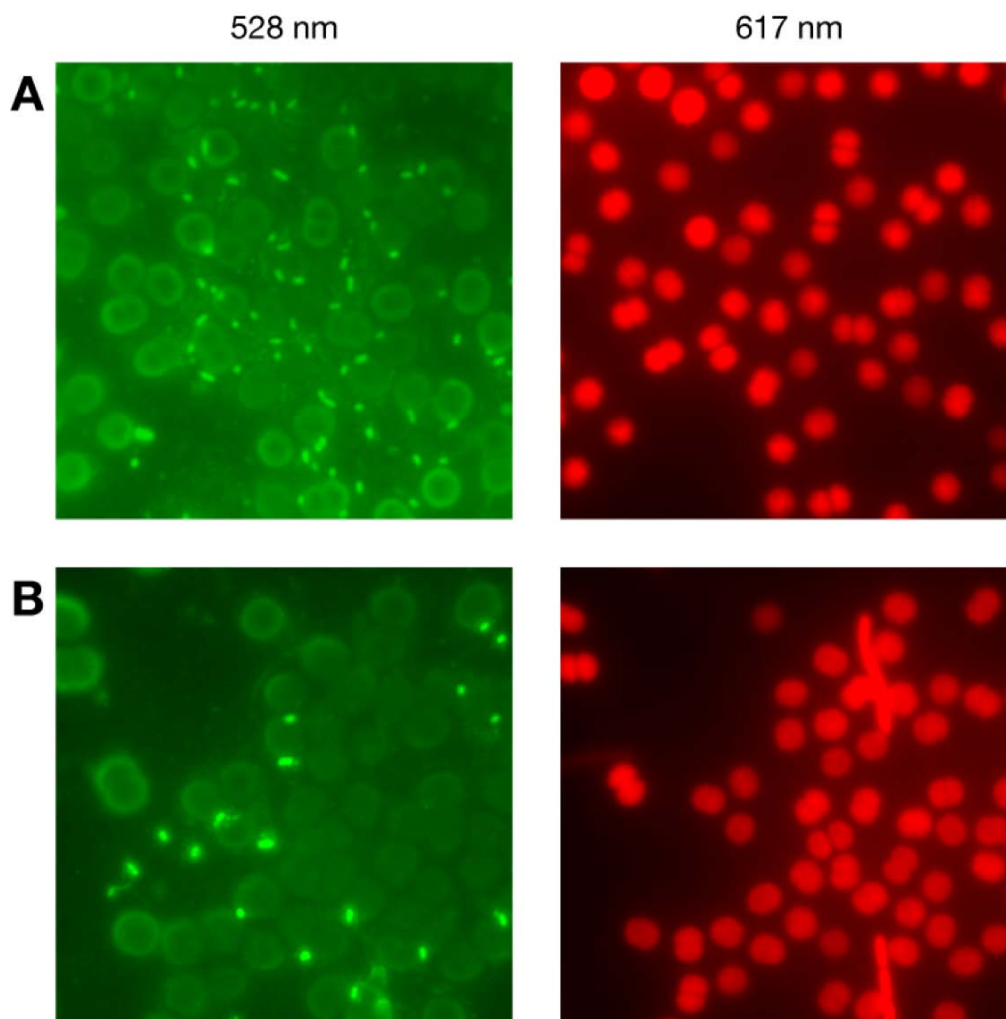
Fig. 36: Detection of MrpC by immunofluorescence microscopy on samples from the Braakman reservoir. In some colonies that expressed MrpC the antibody also recognised heterotrophic bacteria associated with the *Microcystis* colonies. Possibly the antibody recognised carbohydrate structures that are similar between the *Microcystis* cells and the associated bacteria.

4 DISCUSSION

Over the last twenty years much attention was drawn to research on toxic cyanobacteria. The occurence of toxic waterblooms of *Microcystis* is a worldwide phenomenon and being a feeding deterrent seemed to be the main function of microcystins for a long time. In recent years the idea that microcystins might be primarily of considerable physiological benefit for the producing strain, rather than being a toxin first and foremost has become widely accepted. A function apart from the toxicity of microcystin seems to be reasonable for different reasons. First of all, it was shown that the biosynthesis genes and the progenitor of today's microcystin are older than the eukaryotic predators of *Microcystis* (Rantala *et al.*, 2004). Some studies found evidence for microcystin to be an intercellular signal molecule (Dittmann *et al.*, 2001; Schatz *et al.*, 2007), while others reported an implication of adaptation to low carbon concentrations (Jähnichen *et al.*, 2007). The most comprehensive study so far compared the proteomic differences of *M. aeruginosa* PCC 7806 wild type and the microcystin-deficient mutant *mcy*B (Zilliges, 2007). In this study up to 20 % of the proteins showed a differential abundance. One of the heavily affected proteins was the extracellular glycoprotein MrpC. Morphological colony characteristics were already correlated with peptide production (Fastner *et al.*, 2001; Kurmayer *et al.*, 2002; Via-Ordorika *et al.*, 2004; Welker *et al.*, 2004). The formation of distinctly shaped colonies necessarily implies the presence of strain specific extracellular matrix compounds such as oligisaccharides, glycoproteins and lectins that were shown to play substantial roles in bacterial cell-cell attachment. The identification of the putative lectin Mvn (Kehr, 2003) and the glycoprotein MrpC (Zilliges *et al.*, 2008), both affected by microcystin in their expression, urged to focus on the function of these proteins to extend the understanding of the function of microcystin.

4.1 Limitations

Before the results will be discussed in detail a brief comment has to be made on the drawbacks that arose from the work with the strain *M. aeruginosa* PCC 7806 and other strains cultivated in the laboratory in general. This study was focused on the characterisation of the putative lectin Mvn for which an extracellular localisation and function seemed obvious. As already mentioned, the PCC 7806 strain has lost the ability to form colonies, a phenomenon observed in virtually all *Microcystis* strains that have undergone a continuous culturing in the laboratory. This might explain why the disruption of the *mvn* gene did not result in a macroscopically noticeable

phenotype. Thus an extensive biochemical charaterisation was carried out and first attempts were made to study Mvn in field samples. The data will be discussed in the following sections and a function of microvirin will be suggested.

4.2 General characterisation of microvirin

The characterisation of microvirin was mainly done using the heterologously expressed protein from *E. coli*. According to the homology to cyanovirin-N, initial assumptions were made and verified experimentally. Cyanovirin-N possesses internal disulphide bonds, forms dimers and is a mannan-specific lectin (Barrientos and Gronenborn, 2002; Barrientos *et al.*, 2002; Bewley, 2001; Bewley and Otero-Quintero, 2001; Botos *et al.*, 2002; Gustafson *et al.*, 1997; Shenoy *et al.*, 2002; Yang *et al.*, 1999). It was shown that all members of the CV-N domain family – despite the differences on the level of the primary sequence – bear a high level of tertiary structure conservation (Koharudin *et al.*, 2008). In the study of Koharudin *et al.* the CV-N homologues formed three phylogenetic groups and NMR as well as crystal structures were obtained from one member of each group. Mvn clustered with CV-N in this analysis. Therefore, it can be assumed that Mvn also shares many of the features of CV-N.

Microvirin was analysed by mass spectrometry and the presence of two intramolecular disulphide bonds was confirmed. Carbohydrate binding was proven and specificity for mannan, an oligosaccharide composed of $\alpha(1,2)$-linked mannose units, was corroborated. The outer cell surface localisation of Mvn was strongly suggested by phylogenetic analysis and proven by immunofluorescence microscopy. However, a translocation signal could not be identified whitin the protein sequence. Since all known prokaryotic protein secretion machineries depend on such a signal - whether cleaved from the precursor protein upon secretion or not - the mechanism of Mvn export remains elusive. However, the membrane translocation of the large subunit of the hydrogenase 2 devoid of a signal peptide was shown to be co-translocated with the small subunit possessing a twin-arginine motif (Rodrigue *et al.*, 1999). Therefore, it might be speculated that Mvn is translocated in a similar manner e.g. associated with a glycoprotein.

Mannan oligosaccharides were detected on the cell surface of *M. aeruginosa* by lectin binding analysis. Further analysis identified lipopolysaccharides as an interaction partner of Mvn. The protein did not bind to mutant cells defective in O-antigen production and a gel-shift was observed after incubation of Mvn with isolated LPS. However, the shifted 28 kDa band did not

fit to the 50 kDa and 54 kDa bands detected when cell extracts of *M. aeruginosa* PCC 7806 were analysed and also have been confirmed by gel filtration (see chapter 3.1.4).

Mvn most likely contains two carbohydrate-binding sites as inferred from homology to cyanovirin-N and thus an additional binding partner may exist *in vivo*. One possible canditate could be the glycoprotein MrpC that is attached to the cell surface of *M. aeruginosa* PCC 7806. Gel filtration analysis of *M. aeruginosa* extracts showed that the protein appears in high molecular weight forms between 120 and 170 kDa (Zilliges *et al.*, 2008). A corresponding gel filtration was also performed in this study and Mvn was detected in a size range between 55 kDa and 65 kDa agreeing with the bands observed on immunoblots of *M. aeruginosa* extracts. Hence, a tight interaction of MrpC and Mvn *in vivo* seems unlikely.

4.3 Microcystin binds to proteins

4.3.1 MICROCYSTIN BINDS COVALENTLY TO CYSTEINE SH-GROUPS OF MVN

The results presented in chapter 3.2 unambiguously show that microcystin is capable of binding to microvirin. The attachment of microcystin to a number of proteins in the cells of *M. aeruginosa* was proposed in several reports previously and was corroborated using diverse strategies. Immunogold electron microscopy was used to locate microcystin at distinct sites of the cell (Gerbersdorf, 2006; Young *et al.*, 2005), while others co-isolated microcystin together with thylakoids and phycobilisomes (Jüttner and Luthi, 2008). Zilliges (2007) identified microcystin to be bound to proteins that were separated by 2D electrophoresis. Furthermore the binding of microcystin to RubisCO was confirmed *in vitro*. For the first time, this study proposes a detailed mechanism of how microcystin binds to proteins and will suggest an expanded model of the function of microcystins in *Microcystis aeruginosa* based on the findings of Zilliges (2007).

Based on the rich data on the relation of microvirin and microcystin, such as the differential expression of microvirin in the *M. aeruginosa* PCC 7806 wild type and microcystin-deficient mutants, the microcystin phenotype of the Δ*mvn* mutant and the above mentioned literature about microcystin binding to proteins, the possibility that microvirin might also be a direct target of microcystin was considered. It was further assumed that binding of microcystin might occur by interaction with the thiol groups of cysteine residues which was described for the interaction of the toxin with eukaryotic protein phosphatases before (MacKintosh *et al.*, 1995; Runnegar *et al.*, 1993).

The presence of internal disulphide bonds in the heterologously expressed Mvn was confirmed by MALDI-TOF analysis (see chapter 3.1.3) and showed the general ability of Mvn to form disulphide bonds. However, the exact number and positions of disulphide bonds formed in the native protein in *M. aeruginosa* may differ from those in the recombinant protein. At least the presence of disulphide bonds within the molecule had to be taken into account before designing the microcystin binding experiments, because the basic prerequisites are different if thiols are freely accessible or involved in disulphide formation. The results of the initial binding assay summarised in **Fig. 19** (p. 70) clearly indicate that free thiols are a precondition for the binding of microcystin. This result supported the initial hypothesis that microcystin binds to cysteine residues and the fact that the attachment of microcystin endured the SDS-PAGE is a strong evidence for a covalent bond. However, an additional assay was designed to finally prove the hypothesis. It was shown that the binding of microcystin could be prevented if the free thiol groups of the reduced Mvn were blocked by a treatment with DTNB. As a consequence of DTNB treatment the establishment of disulphide bonds was impaired leaving the protein in a "quasi-reduced" state, but without free thiol groups. The absence of microcystin binding in this case showed beyond doubt that microcystin interacts with reduced cysteine residues.

It was further proven that the vinyl group of the *N*-methyl-dehydroalanine residue is the particular group that reacts with thiols of cysteine residues forming a thioether. Interestingly, microcystin remained bound to proteins under reducing conditions as it was shown for *M. aeruginosa* PCC 7806 wild type protein extracts separated by reducing SDS-PAGE (see **Fig. 25**). Generally, two cysteine residues can form a reversible disulphide bond, but the apparently irreversible attachment of a microcystin molecule to a free thiol group of a cysteine residue would lead to a dead end regarding all processes that depend on conditional disulphide bond formation.

4.3.2 GENERAL CONSIDERATIONS ON *IN VIVO* MICROCYSTIN BINDING

The binding of microcystin to cysteine-thiols strongly implies a role in redox regulation. It is widely known that many proteins in cyanobacteria are subjected to a tight redox regulation. These proteins contain conserved cysteine residues and regulation occurs through changes in the oxidation states of the proteins. RubisCO is the best investigated protein that is regulated by redox modulation. Oxidative conditions lead to the inactivation of RubisCO. This is achieved by oxidative cross-linking of RubisCO subunits by disulphide bonds (Garcia-Ferris and Moreno,

1994) and conformational changes render the enzyme sensitive to proteolytic degradation (Marcus *et al.*, 2003). Usually the oxidation of cysteine-thiols is reversible and depends on the ratio of reduced/oxidised redox-carriers such as thioredoxin (Trost *et al.*, 2006). **Tab. 7** summarises redox-regulated proteins that were identified by proteomic studies (Florencio *et al.*, 2006). In addition, it is indicated which proteins were shown to be differentially expressed in *M. aeruginosa* PCC 7806 wild type and Δ*mcy*B mutant (Zilliges, 2007). In the study of Zilliges around 500 proteins were identified and thus complete coverage of the proteome is not given. Therefore it might be expected that more compliance with thioredoxin targets will be gained if higher resolution proteomics are employed. The membrane fraction was excluded from this study in particular. This fraction includes all transmembrane proteins and proteins that are only partially inserted in the membrane whereas parts of them are exposed on either side of the membrane.

Tab. 7: Redox-regulated cyanobacterial proteins ([1](Lindahl and Florencio, 2003) and [2](Pèrez-Pèrez *et al.*, 2006). Those proteins that were differentially expressed in the microcystin-deficient mutant (Zilliges, 2007) are indicated (+). Additionally differentially expressed proteins that are involved in known redox-redgulated pathways are listed (e.g. CcmK).

ORF	Protein name	References	Δmicrocystin
Carbon dioxide fixation			
slr0009	Rubisco large subunit (RbcL)	1	+
sll1031	Carboxysomal protein (CcmM)	1	CcmK
sll1525	Phosphoribulokinase	2	+
Glycolysis and pentose phosphate pathway			
sll0018	Fructose-1,6-bisphosphate aldolase, class II	2	+
sll1342	Glyceraldehyde-3-phosphate dehydrogenase (Gap2)	2	+
slr0394	Phosphoglycerate kinase	2	
sll1841	Pyruvate dehydrogenase subunit E2	2	
sll1070	Transketolase	2	+
Glycogen metabolism			
sll0726	Phosphoglucomutase	1	
slr1176	ADP-glucose pyrophosphorylase	1	+
sll1393	Glycogen synthase (Glg2)	1	
sll0158	Glucan branching enzyme (GlgB)	1	
slr1367	Glycogen phosphorylase	2	
Sugar-nucleotide metabolism			
sll1212	GDP-mannose dehydratase	1	
sll0576	Sugar-nucleotide epimerase	1	
Sulphur metabolism			
slr1165	Sulphate adenylyltransferase	1	
slr0963	Ferrredoxin-sulphite reductase	1	
Nitrogen metabolism			
sll1499	Ferredoxin-GOGAT (GlsF)	1	GlnA
sll1502	NADH-GOGAT (GltB)	1	
slr0585	Argininosuccinate synthetase	1	
slr1133	Argininosuccinate lyase	2	
Tetrapyrrole biosynthesis			
sll1994	Porphobilinogen synthase	1	
Oxidative stress response			
slr1198	1-Cys peroxiredoxin (1-Cys-Prx)	1	+
sll1621	YLR109-homologue (Type II Prx)	1	
sll1987	Catalase-peroxidase (KatG)	2	
Light harvesting			
ssr3383	Phycobilisome core linker (L_C)	1	
slr0335	Phycobilisome core-membrane linker (L_{CM})	1	
sll1577	Phycocyanin β-subunit	2	
RNA metabolism			
sll1789	RNA polymerase β'-subunit	1	
sll1787	RNA polymerase β-subunit	1	
sll1043	Polyribonucleotide nucleotidyl transferase	2	
Protein synthesis and folding			
slr0557	Valyl-tRNA synthetase	1	
slr1550	Lysyl-tRNA synthetase	2	
slr1463	Translation elongation factor EF-G	1	
sll1099	Translation elongation factor EF-Tu	1	1
sll1804	30S ribosomal protein S3	1	
slr2076	60-kDa chaperonin 1 (GroEL)	1	
Redox regulation			
slr0623	Thioredoxin (TrxA)	2	
Unknown			
slr1855	Hypothetical protein	2	

The finding that microcystin might interfere with thioredoxin-dependent redox regulation results in fundamental consequences. Apparently, microcystin itself is not a redox active compound since microcystin binding requires reductive conditions and binding of microcystin to oxidated cysteine residues was never observed in any *in vitro* assay. The attachment of DTNB is reversible if a reductive agent is present, but microcystin was not able to displace the bound DTNB. Hence, the binding *in vivo* occurs under the same conditions as thioredoxin interacts with its targets. Reductive conditions exist under light when thioredoxins are reduced using the reducing power available from NADPH through NADPH-linked thioredoxin reductases (Florencio *et al.*, 1988) or via ferredoxin through ferrodoxin-thioredoxin reductase (Jacquot *et al.*, 1997).
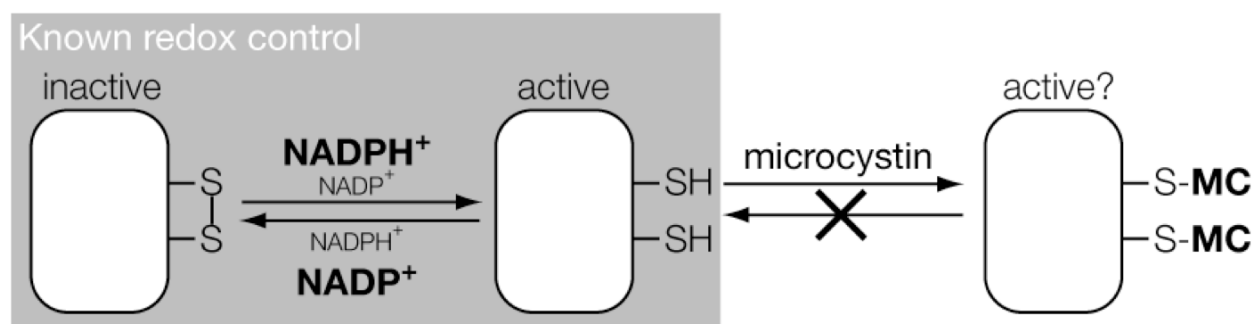
Fig. 37: Schematic representation of redox control through the oxdidation state of cysteine residues. Microcystin bound to cysteine residues might prevent the oxidative disulphide bond formation that renders enzymes inactive.

As mentioned above, microcystin established a rather stable thioether bond with targeted cysteine-thiols that endured the harsh treatment of reducing SDS-PAGE conditions. Thus, once bound to the target protein, microcystin remains attached and will circumvent the establishment of disulphide bonds (see **Fig. 37**). This might be beneficial under enhanced oxidative stress conditions or conditions where not enough reducing power is available to completely maintain active enzymes. In the case of RubisCO, bound microcystin could inhibit the cross-linking of subunits and prevent proteolytic degradation of the enzyme.

The results of the *in vitro* binding assays imply an irreversible binding of microcystin to target proteins. However, the *in vivo* situation might be different and reversibility of microcystin binding could be achieved by factors absent from the *in vitro* assays. Given the stable attachment of microcystin to Mvn it can be speculated whether such an antagonist could be an enzyme specifically cleaving the thioether. On the other hand microcystin-free proteins might simply be generated by *de novo* synthesis. This question has to be addressed in future studies.

4.4 Impact of microcystin on microvirin

The impact of microcystin on microvirin might be similar to what has been discussed about the general function of microcystin, but additional and more detailed information could be included here. The alteration of the oxidation state of the cysteine-thiols can influence the structure of a protein as it was shown for the example of RubisCO before. Information was gathered that this also applies for Mvn. The gel filtration experiments (see chapter 3.2.4) clearly demonstrated that microcystin bound to Mvn partially results in different multimeric isoforms of the protein. The anti-Mvn antibody exhibited a decreased reactivity against Mvn under these conditions indicating that the protein had undergone extensive structural changes. In addition, the immunoblots of the oxidative stress experiment (see chapter 3.3) confirmed that additional Mvn signals appear under conditions that also facilitate an enhanced binding of microcystins to proteins in general. Since lectins usually do not exhibit any enzymatic activity the question arises, which functional relevance these structural changes have.

4.4.1 MICROCYSTIN COULD AFFECT THE CARBOHYDRATE BINDING PROPERTIES OF MVN IN MULTIPLE WAYS

Earlier studies (Kehr, 2003) showed that the differences in the abundance of Mvn in wild type and ΔmcyB mutant cannot be related to differences on the level of transcription, which implies that regulation occurs posttranslationally in a way that microcystin affects the stability of the protein. This might happen in manner similar to the mechanisms discussed above.

Structural changes as a result of microcystin binding might also alter the carbohydrate binding properties of microvirin. Aromatic residues like tryptophan and phenylalanine are often present in carbohydrate binding pockets of lectins, because they can interact with nonpolar patches formed by protons and carbons at the epimeric centers of sugars. The introduction of a tryptophan residue into the rat serum mannose-binding protein by site-directed mutagenesis changed the specificity towards galactose (Iobst and Drickamer, 1994). The tryptophan residue packed with the apolar face of the galactose, whereas it was incompatible with the mannose (Kolatkar and Weis, 1996). Microcystin, which contains the aromatic Adda residue, might have a similar effect on the carbohydrate binding properties of Mvn by binding to the protein.

A second influence on the carbohydrate binding properties might result from changes in the oligomerisation of Mvn upon microcystin binding. The gel filtration experiments performed with

Mvn associated with and without microcystin showed that microcystin influences the oligomerisation of the protein (see chapter 3.2.4). The number of monomers that make up a multimeric lectin determines the number of carbohydrate binding sites in such a multimer. Hence, an influence of microcystin on the multimerisation would have direct influence on the sugar binding capabilities of Mvn. It can be assumed that an increasing number of binding sites results in multiple interactions and thus in a stronger binding or a stronger cross-linking of multiple binding partners. It was shown that multivalent carbohydrate binding and crosslinking is responsible for the HIV-inactivating activtity of CV-N (Barrientos *et al.*, 2006). Mutants of CV-N unable to undergo domain swapped dimerisation were devoid of crosslinking activity (Matei *et al.*, 2008).

Domain swapping as in CV-N (Barrientos and Gronenborn, 2002; Kelley *et al.*, 2002) might also be the mechanism that drives the multimerisation of Mvn. Domain swapping describes a process in which a structural element or domain of one peptide chain is interchanged with the corresponding element of a second identical peptide chain, resulting in an intertwined homooligomer (Bennett *et al.*, 1995). Domain-swapped dimers or open-ended fibrils are formed upon the destabilisation of the monomer via an intermediate open monomer conformation (Bennett and Eisenberg, 2004; Nagradova, 2002). The energy barrier stabilising the monomer can be overcome by changes in physiological conditions (Liu and Eisenberg, 2002) like pH (Zegers *et al.*, 1999) or the reduction state of the protein (Lee and Eisenberg, 2003). In the case of microvirin, the binding of microcystin to the protein might help to overcome the energy barrier by disrupting the ordered structure of the monomer. Indeed, preliminary small-angle X-ray scattering (SAXS) measurements – a method used to determine the shape of macromolecules in solution (Forster *et al.*, 2005) – strongly suggested an influence of microcystin on the oligomerisation of Mvn (data not shown; Prof. Dr. A. Thünemann, Bundesamt für Materialforschung und -prüfung, personal communication).

4.4.2 INFLUENCE OF MICROCYSTIN-BINDING ON DISULPHIDE BOND FORMATION

Microcystin binds to free thiols of cysteine residues and normally the cysteine residues of cytoplasmic proteins are in a reduced state. The situation is entirely different for extracellular proteins that are commonly released to an oxidative environment. Gram-negative bacteria possess the DsbAB oxidoreductases located in the periplasmic space that facilitates the formation of disulphide bonds prior to the secretion of proteins (Collet and Bardwell, 2002). Just very recently such a system was identified in cyanobacteria (Singh *et al.*, 2008) and it can be

speculated whether the binding of microcystin disturbs the correct formation of disulphide bonds. Indeed, differently migrating forms of Mvn were oberved on immunoblots of samples from the wild type under oxidative stress conditions. In particular the ratio of the 54 kDa and 50 kDa forms of Mvn was shifted towards the 50 kDa form (see **Fig. 26**). This effect was not observed in the ΔmcyB mutant and thus has to be attributed to the absence of microcystin. Interestingly, the majority of DsbA mutants, which are unable to establish disulphide bonds, showed defects in the secretion of extracellular proteins (Coulthurst et al., 2008; Ha et al., 2003) and led to the alteration of colony morphology (Mavrodi et al., 2006). In some species the biofilm formation and attachment was impaired in a ΔdsbA background (Genevaux et al., 1999; Lee et al., 2008). An impaired secretion of Mvn due to the binding of microcystin might therefore modulate Mvn-mediated cell-cell interactions.

4.5 Implications on microcystin detection and risk assessment

Apart from the physiological function of microcystin discussed here it is a matter of fact that dissolved microcystin in water bodies represents a serious health threat (Christoffersen, 1996; Codd et al., 2003; Codd et al., 2005; Falconer et al., 1983; Falconer and Runnegar, 1987; Runnegar et al., 1988; Runnegar et al., 1993). A reliable quantification is necessary to ensure the employment of a lake as e.g. drinking water supply or for recreational purposes without harm. The binding of microcystin raises fundamental questions on the detection of microcystins in the environment. Here it was shown that the degree of microcystin binding depends on the conditions of culturing. Proteins isolated from cells that were subjected to oxidative stress conditions due to iron depletion or high light exposure were associated with microcystin to a higher degree than the controls. The outcome of this work and previous studies (Gerbersdorf, 2006; Jüttner and Luthi, 2008; Young et al., 2005; Zilliges, 2007) defined new prerequisites for the detection of microcystins. Common methods for the monitoring of microcystin contamination include enzyme linked immunosorbent assays (ELISA), protein phosphatase inhibition assays (PPIA) and HPLC (McElhiney and Lawton, 2005; Msagati et al., 2006; Sivonen, 2008). Depending on the method used, different amounts of microcystin might be detected. In this work the microcystin bound to proteins was detected using an antibody that is also used for the detection of microcystins by ELISA and thus samples evaluated by ELISA include bound and free microcystin. Contrarily HPLC would only recognise free microcystin, which most likely applies for PPIA, too. In order to evaluate the toxicity of a given sample the latter methods are more suitable, whereas ELISA would overestimate it. On the other hand, ELISA would give a

more reliable estimation of the total microcystin content. The ratio of free and bound microcystin might change over time depending on the physiological state of the cells. Therefore, a long term monitoring is recommended. The quantification of the total microcystin by ELISA should be used for samples taken over a period of time to assess changes in the net production in a monitored lake. The results of this work imply that the attachment of microcystin to proteins is irreversible, but most experiments were performed using an *in vitro* system and even the whole cell extracts of *M. aeruginosa* investigated represent only a snapshot. It cannot be excluded that microcystin is released from the proteins upon changes in the physiological state of the cell. Thus the conclusions made in this study must be confirmed by further culture experiments and field studies.

In previous experiments where the effect of an external microcystin addition on *M. aeruginosa* was studied, a drop of the microcystin level below the concentration of added microcystin was observed (E. Dittmann, Humboldt-University Berlin, unpublished results). The recent results shed new light on those experiments, which may now serve as starting point for future research. It would be worthwhile to compare the microcystin content determined with ELISA and HPLC before and after cultures were subjected to oxidative stress conditions.

4.6 Implications from field studies

The analysis of cyanobacterial field samples using fluorescence microscopy techniques has not been done previously. This study showed that this technique might be used to achieve a better understanding of *Microcystis´* ecostrategy and its communal behaviour for reasons discussed in the following. It is widely known that *Microcystis* strains differ in their morphological characteristics and here it was unambigously shown that the molecular basis for these differences is to be found in the presence of strain-specific proteins and carbohydrates released to the proximate environment of the cell. Both Mvn (see chapter 3.4.1) and MrpC (Zilliges *et al.*, 2008) were shown to be strain specific, which also applied for the high mannan recognised by Mvn. Although no proof was given in this study it seems that Mvn and the specific mannan only occur in combination.

The analysis emphasised that there are considerable differences between strains that have been cultivated in laboratory for a long time and those strains freshly isolated from the lake. The loss of colony formation after prolonged cultivation is a widely described phenomenon and easily recognised macroscopically, but the here-introduced methods provide tools to track

down the molecular mechanisms. Furthermore, the detailed investigation of cell surface carbohydrates and the associated proteins as well as the carbohydrate composition of the extracellular mucilage might elucidate the molecular mechanisms that are the basis for the different morphotypes of *Microcystis*.

Dynamics were observed in the expression of cell surface oligosaccharides in response to oxidative stress in the laboratory strain as it was shown by the LBA technique (see chapter 3.3). This might be one factor contributing to the stress adaptation in the lake. The gradient of MrpC abundance in single colonies that was observed suggests that there might be a "division of labour" between cells of a colony. A gradient similar to the MrpC expression pattern was described previously for the fixation of CO_2 by *Microcystis* colonies. A partitioning of CO_2 fixation between peripheral and internal cells (**Fig. 38**) was shown using ^{14}C-labelling (Paerl, 1983). Moreover, a correlation between carbon availabilty and microcystin production was described (Jähnichen *et al.*, 2007) that connects both phenomena. Jähnichen *et al.* showed that the microcystin net production was low upon high C_i availability whereas Zilliges *et al.* showed that MrpC abundance was drastically increased in the ΔmcyB mutant and hence in the absence of microcystin. It may thus be assumed that the gradient of carbon availabilty in **Fig. 38** might cause a microcystin gradient, which in turn would perfectly explain the MrpC gradient observed in the field samples (see **Fig. 35**).
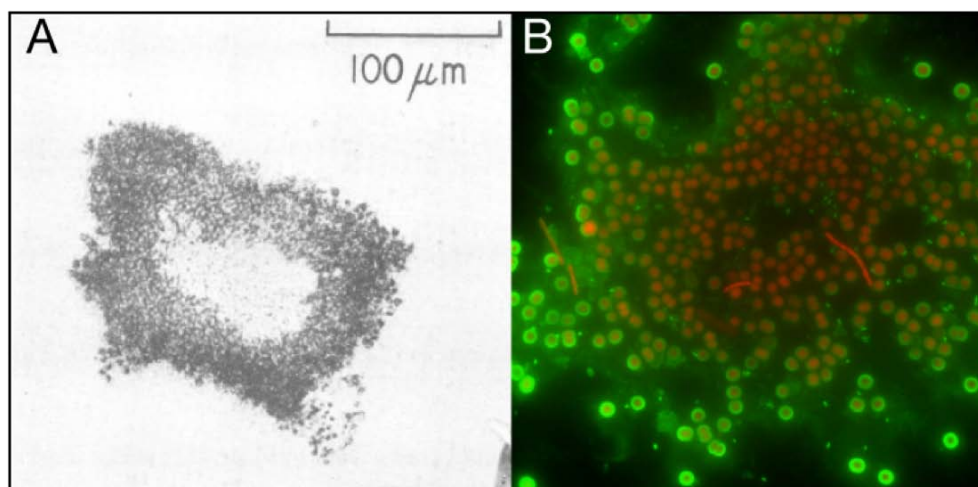
Fig. 38: Gradients of A) CO$_2$-fixation activitiy and B) MrpC expression in *M. aeruginosa* colonies. The fixation of CO$_2$ was followed using radiactively-labelled CO2 and the highest activtiy was observed at the periphery of the colonies (Paerl, 1983). MrpC was detected by IFM (see chapter 3.5.4).

However, these observations need to be verified by a systematic approach. Such an experiment could involve lake sampling and the isolation of colonies of varying diameter from different water layers during a longer time period. It would be interesting to establish an *in situ* method for the detection of microcystin in colonies to elucidate the distribution of micocystin within these.

4.7 Hypothesis for the function of microvirin

4.7.1 MVN IN INTRASPECIES INTERACTIONS AND MORPHOTYPE DETERMINATION

The rich data provided on the biochemical properties and the expression of microvirin in *M. aeruginosa* PCC 7806 wild type and microcystin-deficient mutant allows drawing a model of Mvn function *in vivo*. Referring to the current knowledge of the roles of bacterial lectins presented in the literature, it becomes obvious that the focus lies on the interaction of pathogens with their respective hosts mediated by lectin-carbohydrate interactions (Cambi *et al.*, 2005; Davies *et al.*, 2001). However, the mechanisms described for these interactions certainly apply for lectins of non-pathogenic bacteria. A well-investigated example is the initiation of symbiosis between *Rhizobia* and legumes (Hirsch, 1999; Karr *et al.*, 2000; Sharon and Lis, 2002). What are the fundamental mechanisms of these interactions? Generally,

attachment of bacteria to host cells is facilitated by lectins provided by one partner, which recognise carbohydrate structures on the other partner. In the case of host defence, immune lectins bind to capsular polysaccharides or LPS on the bacterial cell surface (Kurata *et al.*, 2006; Lloyd *et al.*, 2007; Moran *et al.*, 2005; Sahly *et al.*, 2008). The same mechanism drives the initial recognition of symbionts by the legume host (Bolanos *et al.*, 2004; Garcia-Pino *et al.*, 2007; Hirsch, 1999). Apart from interspecies interactions bacterial lectins play an important role in the intraspecies recognition and attachment like those occuring during the formation of biofilms (Banas *et al.*, 2007; Diggle *et al.*, 2006; Greiner *et al.*, 2005; Lynch *et al.*, 2007; Tielker *et al.*, 2005).

Here, the presence of a lectin and the corresponding carbohydrate was shown in the same organism, which implies that this pair is implicated in intraspecies rather than interspecies interactions. It was already mentioned that *Microcystis* assembles to complex cell communities that show characteristic colony morphology for distinct species. These morphological differences between the species must have their origin in an individual set of molecular factors, most likely polysaccharides, glycoproteins and lectins. A few reports deal with these factors in *Microcystis* (Jürgens *et al.*, 1989; Papageorgiou *et al.*, 2004; Raziuddin *et al.*, 1983; Weckesser *et al.*, 1979), but to date no broader study was conducted that has systematically compared the extracellular proteome and glycome of different *Microcystis* species. Here, the lectin microvirin was characterised and it was shown that this protein is strain specific. The *mvn* gene was detected in a subset of *Microcystis* strains from culture collections. The protein and the corresponding polysaccharide were shown to be present in few colonies isolated from field samples. Although the morphotypes present in the environmental samples could not be determined without doubt, the absence of Mvn in cleary assigned *M. wesenbergii* colonies was demonstrated in all cases. Further investigations have to be done to identify lectins and determine carbohydrate structures of different *Microcystis* species. Such analysis might reveal factors, which are unique characteristics of a certain species. Indeed, lectins were isolated from various *Microcystis* strains previously, but the function of these has not been revealed yet (Jimbo *et al.*, 2000; Yamaguchi *et al.*, 1998; Yamaguchi *et al.*, 2000; Yamaguchi *et al.*, 1999). Especially the lectin binding analysis introduced here for the use on cyanobacteria provides a sophisticated way to characterise surface carbohydrates. Many lectins with a broad range of carbohydrate specificities are readily available as fluorescence conjugates (Roberts *et al.*, 2006; Tien *et al.*, 2005) and could be used to characterise *Microcystis* samples. Fluorescently labelled lectins were already used successfully to characterise biofilms (Johnsen *et al.*, 2000; Neu *et al.*, 2002; Neu *et al.*, 2004; Wigglesworth-Cooksey and Cooksey, 2005).

4.7.2 Mvn and buoyancy regulation

Buoyancy regulation was shown to be a key factor for the adaptation to changing environmental conditions in the habitat of *Microcystis* (Brookes and Ganf, 2001; Chu *et al.*, 2007; Ibelings *et al.*, 1991; Konopka *et al.*, 1987; Rabouille *et al.*, 2003). Apart from the accumulation of fixed carbon and thus the increasing cell density, the colony size has impact on the buoyancy. This study provides evidence that microvirin might be involved in the control of buoyancy for several reasons. First of all, it is localised at the cell surface and therefore it can interact with multiple cells contributing to the formation of colonies. Its function is obviously affected by microcystin in a way that it binds to microvirin under certain conditions. Initial experiments showed that the binding of microcystin to proteins is promoted by oxidative stress conditions that occur upon the limitation of iron or the exposition to light stress. The lectin binding analysis showed that these conditions also increased the release of extracellular carbohydrates that are recognised by Mvn. All these factors might accelerate the attachement of *Microcystis* cells and contribute to an adaptation of *Microcystis* in case of e.g. light stress enabling the colonies to rapidly move to deeper water layers. Due to the limitations discussed at the beginning such a behaviour could not be observed in the PCC 7806 strain.

4.7.3 Microcystin and Mvn in stress adaptation

The investigation of culture collection strains was limited to *M. aeruginosa* species and it became obvious that Mvn is not a general feature of *M. aeruginosa* strains. The interaction of Mvn and microcystin examined in this study support a functional correlation of the lectin and the peptide. Indeed, a strong bias to the presence of Mvn in microcystin producers was observed (see **Tab. 6**). Only one microcystin-producing strain (NIES-843) was found not to contain the *mvn* gene. However, the gene might have got lost during cultivation of the strain. Evidence for this was found by the direct comparison of the *mvn* encoding genomic region of the PCC 7806 strain with the corresponding locus of the NIES 843 genome (see chapter 3.4.2). Indeed, it was revealed that rearrangement occurred around this locus and repetetive sequences could be identified that might have served as hotspots for recombination events. Several reports describe spontaneous mutants of *Microcystis* and other cyanobacteria. A gas vesicle mutant of *M. aeruginosa* PCC 7806 generated by the insertion of IS elements resulting in the rearrangement of the gas vesicle gene cluster lost its buoyancy (Mlouka *et al.*, 2004; Mlouka *et al.*, 2004). Another mutation that occurred during prolonged laboratory cultivation affected the *M. aeruginosa* MRC strain in its ability to produce microcystin. This strain does not produce

microcystin anymore, but still contains the full set of genes required for the synthesis of microcystin (Kaebernick *et al.*, 2001; Tillett *et al.*, 2001). The loss of colony formation and the reduced production of extracellular carbohydrates have been addressed previously in this work and most likely result from mutations.

Apparently, all mutations and alterations described in *Microcystis* laboratory strains so far seem to be related to processes that are needed for the adaptation in their natural environment, especially the buoyancy regulation. Buoyancy regulation is useless for cultivated *Microcystis* strains for several reasons. First of all the size of culture vessels is too small for the colonies to perform a true vertical migration that would result in changes of light conditions for the cells. Furthermore, the bacteria are usually kept under ambient light conditions and supplied with optimal nutrition. Therefore it is plausible that the *mvn* gene got lost in some strains of culture collections. This is further supported by the recent findings that the genomes of *Microcystis* strains PCC 7806 and NIES 843 exhibit a remarkable degree of plasticity (Frangeul *et al.*, 2008; Kaneko *et al.*, 2007). Both genomes contain a high number of both insertion sequences and miniature inverted-repeat transposable elements that contribute to around 12% of the whole genome sequence. Recombination events were further detected in the adenylation domains of the microcystin biosynthesis genes (Fewer *et al.*, 2007; Tooming-Klunderud *et al.*, 2008; Tooming-Klunderud *et al.*, 2008). Hence, the absence or the inactivation of genes from cultivated *Microcystis* strains reflects the adaptation to the ambient laboratory conditions. Indeed, it was shown in competition experiments that microcystin production does not provide an advantage under light limited conditions and the non-toxic strain even outcompeted the toxic strain (Kardinaal *et al.*, 2007). The *in vivo* as well as the *in vitro* results presented here corroborate that microcystin serves the adaptation to oxidative stress conditions, which are characterised by very high light or iron depletion. Iron depletion was already shown to stimulate microcystin production (Sevilla *et al.*, 2008). Therefore, an advantage of the *M. aeruginosa* wild type over the microcystin-deficient Δ*mcy*B mutant might become manifest under stress conditions.

4.8 Phylogenetic aspects of Mvn

From an evolutionary perspective the occurrence of microvirin in *M. aeruginosa* gives rise to interesting considerations. Mvn belongs to the CV-N domain family (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR011058) whose members are predominantly

present in filamentous ascomycetes of the subphylum *Pezizomycotina* and rarely occur in other taxa. To date (September, 2008), 1270 fully sequenced bacterial genomes from 896 species are listed in the ENTREZ database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome) and only six CV-N family proteins including Mvn from *M. aeruginosa* are known in prokaryotes. In eukaryotes the only known non-fungal member of this family was found in the fern *Ceratopteris richardii*. This patchy distribution implies that some organisms acquired the CV-N domain by horizontal gene transfer (HGT) events. The total genome sequences of 33 cyanobacteria were available at the time of this study and only the genome of *M. aeruginosa* PCC7806 contains the *mvn* gene sequence. Horizontal gene transfer (or "lateral gene transfer") is widely accepted as a force in genome shaping and considered a significant factor in evolution (Doolittle, 1999; Gogarten and Townsend, 2005; Tepfer *et al.*, 2003). Comparative genome analyses confirmed that these mechanisms also act in cyanobacteria (Zhaxybayeva *et al.*, 2006).

It was estimated that individual cyanobacterial genomes have acquired between 9.5% and 16.6% of their genes by HGT (Nakamura *et al.*, 2004; Ochman *et al.*, 2000). HGT events from eukaryotes to cyanobacteria have been described in detail in previous studies and it was also demonstrated that the genes are expressed. Some marine *Synechococcus* and *Prochlorococcus* strains carry a gene encoding the plastid-targeted fructose bisphosphate aldolase that has been transferred from red algae. This gene resides close to its functional analogue in the genome and in some cases it has even replaced the original version (Rogers *et al.*, 2007). Other examples are the eukaryotic cytoskeletal elements actin and profilin present and expressed in *M. aeruginosa* PCC 7806 that obviously originate from a marine invertebrate (Guljamow *et al.*, 2007).

Interestingly, Nakamura and co-workers (2004) found that the biological functions of transferred genes are biased towards three categories: cell surface, DNA binding and pathogenicity-related functions. Mvn can be considered to belong to two of these categories regarding the implication of lectins in pathogenicity in general. Another survey analysed the distribution of horizontally transferred genes in functional categories according to the classification of the KEGG database. The two categories "cell envelope" and "transport and binding proteins" that describe the Mvn function best together accounted to 30% of the horizontally transferred genes (Shi *et al.*, 2005).

Tab. 8: Distribution of CV-N domains in fully sequenced genomes.

Taxon	Fully sequenced genomes[1]	Mvn orthologues[2]
Ascomycetes (Pezizomycotina)	10	50
Chloroflexi	0	1
Cyanobacteria	33	2
α-Proteobacteria	85	1
2 *Caulobacter*		

[1] http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome
[2] http://pfam.sanger.ac.uk/family?acc=PF08881

The almost exclusive occurrence of CV-N domain proteins in ascomycetes (see **Tab. 8**) poses the question whether Mvn is of fungal origin. All ascomycetes species possessing CV-N domain proteins belong to the subphylum of *Pezizomycotina* and this phylum includes all lichen-forming ascomycetes (Liu and Hall, 2004). This is extremely interesting because members of the genus *Nostoc* are known to be symbiotic partners of fungi in the formation of lichens. Examples are fungi of the genus *Nephroma* or *Peltigera* which both belong to the *Lecanoromycetes*, a subphylum of the *Pezizomycotina* (Paulsrud and Lindblad, 1998; Paulsrud *et al.*, 1998; Paulsrud *et al.*, 2000). Unfortunately little sequence information is available from members of the *Lecanoromycetes* and nothing is known aabout the presence of CV-N domain proteins in these fungi. However, the ascomycetes species containing CV-N domain proteins – although they are not lichen-forming fungi – are closely related to the *Lecanoromycetes* (Liu and Hall, 2004) and therefore it can be speculated that CV-N was transferred from a fungus to a symbiotic *Nostoc* or one of its ancestors. While *Nostoc* clearly shares a habitat with filamentous fungi this is not the case for *Microcystis*. But phylogenetic analyses of 16S rRNA genes of cyanobacteria corroborated that *Nostoc* and *Microcystis* share a common ancestor (Lyra *et al.*, 2001). Thus this common ancestor of *Microcystis* and *Nostoc* might have acquired *mvn*-encoding genes and then the gene was lost in some of its descendants. Interestingly, this study has shown that there is a strong bias for the presence of Mvn in microcystin-producing strains. Taking into account that microcystin interacts with microvirin, it can be speculated that in particular the binding of microcystin to Mvn contributed to the preservation of the gene in *Microcystis*.

It was inferred from phylogenetic analysis (see **Fig. 39**) that found a high degree of congruence between the phylogeny of microcystin synthetase genes and housekeeping genes (16S rRNA

and *rpoC1*) of the genera *Planktothrix*, *Microcystis*, *Anabaena* and *Nostoc*, that microcystin synthetase genes were present in the last common ancestor of said species (Jungblut and Neilan, 2006; Rantala *et al.*, 2004). It was further discussed that the microcystin synthetase genes have been lost through the proceeding divergence of the lineage.
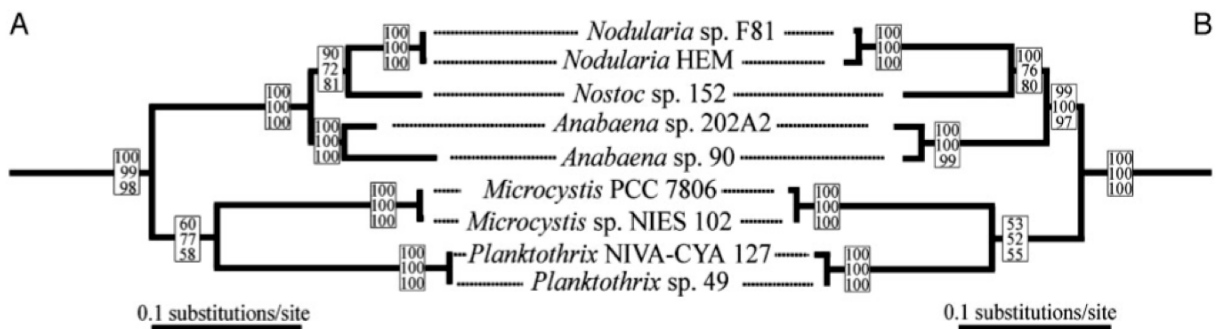
Fig. 39: Congruence between the 16S rRNA and rpoC1 data set and the microcystin synthetase gene data set. A) A maximum-likelihood tree based on the 16S rRNA and *rpo*C1 data set. B) A maximum-likelihood tree based on the *mcy*A, *mcy*D, and *mcy*E data set. From (Rantala *et al.*, 2004).

Although this study did not provide data on the presence and distribution of *mvn* genes in other microcystin-producing genera it seems that at least in *Microcystis* Mvn has evolved with microcystin and the loss of the microcystin synthetase genes also led to the loss of Mvn in non-toxic strains. It would be interesting to screen for *mvn* encoding genes in *Planktothrix, Anabaena* and *Nostoc* to test whether *mvn* is present in these genera and a bias towards toxic strains can be observed. The toxic *Planktothrix argardhii* CYA 126 was shown to contain the *mvn* gene and the expression was proven by immunoblotting (Kehr, 2003).

4.9 Concluding remarks and outlook

Recent studies have suggested new functions of microcystins in the primary metabolism of its producers. This study showed that microcystin impact might result from its immediate interaction with proteins. The conditions that promote these interactions as well as the consequences arising from the binding of microcystin were elucidated for the first time. The results strongly suggest microcystin being a redox-sensitive molecule that is involved in the adaptation to oxidative stress conditions. These findings provide a basis for a detailed investigation of microcystin targets and function.

Several questions result from this study that have to be addressed in the future. It needs to be analysed if additional factors are present *in vivo* that allow a reversible binding of microcystin to its targets. In addition the stability and halflife of proteins such as RubisCO that are targets of microcystin have to be compared in the *M. aeruginosa* wild type and microcystin-deficient mutant under oxidative stress conditions. Competition experiments under the respective condtions might prove this hypothesis further and reveal phenotypic differences between the wild type and mutant.

Furthermore this study has revealed that the adaptation to oxidative stress conditions involves changes of the cell surface composition of *M. aeruginosa* cells. The extracellular lectin Mvn was shown to be directly involved in these processes. Techniques such as the lectin binding analysis and immunofluorescence microscopy were developed to investigate morphological changes *in situ*, which provide a toolkit to follow stress adaptation in the field. Field studies have to be conducted to overcome the restrictions imposed upon the work with laboratory strains.

Furthermore, the presence of certain extracellular factors was shown to be strain-specific. It might be proposed that a broad survey of *Microcystis* surface characteristics leads to the identification of markers suitable to discriminate toxic and non-toxic strains.

REFERENCES

Adams, E. W.; Ratner, D. M.; Bokesch, H. R.; McMahon, J. B.; O'Keefe, B. R. and Seeberger, P. H. (2004): Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology: glycan-dependent gp120/protein interactions, Chemistry & Biology 11 [6], pp. 875-881.

Apweiler, Rolf; Hermjakob, Henning and Sharon, Nathan (1999): On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database, Biochimica et Biophysica Acta (BBA) - General Subjects 1473 [1], pp. 4-8.

Balzarini, J.; Hatse, S.; Vermeire, K.; Princen, K.; Aquaro, S.; Perno, C.-F.; De Clercq, E.; Egberink, H.; Vanden Mooter, G.; Peumans, W.; Van Damme, E. and Schols, D. (2004): Mannose-specific plant lectins from the *Amaryllidaceae* family qualify as efficient microbicides for prevention of human immunodeficiency virus infection, Antimicrobial Agents and Chemotherapy 48 [10], pp. 3858-3870.

Balzarini, J.; Van Laethem, K.; Hatse, S.; Froeyen, M.; Van Damme, E.; Bolmstedt, A.; Peumans, W.; De Clercq, E. and Schols, D. (2005): Marked depletion of glycosylation sites in HIV-1 gp120 under selection pressure by the mannose-specific plant lectins of *Hippeastrum Hybrid* and *Galanthus nivalis*, Molecular Pharmacology 67 [5], pp. 1556-1565.

Balzarini, J.; Van Laethem, K.; Hatse, S.; Vermeire, K.; De Clercq, E.; Peumans, W.; Van Damme, E.; Vandamme, A.; Bohlmstedt, A. and Schols, D. (2004): Profile of Resistance of Human Immunodeficiency Virus to Mannose-Specific Plant Lectins, Journal of Virology 78 [19], pp. 10617-10627.

Banas, J. A.; Fountain, T. L.; Mazurkiewicz, J. E.; Sun, K. and Vickerman, M. (2007): Streptococcus mutans glucan-binding protein-A affects *Streptococcus gordonii* biofilm architecture, FEMS Microbiology Letters 267 [1], pp. 80-88.

Barrientos, L. G. and Gronenborn, A. M. (2002): The domain-swapped dimer of cyanovirin-N contains two sets of oligosaccharide binding sites in solution, Biochemical and Biophysical Research Communications 298 [4], pp. 598-602.

Barrientos, L. G.; Louis, J. M.; Botos, I.; Mori, T.; Han, Z.; O'Keefe, B. R.; Boyd, M. R.; Wlodawer, A. and Gronenborn, A. M. (2002): The domain-swapped dimer of cyanovirin-N is in a metastable folded state: reconciliation of X-ray and NMR structures, Structure 10 [5], pp. 673-686.

Barrientos, L. G.; Matei, E.; Lasala, F.; Delgado, R. and Gronenborn, A. M. (2006): Dissecting carbohydrate-cyanovirin-N binding by structure-guided mutagenesis: functional implications for viral entry inhibition, Protein Engineering, Design & Selection 19 [12], pp. 525-535.

Bennett, M. J. and Eisenberg, D. (2004): The evolving role of 3D domain swapping in proteins, Structure 12 [8], pp. 1339-1341.

Bennett, M. J.; Schlunegger, M. P. and Eisenberg, D. (1995): 3D Domain swapping: A mechanism for oligomer assembly, Protein Science 4 [12], pp. 2455-2468.

Benz, I. and Schmidt, M. A. (2002): Never say never again: protein glycosylation in pathogenic bacteria, Molecular Microbiology 45 [2], pp. 267-276.

Bewley, C. A. (2001): Solution structure of a cyanovirin-N:Man alpha 1-2Man alpha complex: structural basis for high-affinity carbohydrate-mediated binding to gp120, Structure 9 [10], pp. 931-940.

Bewley, C. A.; Gustafson, K. R.; Boyd, M. R.; Covell, D. G.; Bax, A.; Clore, G. M. and Gronenborn, A. M. (1998): Solution structure of cyanovirin-N, a potent HIV-inactivating protein, Nature Structural Biology 5 [7], pp. 571-578.

Bewley, C. A.; Kiyonaka, S. and Hamachi, I. (2002): Site-specific discrimination by cyanovirin-N for alpha-linked trisaccharides comprising the three arms of Man(8) and Man(9), Journal of Molecular Biology 322 [4], pp. 881-889.

Bewley, C. A. and Otero-Quintero, S. (2001): The potent anti-HIV protein cyanovirin-N contains two novel carbohydrate binding sites that selectively bind to Man(8) D1D3 and Man(9) with nanomolar affinity: implications for binding to the HIV envelope protein gp120, Journal of the American Chemical Society 123 [17], pp. 3892-3902.

Boeckelmann, U.; Manz, W.; Neu, T. R. and Szewzyk, U (2002): Investigation of lotic microbial aggregates by a combined technique of fluorescent in situ hybridization and lectin-binding-analysis, Journal of Microbiological Methods 49 [1], pp. 75-87.

Bokesch, H. R.; O'Keefe, B. R.; McKee, T. C.; Pannell, L. K.; Patterson, G. M. L.; Gardella, R. S.; Sowder, R. C.; Turpin, J.; Watson, K.; Buckheit, R. W. and Boyd, M. R. (2003): A potent novel anti-HIV protein from the cultured cyanobacterium *Scytonema varium*, Biochemistry 42 [9], pp. 2578-2584.

Bolanos, L.; Redondo-Nieto, M.; Rivilla, R.; Brewin, N. J. and Bonilla, I. (2004): Cell surface interactions of *Rhizobium* bacteroids and other bacterial strains with symbiosomal and peribacteroid membrane components from pea nodules, Molecular Plant-Microbe Interactions 17 [2], pp. 216-223.

Bolmstedt, A. J.; O'Keefe, B. R.; Shenoy, S. R.; McMahon, J. B. and Boyd, M. R. (2001): Cyanovirin-N defines a new class of antiviral agent targeting N-linked, high-mannose glycans in an oligosaccharide-specific manner, Molecular Pharmacology 59 [5], pp. 949-954.

Botes, D. P.; Tuinman, A. A.; Wessels, P. L.; Viljoen, C. C. and Kruger, H. (1984): The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*, Journal of the Chemical Society. Perkin Transactions 1 4 [223], pp. 2311-2318.

Botos, I.; Mori, T.; Cartner, L. K.; Boyd, M. R. and Wlodawer, A. (2002): Domain-swapped structure of a mutant of cyanovirin-N, Biochemical and Biophysical Research Communications 294 [1], pp. 184-190.

Botos, I.; O'Keefe, B. R.; Shenoy, S. R.; Cartner, L. K.; Ratner, D. M.; Seeberger, P. H.; Boyd, M. R. and Wlodawer, A. (2002): Structures of the complexes of a potent anti-HIV protein cyanovirin-N and high mannose oligosaccharides, The Journal of Biological Chemistry 277 [37], pp. 34336-34342.

Boyd, M. R.; Gustafson, K. R.; McMahon, J. B.; Shoemaker, R. H.; O'Keefe, B. R.; Mori, T.; Gulakowski, R. J.; Wu, L.; Rivera, M. I.; Laurencot, C. M.; Currens, M. J.; Cardellina, J. H., 2nd; Buckheit, R. W., Jr.; Nara, P. L.; Pannell, L. K.; Sowder, R. C., 2nd and Henderson, L. E. (1997): Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development, Antimicrobial Agents and Chemotherapy 41 [7], pp. 1521-1530.

Brookes, J. D. and Ganf, G. G. (2001): Variations in the buoyancy response of Microcystis aeruginosa to nitrogen, phosphorus and light, Journal of Plankton Research 23 [12], pp. 1399-1411.

Bulgheresi, S.; Schabussova, I.; Chen, T.; Mullin, N. P.; Maizels, R. M. and Ott, J. A. (2006): A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine Nematode, Applied and Environmental Microbiology 72 [4], pp. 2950-2956.

Cambi, A.; Koopman, M. and Figdor, C. G. (2005): How C-type lectins detect pathogens, Cellular Microbiology 7 [4], pp. 481-488.

Carmichael, W. W.; Azevedo, S. M.; An, J. S.; Molica, R. J.; Jochimsen, E. M.; Lau, S.; Rinehart, K. L.; Shaw, G. R. and Eaglesham, G. K. (2001): Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins, Environmental Health Perspectives 109 [7], pp. 663-668.

Chang, L. C. and Bewley, C. A. (2002): Potent inhibition of HIV-1 fusion by cyanovirin-N requires only a single high affinity carbohydrate binding site: characterization of low affinity carbohydrate binding site knockout mutants, Journal of Molecular Biology 318 [1], pp. 1-8.

Chrispeels, M. J. and Raikhel, N. V. (1991): Lectins, lectin genes, and their role in plant defense, Plant Cell 3 [1], pp. 1-9.

Christiansen, G.; Dittmann, E.; Via Ordorika, L.; Rippka, R.; Herdman, M. and Börner, T. (2001): Nonribosomal peptide synthetase genes occur in most cyanobacterial genera as evidenced by their distribution in axenic strains of the PCC, Archives of Microbiology 176 [6], pp. 452-458.

Christoffersen, K. (1996): Ecological implications of cyanobacterial toxins in aquatic ecosystems, Phycologia 36, pp. 42-50.

Chu, Z.; Jin, X.; Yang, B. and Zeng, Q. (2007): Buoyancy regulation of Microcystis flos-aquae during phosphorus-limited and nitrogen-limited growth, Journal of Plankton Research 29 [9], pp. 739-745.

Codd, G. A.; Metcalf, J. S.; Morrison, L. F.; Krienitz, L.; Ballot, A.; Pflugmacher, S.; Wiegand, C. and Kotut, K. (2003): Susceptibility of flamingos to cyanobacterial toxins via feeding, The Veterinary Record 152 [23], pp. 722-723.

Codd, G. A.; Morrison, L. F. and Metcalf, J. S. (2005): Cyanobacterial toxins: risk management for health protection, Toxicology and Applied Pharmacology 203 [3], pp. 264-272.

Collet, J. F. and Bardwell, J. C. A. (2002): Oxidative protein folding in bacteria, Molecular Microbiology 44 [1], pp. 1-8.

Coulthurst, S. J.; Lilley, K. S.; Hedley, P. E.; Liu, H.; Toth, I. K. and Salmond, G. P. C. (2008): DsbA plays a critical and multi-faceted role in the production of secreted virulence factors by the phytopathogen, *Erwinia carotovora* subsp. *atroseptica*, The Journal of Biological Chemistry, p. M801829200.

Davies, J.; Turner, M. and Klein, N. (2001): The role of the collectin system in pulmonary defence, Paediatric Respiratory Reviews 2 [1], pp. 70-75.

DePriest, P. T. (2004): Early molecular investigations of lichen-forming symbionts: 1986-2001, Annual Review of Microbiology 58 [1], pp. 273-301.

Dey, B.; Lerner, D. L.; Lusso, P.; Boyd, M. R.; Elder, J. H. and Berger, E. A. (2000): Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor and inhibition of diverse enveloped viruses, Journal of Virology 74 [10], pp. 4562-4569.

Diggle, S. P.; Stacey, R. E.; Dodd, C.; Camara, M.; Williams, P. and Winzer, K. (2006): The galactophilic lectin, LecA, contributes to biofilm development in *Pseudomonas aeruginosa*, Environmental Microbiology 8 [6], pp. 1095-1104.

Dismukes, G. C.; Klimov, V. V.; Baranov, S. V.; Kozlov, Y. N.; DasGupta, J. and Tyryshkin, A. (2001): The origin of atmospheric oxygen on Earth: the innovation of oxygenic photosynthesis, Proceedings of the National Academy of Sciences 98 [5], pp. 2170-2175.

Dittmann, E.; Erhard, M.; Kaebernick, M.; Scheler, C.; Neilan, B. A.; von Döhren, H. and Börner, T. (2001): Altered expression of two light-dependent genes in a microcystin-lacking mutant of *Microcystis aeruginosa* PCC 7806, Microbiology 147 [Pt 11], pp. 3113-3119.

Dittmann, E.; Neilan, B. A.; Erhard, M.; von Döhren, H. and Börner, T. (1997): Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806, Molecular Microbiology 26 [4], pp. 779-787.

Dommett, R. M.; Klein, N. and Turner, M. W. (2006): Mannose-binding lectin in innate immunity: past, present and future, Tissue Antigens 68 [3], pp. 193-209.

Doolittle, W. F. (1999): Phylogenetic classification and the universal tree, Science 284, pp. 2124 - 2129.

Downing, T. G.; Meyer, C.; Gehringer, M. M. and van de Venter, M. (2005): Microcystin content of *Microcystis aeruginosa* is modulated by nitrogen uptake rate relative to specific growth rate or carbon fixation rate, Environmental Toxicology 20 [3], pp. 257-262.

Downing, T. G.; Sember, C. S.; Gehringer, M. M. and Leukes, W. (2005): Medium N:P ratios and specific growth rate comodulate microcystin and protein content in *Microcystis aeruginosa* PCC7806 and *M. aeruginosa* UV027, Microbial Ecology 49 [3], pp. 468-473.

Dühring, U.; Axmann, I. M.; Hess, W. R. and Wilde, A. (2006): From the Cover: An internal antisense RNA regulates expression of the photosynthesis gene isiA, Proceedings of the National Academy of Sciences 103 [18], pp. 7054-7058.

Esser, M. T.; Mori, T.; Mondor, I.; Sattentau, Q. J.; Dey, B.; Berger, E. A.; Boyd, M. R. and Lifson, J. D. (1999): Cyanovirin-N binds to gp120 to interfere with CD4-dependent human immunodeficiency virus type 1 virion binding, fusion, and infectivity but does not affect the CD4 binding site on gp120 or soluble CD4-induced conformational changes in gp120, Journal of Virology 73 [5], pp. 4360-4371.

Falconer, I. R.; Beresford, A. M. and Runnegar, M. T. (1983): Evidence of liver damage by toxin from a bloom of the blue-green alga, *Microcystis aeruginosa*, The Medical Journal of Australia 1 [11], pp. 511-514.

Falconer, I. R. and Runnegar, M. T. (1987): Effects of the peptide toxin from *Microcystis aeruginosa* on intracellular calcium, pH and membrane integrity in mammalian cells, Chemico-Biological Interactions 63 [3], pp. 215-225.

Falconer, I. R. and Yeung, D. S. (1992): Cytoskeletal changes in hepatocytes induced by *Microcystis* toxins and their relation to hyperphosphorylation of cell proteins, Chemico-Biological Interactions 81 [1-2], pp. 181-196.

Fastner, J.; Erhard, M. and von Döhren, H. (2001): Determination of oligopeptide diversity within a natural population of *Microcystis spp.* (cyanobacteria) by typing single colonies by matrix-assisted laser desorption ionization-time of flight mass spectrometry, Applied and Environmental Microbiology 67 [11], pp. 5069-5076.

Felsenstein, J. (2005): Using the quantitative genetic threshold model for inferences between and within species, Philosophical Transactions of the Royal Society B: Biological Sciences 360 [1459], pp. 1427-1434.

Fewer, D. P.; Rouhiainen, L.; Jokela, J.; Wahlsten, M.; Laakso, K.; Wang, H. and Sivonen, K. (2007): Recurrent adenylation domain replacement in the microcystin synthetase gene cluster, BMC Evolutionary Biology 7 [1], p. 183.

Finking, R. and Marahiel, M. A. (2004): Biosynthesis of nonribosomal peptides, Annual Review of Microbiology 58 [1], pp. 453-488.

Florencio, F. ; Pérez-Pérez, M.; López-Maury, L.; Mata-Cabana, A. and Lindahl, M. (2006): The diversity and complexity of the cyanobacterial thioredoxin systems, Photosynthesis Research 89 [2], pp. 157-171.

Florencio, F. J.; Yee, B. C.; Johnson, T. C. and Buchanan, B. B. (1988): An NADP/thioredoxin system in leaves: purification and characterization of NADP-thioredoxin reductase and thioredoxin h from spinach, Archives of biochemistry and biophysics 266 [2], pp. 496-507.

Forster, S.; Timmann, A.; Konrad, M.; Schellbach, C.; Meyer, A.; Funari, S. S.; Mulvaney, P. and Knott, R. (2005): Scattering Curves of Ordered Mesoscopic Materials, The Journal of Physical Chemistry B 109 [4], pp. 1347-1360.

Frangeul, L.; Quillardet, P.; Castets, A. M.; Humbert, J. F.; Matthijs, H.; Cortez, D.; Tolonen, A. C.; Zhang, C. C.; Gribaldo, S.; Kehr, J. C.; Zilliges, Y.; Ziemert, N.; Becker, S.; Talla, E. I.; Latifi, A.; Billault, A.; Lepelletier, A.; Dittmann, E.; Bouchier, C. and Tandeau de Marsac, N. (2008): Highly plastic genome of *Microcystis aeruginosa* PCC 7806, a ubiquitous toxic freshwater cyanobacterium, BMC Genomics 9 [1], p. 274.

Fromme, R.; Katiliene, Z.; Giomarelli, B.; Bogani, F.; Mc Mahon, J.; Mori, T.; Fromme, P. and Ghirlanda, G. (2007): A monovalent mutant of cyanovirin-N provides insight into the role of multiple interactions with gp120 for antiviral activity, Biochemistry 46 [32], pp. 9199-9207.

Gabius, H. J. ; Siebert, H. C.; Andre, S.; JimÈnez-Barbero, J. and Rüdiger, H. (2004): Chemical biology of the sugar code, ChemBioChem : a European Journal of Chemical Biology 5 [6], pp. 740-764.

Gabius, H. J.; Andre, S.; Kaltner, H. and Siebert, H. C. (2002): The sugar code: functional lectinomics, Biochimica et Biophysica Acta (BBA) - General Subjects 1572 [2-3], pp. 165-177.

Garcia-Ferris, C. and Moreno, J. (1994): Oxidative modification and breakdown of ribulose-1,5-bisphosphate carboxylase/oxygenase induced in *Euglena gracilis* by nitrogen starvation, Planta 193 [2], pp. 208-215.

Garcia-Pino, A.; Buts, L.; Wyns, L.; Imberty, A. and Loris, R. (2007): How a plant lectin recognizes high mannose oligosaccharides, Plant Physiology 144 [4], pp. 1733-1741.

Genevaux, P.; Bauda, P.; DuBow, M. S. and Oudega, B. (1999): Identification of Tn10 insertions in the dsbA gene affecting *Escherichia coli* biofilm formation, FEMS Microbiology Letters 173 [2], pp. 403-409.

Gerbersdorf, S. U. (2006): An advanced technique for immuno-labelling of microcystins in cryosectioned cells of *Microcystis aeruginosa* PCC 7806 (cyanobacteria): Implementations of an experiment with varying light scenarios and culture densities, Toxicon 47 [2], pp. 218-228.

Gogarten, P. J. and Townsend, J. P. (2005): Horizontal gene transfer, genome innovation and evolution, Nature Reviews Microbiology 3 [9], pp. 679-687.

Göldner, K. (2007): Untersuchungen zur Charakterisierung von Lektinen und Lipopolysacchariden in *Microcystis aeruginosa* PCC 7806, Diploma Thesis, Molecular Ecology, Humboldt-Universität, Berlin.

Greiner, L. L.; Edwards, J. L.; Shao, J.; Rabinak, C.; Entz, D. and Apicella, M. A. (2005): Biofilm Formation by *Neisseria gonorrhoeae*, Infection and Immunity 73 [4], pp. 1964-1970.

Guljamow, A.; Jenke-Kodama, H.; Saumweber, H.; Quillardet, P.; Frangeul, L.; Castets, A. M.; Bouchier, C.; Tandeau de Marsac, N. and Dittmann, E. (2007): Horizontal gene transfer of two cytoskeletal elements from a eukaryote to a cyanobacterium, Current Biology 17 [17], pp. R757-759.

Gustafson, K. R.; Sowder, R. C., 2nd; Henderson, L. E.; Cardellina, J. H., 2nd; McMahon, J. B.; Rajamani, U.; Pannell, L. K. and Boyd, M. R. (1997): Isolation, primary sequence determination, and disulfide bond structure of cyanovirin-N, an anti-HIV (human immunodeficiency virus) protein from the cyanobacterium *Nostoc ellipsosporum*, Biochemical and Biophysical Research Communications 238 [1], pp. 223-228.

Ha, Un-Hwan; Wang, Yanping and Jin, Shouguang (2003): DsbA of *Pseudomonas aeruginosa* is essential for multiple virulence factors, Infection and Immunity 71 [3], pp. 1590-1595.

Hall, T. A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucleic Acids Symposium Series 41, pp. 95-98.

Han, Z.; Xiong, C.; Mori, T. and Boyd, M. R. (2002): Discovery of a stable dimeric mutant of cyanovirin-N (CV-N) from a T7 phage-displayed CV-N mutant library, Biochemical and Biophysical Research Communications 292 [4], pp. 1036-1043.

Harper, M.; Cox, A. D.; St. Michael, F.; Wilkie, I. W.; Boyce, J. D. and Adler, B. (2004): A heptosyltransferase mutant of *Pasteurella multocida* produces a truncated lipopolysaccharide structure and is attenuated in virulence, Infection and Immunity 72 [6], pp. 3436-3443.

Hedemann, M. S.; Hojsgaard, S. and Jensen, B.B. (2007): Lectin histochemical characterisation of the porcine small intestine around weanin, Research in veterinary science 82 [2], pp. 257-262.

Helenius, A. and Aebi, M. (2004): Roles of N-linked glycans in the endoplasmic reticulum, Annual Review of Biochemistry 73 [1], pp. 1019-1049.

Hellstrom, U.; Hallberg, E. C.; Sandros, J.; Rydberg, L. and Backer, A. E. (2004): Carbohydrates act as receptors for the periodontitis-associated bacterium *Porphyromonas gingivalis*: a study of bacterial binding to glycolipids, Glycobiology 14 [6], pp. 511-519.

Henikoff, S. and Henikoff, J. G. (1993): Performance evaluation of amino acid substitution matrices, Proteins: Structure, Function, and Genetics 17 [1], pp. 49-61.

Hesse, K. and Kohl, J. G. (2001): Effects of light and nutrient supply on growth and microcystin content of different strains of *Microcystis aeruginosa*, Chorus, I., Cyanotoxins: occurrence, causes, consequences pp. 152-158, Springer-Verlag KG, Berlin.

Hirel, P. H.; Schmitter, M. J.; Dessen, P.; Fayat, G. and Blanquet, S. (1989): Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid, Proceedings of the National Academy of Sciences 86 [21], pp. 8247-8251.

Hirsch, A. M. (1999): Role of lectins (and rhizobial exopolysaccharides) in legume nodulation, Current Opinion in Plant Biology 2 [4], pp. 320-326.

Hisbergues, M.; Christiansen, G.; Rouhiainen, L.; Sivonen, K. and Börner, T. (2003): PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera, Archives of Microbiology 180 [6], pp. 402-410.

Hitchen, P.I G. and Dell, A. (2006): Bacterial glycoproteomics, Microbiology 152 [6], pp. 1575-1580.

Holmskov, U.; Thiel, S. and Jensenius, J. C. (2003): Collectins and ficolins: humoral lectins of the innate immune defense, Annual Review of Immunology 21 [1], pp. 547-578.

Huelsenbeck, J. P. and Ronquist, F. (2001): MRBAYES: Bayesian inference of phylogenetic trees, Bioinformatics 17 [8], pp. 754-755.

Hynes, S. O.; Teneberg, S.; Roche, N. and Wadstrom, T. (2003): Glycoconjugate binding of gastric and enterohepatic *Helicobacter spp*, Infection and Immunity 71 [5], pp. 2976-2980.

Ibelings, B. W.; Mur, L. R. and Walsby, A. E. (1991): Diurnal changes in buoyancy and vertical distribution in populations of *Microcystis* in two shallow lakes, Journal of Plankton Research 13 [2], pp. 419-436.

Iobst, S. T. and Drickamer, K. (1994): Binding of sugar ligands to Ca(2+)-dependent animal lectins. II. Generation of high-affinity galactose binding by site-directed mutagenesis, The Journal of Biological Chemistry 269 [22], pp. 15512-15519.

Jacquot, J. P.; Stein, M.; Suzuki, A.; Liottet, S.; Sandoz, G. and Miginiac-Maslow, M. (1997): Residue Glu-91 of *Chlamydomonas reinhardtii* ferredoxin is essential for electron transfer to ferredoxin-thioredoxin reductase, FEBS Letters 400 [3], pp. 293-296.

Jähnichen, S.; Ihle, T.; Petzoldt, T. and Benndorf, J. (2007): Impact of inorganic carbon availability on microcystin production by *Microcystis aeruginosa* PCC 7806, Applied and Environmental Microbiology 73 [21], pp. 6994-7002.

Jähnichen, S.; Petzoldt, T. and Benndorf, J. (2001): Evidence for control of microcystin dynamics in Bautzen reservoir (Germany) by cyanobacterial population growth rates and dissolved inorganic carbon, Archiv für Hydrobiologie 150 [2], pp. 177-196.

Jimbo, M.; Yamaguchi, M.; Muramoto, K. and Kamiya, H. (2000): Cloning of the *Microcystis aeruginosa* M228 lectin (MAL) gene, Biochemical and Biophysical Research Communications 273 [2], pp. 499-504.

Jochimsen, E. M.; Carmichael, W. W.; An, J. S.; Cardo, D. M.; Cookson, S. T.; Holmes, C. E.; Antunes, M. B.; de Melo Filho, D. A.; Lyra, T. M.; Barreto, V. S.; Azevedo, S. M. and Jarvis, W. R. (1998): Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil, The New England Journal of Medicine 338 [13], pp. 873-878.

Johnsen, A. R.; Hausner, M.; Schnell, A. and Wuertz, S. (2000): Evaluation of fluorescently labeled lectins for noninvasive localization of extracellular polymeric substances in *Sphingomonas* biofilms, Applied and Environmental Microbiology 66 [8], pp. 3487-3491.

Jones, D. T.; Taylor, W. R. and Thornton, J. M. (1992): The rapid generation of mutation data matrices from protein sequences, Computer applications in the biosciences 8 [3], pp. 275-282.

Jungblut, A. D. and Neilan, B. A. (2006): Molecular identification and evolution of the cyclic peptide hepatotoxins, microcystin and nodularin, synthetase genes in three orders of cyanobacteria, Archives of Microbiology 185 [2], pp. 107-114.

Jürgens, U. J.; Martin, C. and Weckesser, J. (1989): Cell wall constituents of *Microcystis sp*. PCC 7806 FEMS Microbiology Letters 53 [1-2], pp. 47-51.

Jüttner, F. and Luthi, H. (2008): Topology and enhanced toxicity of bound microcystins in *Microcystis* PCC 7806, Toxicon 51 [3], pp. 388-397.

Kaebernick, M.; Dittmann, E.; Börner, T. and Neilan, B. A. (2002): Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide, Applied and Environmental Microbiology 68 [2], pp. 449-455.

Kaebernick, M.; Neilan, B. A.; Börner, T. and Dittmann, E. (2000): Light and the transcriptional response of the microcystin biosynthesis gene cluster, Applied and Environmental Microbiology 66 [8], pp. 3387-3392.

Kaebernick, M.; Rohrlack, T.; Christoffersen, K. and Neilan, B. A. (2001): A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*, Environmental Microbiology 3 [11], pp. 669-679.

Kaneko, T.; Nakajima, N.; Okamoto, S.; Suzuki, I.; Tanabe, Y.; Tamaoki, M.; Nakamura, Y.; Kasai, F.; Watanabe, A.; Kawashima, K.; Kishida, Y.; Ono, A.; Shimizu, Y.; Takahashi, C.; Minami, C.; Fujishiro, T.; Kohara, M.; Katoh, M.; Nakazaki, N.; Nakayama, S.; Yamada, M.; Tabata, S. and Watanabe, M. M. (2007): Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843, DNA Research 14 [6], pp. 247-256.

Kardinaal, W. E.; Tonk, L.; Janse, I.; Hol, S.; Slot, P.; Huisman, J. and Visser, P. M. (2007): Competition for light between toxic and nontoxic strains of the harmful cyanobacterium *Microcystis*, Applied and Environmental Microbiology 73 [9], pp. 2939-2946.

Karlyshev, A. V.; Everest, P.; Linton, D.; Cawthraw, S.; Newell, D. G. and Wren, B. W. (2004): The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks, Microbiology 150 [6], pp. 1957-1964.

Karr, D. B.; Liang, R. T.; Reuhs, B. L. and Emerich, D. W. (2000): Altered exopolysaccharides of *Bradyrhizobium japonicum* mutants correlate with impaired soybean lectin binding, but not with effective nodule formation, Planta 211 [2], pp. 218-226.

Kehr, J. C. (2003): Untersuchungen zu Microvirin - einem potentiellen mannosebindenden Lektin aus *Microcystis aeruginosa* PCC 7806, Institute für Biologie / Molekulare Ökologie, Humboldt-Univeristät, Berlin.

Kelley, B. S.; Chang, L. C. and Bewley, C. A. (2002): Engineering an obligate domain-swapped dimer of cyanovirin-N with enhanced anti-HIV activity, Journal of the American Chemical Society 124 [13], pp. 3210-3211.

Kishore, U.; Greenhough, T. J.; Waters, P.; Shrive, A. K.; Ghai, R.; Kamran, M. F.; Bernal, A. L.; Reid, K. B. M.; Madan, T. and Chakraborty, T. (2006): Surfactant proteins SP-A and SP-D: Structure, function and receptors, Molecular Immunology 43 [9], pp. 1293-1315.

Klena, J. D.; Ashford, R. S., 2nd and Schnaitman, C. A. (1992): Role of *Escherichia coli* K-12 rfa genes and the rfp gene of *Shigella dysenteriae* 1 in generation of lipopolysaccharide core heterogeneity and attachment of O antigen, Journal of Bacteriology 174 [22], pp. 7297-7307.

Koharudin, L. M.; Viscomi, A.R.; Jee, J.G.; Ottonello, S. and Gronenborn, A. M. (2008): The evolutionarily conserved family of cyanovirin-N homologs: structures and carbohydrate specificity, Structure 16 [4], pp. 570-584.

Kolatkar, A. R. and Weis, W. I. (1996): Structural basis of galactose recognition by C-type animal lectins, The Journal of Biological Chemistry 271 [12], pp. 6679-6685.

Kondo, R.; Yoshida, T.; Yuki, Y. and Hiroishi, S. (2000): DNA-DNA reassociation among a bloom-forming cyanobacterial genus, *Microcystis*, International Journal of Systematic and Evolutionary Microbiology 50 [2], pp. 767-770.

Konopka, A.; Kromkamp, J. C. and Mur, L. R. (1987): Buoyancy regulation in phosphate-limited cultures of *Microcystis aeruginosa*, FEMS Microbiology Letters 45 [3], pp. 135-142.

Kromkamp, J. C.; Konopka, A. and Mur, L. R. (1988): Buoyancy regulation in light-limited continuous cultures of *Microcystis aeruginosa*, Journal of Plankton Research 10 [2], pp. 171-183.

Kurata, S.; Ariki, S. and Kawabata, S. (2006): Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity, Immunobiology 211 [4], pp. 237-249.

Kurmayer, R.; Dittmann, E.; Fastner, J. and Chorus, I. (2002): Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis spp.* in Lake Wannsee (Berlin, Germany), Microbial Ecology 43 [1], pp. 107-118.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 [5259], pp. 680-685.

Larget, B. and Simon, D. L. (1999): Markov Chasin Monte Carlo algorithms for the bayesian analysis of phylogenetic trees, Molecular Biology and Evolution 16 [6], pp. 750-759.

Lee, S. J. and Eisenberg, D. (2003): Seeded conversion of recombinant prion protein to a disulfide-bonded oligomer by a reduction-oxidation process, Nature Structural & Molecular Biology 10 [9], pp. 725-730.

Lee, S.J.; Jang, M. H.; Kim, H. S.; Yoon, B. D. and Oh, H. M. (2000): Variation of microcystin content of *Microcystis aeruginosa* relative to medium N:P ratio and growth stage, Journal of Applied Microbiology 89 [2], pp. 323-329.

Lee, Y.; Kim, Y.; Yeom, S.; Kim, S.; Park, S.; Jeon, C. O. and Park, W. (2008): The role of disulfide bond isomerase A (DsbA) of *Escherichia coli*, FEMS Microbiology Letters 278 [2], pp. 213-222.

Lengeler, L. W.; Drews, G. and Schlegel, H. G. (1999): Biology of the prokaryotes, Thieme, Stuttgart.

Lindahl, M. and Florencio, F. J. (2003): Thioredoxin-linked processes in cyanobacteria are as numerous as in chloroplasts, but targets are different, Proceedings of the National Academy of Sciences 100 [26], pp. 16107-16112.

Lis, H. and Sharon, N. (1998): Lectins: carbohydrate-specific proteins that mediate cellular recognition, Chemical Reviews 98 [2], pp. 637-674.

Liu, H.; Campbell, L.; Landry, M. R.; Nolla, H. A.; Brown, S. L. and Constantinou, J. (1998): *Prochlorococcus* and *Synechococcus* growth rates and contributions to production in the Arabian Sea during the 1995 Southwest and Northeast Monsoons, Deep Sea Research Part II: Topical Studies in Oceanography 45 [10-11], pp. 2327-2352.

Liu, Y. and Eisenberg, D. (2002): 3D domain swapping: As domains continue to swap, Protein Science 11 [6], pp. 1285-1299.

Liu, Y. J. and Hall, B. D. (2004): Body plan evolution of ascomycetes, as inferred from an RNA polymerase II phylogeny, Proceedings of the National Academy of Sciences 101 [13], pp. 4507-4512.

Lloyd, D. H.; Viac, J.; Werling, D.; Rème, C. A. and Gatto, H. (2007): Role of sugars in surface microbe-host interactions and immune reaction modulation, Veterinary Dermatology 18 [4], pp. 197-204.

Long, B. M.; Jones, G. J. and Orr, P. T. (2001): Cellular Microcystin content in N-limited *Microcystis aeruginosa* can be predicted from growth rate, Applied Environmental Microbiology 67 [1], pp. 278-283.

Lurling, M. (2003): Effects of microcystin-free and microcystin-containing strains of the cyanobacterium *Microcystis aeruginosa* on growth of the grazer *Daphnia magna*, Environmental Toxicology 18 [3], pp. 202-210.

Lyck, S.; Gjolme, N. and Utkilen, H. (1996): Iron starvation increases toxicity of *Microcystis aeruginosa* CYA228/1 (*Chroococcales*, *Cyanophyceae*), Phycologia 35 [6], pp. 120-124.

Lynch, D. J.; Fountain, T. L.; Mazurkiewicz, J. E. and Banas, J. A. (2007): Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture, FEMS Microbiology Letters 268 [2], pp. 158-165.

Lyra, C.; Suomalainen, S.; Gugger, M.; Vezie, C.; Sundman, P.; Paulin, L. and Sivonen, K. (2001): Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera, International Journal of Systematic and Evolutionary Microbiology 51 [2], pp. 513-526.

MacKintosh, C.; Beattle, K. A.; Klumpp, S.; Cohen, P. and Codd, G. A. (1990): Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants, FEBS Letters 264 [2], pp. 187-192.

MacKintosh, R. W.; Dalby, K. N.; Campbell, D. G.; Cohen, P. T. W.; Cohen, P. and MacKintosh, C. (1995): The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1, FEBS Letters 371 [3], pp. 236-240.

Marcus, Y.; Altman-Gueta, H.; Finkler, A. and Gurevitz, M. (2003): Dual role of cysteine 172 in redox regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity and degradation, Journal of Bacteriology 185 [5], pp. 1509-1517.

Matei, E.; Furey, W. and Gronenborn, A. M. (2008): Solution and crystal structures of a sugar binding site mutant of cyanovirin-N: no evidence of domain swapping, Structure 16 [8], pp. 1183-1194.

Mavrodi, O. V.; Mavrodi, D. V.; Park, A. A.; Weller, D. M. and Thomashow, L. S. (2006): The role of dsbA in colonization of the wheat rhizosphere by *Pseudomonas fluorescens* Q8r1-96, Microbiology 152 [3], pp. 863-872.

McElhiney, J. and Lawton, L. A. (2005): Detection of the cyanobacterial hepatotoxins microcystins, Toxicology and Applied Pharmacology 203 [3], pp. 219-230.

McFadden, G. I. (1999): Endosymbiosis and evolution of the plant cell, Current Opinion in Plant Biology 2 [6], pp. 513-519.

McFeeters, R. L.; Xiong, C.; O'Keefe, B. R.; Bokesch, H. R.; McMahon, J. B.; Ratner, D. M.; Castelli, R.; Seeberger, P. H. and Byrd, R. A. (2007): The novel fold of Scytovirin reveals a new twist for antiviral entry inhibitors, Journal of Molecular Biology 369 [2], pp. 451-461.

Mlouka, A.; Comte, K.; Castets, A. M.; Bouchier, C. and Tandeau de Marsac, N. (2004): The gas vesicle gene cluster from *Microcystis aeruginosa* and DNA rearrangements that lead to loss of cell buoyancy, Journal of Bacteriology 186 [8], pp. 2355-2365.

Mlouka, A.; Comte, K. and Tandeau de Marsac, N. (2004): Mobile DNA elements in the gas vesicle gene cluster of the planktonic cyanobacteria *Microcystis aeruginosa*, FEMS Microbiology Letters 237 [1], pp. 27-34.

Moran, A. P.; Khamri, W.; Walker, M. M. and Thursz, M. R. (2005): Role of surfactant protein D (SP-D) in innate immunity in the gastric mucosa: evidence of interaction with *Helicobacter pylori* lipopolysaccharide, Journal of Endotoxin Research 11, p. 357.

Mori, T. and Boyd, M. R. (2001): Cyanovirin-N, a potent human immunodeficiency virus-inactivating protein, blocks both CD4-dependent and CD4-independent binding of soluble gp120 (sgp120) to target cells, inhibits sCD4-induced binding of sgp120 to cell-associated CXCR4, and dissociates bound sgp120 from target cells, Antimicrobial Agents and Chemotherapy 45 [3], pp. 664-672.

Msagati, T. A.; Siame, B. A. and Shushu, D. D. (2006): Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins, Aquatic Toxicology 78 [4], pp. 382-397.

Nagradova, N. K. (2002): Three-Dimensional Domain Swapping in Homooligomeric Proteins and Its Functional Significance, Biochemistry (Moscow) 67 [8], pp. 839-849.

Nakamura, Y.; Itoh, T.; Matsuda, H. and Gojobori, T. (2004): Biased biological functions of horizontally transferred genes in prokaryotic genomes, Nature Genetics 36, pp. 760-766.

Neu, T. R.; Kuhlicke, U. and Lawrence, J. R. (2002): Assessment of fluorochromes for two-photon laser scanning microscopy of biofilms, Applied and Environmental Microbiology 68 [2], pp. 901-909.

Neu, T. R.; Woelfl, S. and Lawrence, J. R. (2004): Three-dimensional differentiation of photo-autotrophic biofilm constituents by multi-channel laser scanning microscopy (single-photon and two-photon excitation), Journal of Microbiological Methods 56 [2], pp. 161-172.

Nishizawa, T.; Nishizawa, A.; Asayama, M.; Harada, K. and Shirai, M. (2007): Diversity within the microcystin biosynthetic gene clusters among the genus *Microcystis*, Microbes and Environments 22 [4], pp. 380-390.

O'Keefe, B. R.; Shenoy, S. R.; Xie, D.; Zhang, W.; Muschik, J. M.; Currens, M. J.; Chaiken, I. and Boyd, M. R. (2000): Analysis of the interaction between the HIV-inactivating protein cyanovirin-N and soluble forms of the envelope glycoproteins gp120 and gp41, Molecular Pharmacology 58 [5], pp. 982-992.

Ochman, H.; Lawrence, J. G. and Groisman, E. A. (2000): Lateral gene transfer and the nature of bacterial innovation, Nature 405 [6784], pp. 299-304.

Otsuka, S.; Suda, S.; Li, R.; Matsumoto, S. and Watanabe, M. M. (2000): Morphological variability of colonies of *Microcystis* morphospecies in culture, The Journal of General and Applied Microbiology 46 [1], pp. 39-50.

Otsuka, S.; Suda, S.; Li, R.; Watanabe, M.; Oyaizu, H.; Matsumoto, S. and Watanabe, M. M. (1999): Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence, FEMS Microbiology Letters 172 [1], pp. 15-21.

Otsuka, S.; Suda, S.; Shibata, S.; Oyaizu, H.; Matsumoto, S. and Watanabe, M. M. (2001): A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kutzing ex Lemmermann 1907 under the rules of the Bacteriological Code, International Journal of Systematic and Evolutionary Microbiology 51 [3], pp. 873-879.

Paerl, H. W. (1983): Partitioning of CO2 fixation in the colonial cyanobacterium *Microcystis aeruginosa*: mechanism promoting formation of surface scums, Applied and Environmental Microbiology 46 [1], pp. 252-259.

Palaniyar, N.; Nadesalingam, J. and Reid, K. B. M. (2002): Pulmonary innate immune proteins and receptors that interact with gram-positive bacterial ligands, Immunobiology 205 [4-5], pp. 575-594.

Papageorgiou, J.; Linke, T. A. ; Kapralos, C.; Nicholson, B. C. and Steffensen, D. A. (2004): Extraction of cyanobacterial endotoxin, Environmental Toxicology 19 [1], pp. 82-87.

Parker, C. T.; Kloser, A. W.; Schnaitman, C. A.; Stein, M. A.; Gottesman, S. and Gibson, B. W. (1992): Role of the rfaG and rfaP genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12, Journal of Bacteriology 174 [8], pp. 2525-2538.

Paulsrud, P. and Lindblad, P. (1998): Sequence variation of the tRNALeu intron as a marker for genetic diversity and specificity of symbiotic cyanobacteria in some lichens, Applied and Environmental Microbiology 64 [1], pp. 310-315.

Paulsrud, P.; Rikkinen, J. and Lindblad, P. (1998): Cyanobiont specificity in some *Nostoc*-containing lichens and in a *Peltigera aphthosa* photosymbiodeme, New Phytologist 139 [3], pp. 517-524.

Paulsrud, P.; Rikkinen, J. and Lindblad, P. (2000): Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens, New Phytologist 146 [2], pp. 291-299.

Pearson, L. A.; Hisbergues, M.; Börner, T.; Dittmann, E. and Neilan, B. A. (2004): Inactivation of an ABC transporter gene, mcyH, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806, Applied and Environmental Microbiology 70 [11], pp. 6370-6378.

Percudani, R.; Montanini, B. and Ottonello, S. (2005): The anti-HIV cyanovirin-N domain is evolutionarily conserved and occurs as a protein module in eukaryotes, Proteins 60 [4], pp. 670-678.

Pèrez-Pèrez, M. E.; Florencio, F. J. and Lindahl, M. (2006): Selecting thioredoxins for disulphide proteomics: Target proteomes of three thioredoxins from the cyanobacterium *Synechocystis* sp. PCC 6803, Proteomics 6 [S1], pp. S186-S195.

Peumans, W. J. and Van Damme, E. J. M. (1995): Lectins as plant defense proteins, Plant Physiology 109 [2], pp. 347-352.

Prakobphol, A.; Boren, T.; Ma, W.; Zhixiang, P. and Fisher, S. J. (2005): Highly glycosylated human salivary molecules present oligosaccharides that mediate adhesion of leukocytes and *Helicobacter pylori*, Biochemistry 44 [6], pp. 2216-2224.

Rabouille, S.; Salencon, M. J. and Thebault, J. M. (2005): Functional analysis of *Microcystis* vertical migration: A dynamic model as a prospecting tool: I--Processes analysis, Ecological Modelling 188 [2-4], pp. 386-403.

Rabouille, S.; Thebault, J. M. and Salencon, M. J. (2003): Simulation of carbon reserve dynamics in *Microcystis* and its influence on vertical migration with Yoyo model, Comptes Rendus Biologies 326 [4], pp. 349-361.

Rantala, A.; Fewer, D. P.; Hisbergues, M.; Rouhiainen, L.; Vaitomaa, J.; Börner, T. and Sivonen, K. (2004): Phylogenetic evidence for the early evolution of microcystin synthesis, Proceedings of the National Academy of Sciences 101 [2], pp. 568-573.

Ratner, D. M.; Adams, E. W.; Disney, M. D. and Seeberger, P. H. (2004): Tools for glycomics: mapping interactions of carbohydrates in biological systems, ChemBioChem : a European Journal of Chemical Biology 5 [10], pp. 1375-1383.

Ratner, D. M.; Adams, E. W.; Su, J. ; O'Keefe, B. R.; Mrksich, M. and Seeberger, P. H. (2004): Probing protein-carbohydrate interactions with microarrays of synthetic oligosaccharides, ChemBioChem : a European Journal of Chemical Biology 5 [3], pp. 379-383.

Ratner, D. M.; Swanson, E. R. and Seeberger, P. H. (2003): Automated synthesis of a protected N-linked glycoprotein core pentasaccharide, Organic Letters 5 [24], pp. 4717-4720.

Raziuddin, S.; Siegelman, H. W. and Tornabene, T. G. (1983): Lipopolysaccharides of the cyanobacterium *Microcystis aeruginosa*, European Journal of Biochemistry 137 [1-2], pp. 333-336.

Reynolds, C. S. ; Jaworski, G. H. M.; Cmiech, H. A. and Leedale, G. F. (1981): On the annual cycle of the llue-green Alga *Microcystis aeruginosa* Kutz. Emend. Elenkin, Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 293 [1068], pp. 419-477.

Rini, J. M. (1995): X-ray crystal structures of animal lectins, Current Opinion in Structural Biology 5 [5], pp. 617-621.

Rippka, R. (1988): Isolation and purification of cyanobacteria, Methods in Enzymology 167, pp. 3-27.

Rippka, R.; Deruelles, J.; Waterbury, J. B.; Herdman, M. and Stanier, R. Y. (1979): Generic assignments, strain histories and properties of pure cultures of cyanobacteria, Journal of General Microbiology 111, pp. 1-61.

Roberts, E. C.; Zubkov, M. V.; Martin-Cereceda, M.; Novarino, G. and Wootton, E. C. (2006): Cell surface lectin-binding glycoconjugates on marine planktonic protists, FEMS Microbiology Letters 265 [2], pp. 202-207.

Robitaille, G.; Moineau, S.; St-Gelais, D.; Vadeboncoeur, C. and Britten, M. (2006): Detection and quantification of capsular exopolysaccharides from *Streptococcus thermophilus* using lectin probes, Journal of Dairy Science 89 [11], pp. 4156-4162.

Rocchetta, H. L.; Burrows, L. L. and Lam, J. S. (1999): Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*, Microbiology and Molecular Biology Reviews 63 [3], pp. 523-553.

Rodrigue, A.; Chanal, A.; Beck, K.; Muller, M. and Wu, L.-F. (1999): Co-translocation of a periplasmic enzyme complex by a hitchhiker mechanism through the bacterial Tat pathway, The Journal of Biological Chemistry 274 [19], pp. 13223-13228.

Rogers, Matthew; Patron, Nicola and Keeling, Patrick (2007): Horizontal transfer of a eukaryotic plastid-targeted protein gene to cyanobacteria, BMC Biology 5 [1], p. 26.

Rohrlack, T.; Dittmann, E.; Börner, T. and Christoffersen, K. (2001): Effects of cell-bound microcystins on survival and feeding of *Daphnia spp.*, Applied and Environmental Microbiology 67 [8], pp. 3523-3529.

Runnegar, M.; Berndt, N.; Kong, S. M.; Lee, E. Y. and Zhang, L. (1995): In vivo and in vitro binding of microcystin to protein phosphatases 1 and 2A, Biochemical and Biophysical Research Communications 216 [1], pp. 162-169.

Runnegar, M. T.; Jackson, A. R. and Falconer, I. R. (1988): Toxicity to mice and sheep of a bloom of the cyanobacterium (blue-green alga) *Anabaena circinalis*, Toxicon 26 [6], pp. 599-602.

Runnegar, M. T.; Kong, S. and Berndt, N. (1993): Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins, The American Journal of Physiology 265 [2 Pt 1], pp. G224-230.

Sahly, H.; Keisari, Y.; Crouch, E.; Sharon, N. and Ofek, I. (2008): Recognition of bacterial surface polysaccharides by lectins of the innate immune system and its contribution to defense against infection: the case of pulmonary pathogens, Infection and Immunity 76 [4], pp. 1322-1332.

Sambrook, J. and Russel, D. W. (2001): Molecular Cloning - A laboratory manual, Irwin, N., Ed, Cold Spring Harbor Laboratory Press, New York.

Sandstrom, C.; Berteau, O.; Gemma, E.; Oscarson, S.; Kenne, L. and Gronenborn, A. M. (2004): Atomic mapping of the interactions between the antiviral agent cyanovirin-N and oligomannosides by saturation-transfer difference NMR, Biochemistry 43 [44], pp. 13926-13931.

Sandvig, K. (2001): Shiga toxins, Toxicon 39 [11], pp. 1629-1635.

Sato, Y.; Okuyama, S. and Hori, K. (2007): Primary structure and carbohydrate binding specificity of a potent anti-HIV lectin isolated from the filamentous cyanobacterium *Oscillatoria agardhii*, The Journal of Biological Chemistry 282 [15], pp. 11021-11029.

Schaffer, C. and Messner, P. (2004): Surface-layer glycoproteins: an example for the diversity of bacterial glycosylation with promising impacts on nanobiotechnology, Glycobiology 14 [8], pp. 31R-42.

Schatz, D.; Keren, Y.; Vardi, A.; Sukenik, A.; Carmeli, S.; Börner, T.; Dittmann, E. and Kaplan, A. (2007): Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins, Environmental Microbiology 9 [4], pp. 965-970.

Schopf, J. W. and Packer, B. M. (1987): Early Archean (3.3-billion to 3.5-billion-year-old) microfossils from Warrawoona Group, Australia, Science 237, pp. 70-73.

Schwarzer, D.; Finking, R. and Marahiel, M. A. (2003): Nonribosomal peptides: from genes to products, Natural Products Reports 20 [3], pp. 275-287.

Sevier, C. S. and Kaiser, C. A. (2002): Formation and transfer of disulphide bonds in living cells, Nature Reviews. Molecular Cell Biology 3 [11], pp. 836-847.

Sevilla, E.; Martin-Luna, B. ; Vela, L. M. ; Bes, T.; Fillat, M. F. and Peleato, M. L. (2008): Iron availability affects *mcyD* expression and microcystin-LR synthesis in *Microcystis aeruginosa* PCC7806, Environmental Microbiology 10 [10], pp. 2476-2483.

Sharon, N. and Lis, H. (1972): Lectins: cell-agglutinating and sugar-specific proteins, Science 177 [53], pp. 949-959.

Sharon, N. and Lis, H. (1989): Lectins as cell recognition molecules, Science 246 [4927], pp. 227-234.

Sharon, N. and Lis, H. (1990): Legume lectins--a large family of homologous proteins, The FASEB journal 4 [14], pp. 3198-3208.

Sharon, N. and Lis, H. (2002): How proteins bind carbohydrates: lessons from legume lectins, Journal of Agricultural and Food Chemistry 50 [22], pp. 6586-6591.

Sharon, N. and Lis, H. (2004): History of lectins: from hemagglutinins to biological recognition molecules, Glycobiology 14 [11], pp. 53R-62R.

Shenoy, S. R.; Barrientos, L. G.; Ratner, D. M.; O'Keefe, B. R.; Seeberger, P. H.; Gronenborn, A. M. and Boyd, M. R. (2002): Multisite and multivalent binding between cyanovirin-N and branched oligomannosides: calorimetric and NMR characterization, Chemistry & Biology 9 [10], pp. 1109-1118.

Shenoy, S. R.; O'Keefe, B. R.; Bolmstedt, A. J.; Cartner, L. K. and Boyd, M. R. (2001): Selective interactions of the human immunodeficiency virus-inactivating protein cyanovirin-N with high-mannose oligosaccharides on gp120 and other glycoproteins, The Journal of Pharmacology and Experimental Therapeutics 297 [2], pp. 704-710.

Sherlock, O.; Vejborg, R. M. and Klemm, P. (2005): The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* Is self recognizing and induces bacterial aggregation and biofilm formation, Infection and Immunity 73 [4], pp. 1954-1963.

Shi, S. Y.; Cai, X. H. and Ding, D. (2005): Identification and categorization of horizontally transferred genes in prokaryotic genomes, Acta Biochimica et Biophysica Sinica 37 [8], pp. 561-566.

Singh, A. K.; Bhattacharyya-Pakrasi, M. and Pakrasi, H. B. (2008): Identification of an atypical membrane protein involved in the formation of protein disulfide bonds in oxygenic photosynthetic organisms, The Journal of Biological Chemistry 283 [23], pp. 15762-15770.

Sivonen, K. (2008): Emerging high throughput analyses of cyanobacterial toxins and toxic cyanobacteria, Advances in Experimental Medicine and Biology 619, pp. 539-557.

Sivonen, K. and Jones, G. (1999): Cyanobacterial toxins, Chorus, I. and Bartram, J., Toxic cyanobacteria in water: a guide to their public health pp. 55-124, E & EF Spon, London and New York.

Smarda, J.; Smajs, D.; Komrska, J. and Krzyzanek, V. (2002): S-layers on cell walls of cyanobacteria, Micron 33 [3], pp. 257-277.

Song, L.; Sano, T.; Li, R.; Watanabe, M. M.; Liu, Y. and Kaya, K. (1998): Microcystin production of *Microcystis viridis* (cyanobacteria) under different culture conditions, Phycological Research 46 [s2], pp. 19-23.

Szymanski, C. M. and Wren, B. W. (2005): Protein glycosylation in bacterial mucosal pathogens, Nature Reviews Microbiology 3 [3], pp. 225-237.

Takeya, K.; Kuwata, A.; Yoshida, M. and Miyazaki, T. (2004): Effect of dilution rate on competitive interactions between the cyanobacterium *Microcystis novacekii* and the green alga *Scenedesmus quadricauda* in mixed chemostat cultures, Journal of Plankton Research 26 [1], pp. 29-35.

Tawfik, D. (2002): Modification of sulfhydryl groups with DTNB, The Protein Protocols Handbook pp. 483-484.

Tepfer, D.; Garcia-Gonzales, R.; Mansouri, H.; Seruga, M.; Message, B.; Leach, F. and Perica, M. C. (2003): Homology-dependent DNA transfer from plants to a soil bacterium under laboratory conditions: Implications in evolution and horizontal gene transfer, Transgenic Research 12 [4], pp. 425-437.

Thomas, R. H. and Walsby, A. E. (1985): Buoyancy regulation in a strain of *Microcystis*, Journal of General Microbiology 131 [4], pp. 799-809.

Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F. and Higgins, D. G. (1997): The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Research 25 [24], pp. 4876-4882.

Tielker, D.; Hacker, S.; Loris, R.; Strathmann, M.; Wingender, J.; Wilhelm, S.; Rosenau, F. and Jaeger, K. E. (2005): *Pseudomonas aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation, Microbiology 151 [5], pp. 1313-1323.

Tien, C. J.; Sigee, D. C. and White, K. N. (2005): Characterization of surface sugars on algal cells with fluorescein isothiocyanate-conjugated lectins, Protoplasma 225 [3], pp. 225-233.

Tillett, D.; Dittmann, E.; Erhard, M.; von Döhren, H.; Börner, T. and Neilan, B. A. (2000): Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system, Chemistry & Biology 7 [10], pp. 753-764.

Tillett, D.; Parker, D. L. and Neilan, B. A. (2001): Detection of toxigenicity by a probe for the microcystin synthetase A gene (mcyA) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (Phycocyanin Intergenic Spacer) phylogenies, Applied and Environmental Microbiology 67 [6], pp. 2810-2818.

Toivola, D. M.; Eriksson, J. E. and Brautigan, D. L. (1994): Identification of protein phosphatase 2A as the primary target for microcystin-LR in rat liver homogenates, FEBS Letters 344 [2-3], pp. 175-180.

Tooming-Klunderud, A.; Fewer, D. P.; Rohrlack, T.; Jokela, J.; Rouhiainen, L.; Sivonen, K.; Kristensen, T. and Jakobsen, K. S. (2008): Evidence for positive selection acting on microcystin synthetase adenylation domains in three cyanobacterial genera, BMC Evolutionary Biology 8 [1], p. 256.

Tooming-Klunderud, A.; Mikalsen, B.; Kristensen, T. and Jakobsen, K. S. (2008): The mosaic structure of the mcyABC operon in *Microcystis*, Microbiology 154 [7], pp. 1886-1899.

Trost, P.; Fermani, S.; Marri, L.; Zaffagnini, M.; Falini, G.; Scagliarini, S.; Pupillo, P. and Sparla, F. (2006): Thioredoxin-dependent regulation of photosynthetic glyceraldehyde-3-phosphate dehydrogenase: autonomous vs. CP12-dependent mechanisms, Photosynthesis Research 89 [2], pp. 263-275.

Upreti, R. K.; Kumar, M. and Shankar, V. (2003): Bacterial glycoproteins: Functions, biosynthesis and applications, Proteomics 3 [4], pp. 363-379.

Via-Ordorika, L.; Fastner, J.; Kurmayer, R.; Hisbergues, M.; Dittmann, E.; Komarek, J.; Erhard, M. and Chorus, I. (2004): Distribution of microcystin-producing and non-microcystin-producing *Microcystis sp*. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies, Systematic and Applied Microbiology 27 [5], pp. 592-602.

Watanabe, M. F. and Oishi, S. (1985): Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions, Applied and Environmental Microbiology 49 [5], pp. 1342-1344.

Weckesser, J.; Drews, G. and Mayer, H. (1979): Lipopolysaccharides of photosynthetic prokaryotes, Annual Review of Microbiology 33 [1], pp. 215-239.

Weis, W. I. and Drickamer, K. (1996): Structural basis of lectin-carbohydrate recognition, Annual Review of Biochemistry 65 [1], pp. 441-473.

Welker, M.; Brunke, M.; Preussel, K.; Lippert, I. and von Döhren, H. (2004): Diversity and distribution of *Microcystis* (cyanobacteria) oligopeptide chemotypes from natural communities studied by single-colony mass spectrometry, Microbiology 150 [6], pp. 1785-1796.

Welker, M. and von Döhren, H. (2006): Cyanobacterial peptides - Nature's own combinatorial biosynthesis, FEMS Microbiology Reviews 30 [4], pp. 530-563.

Westphal, O. and Jann, K. (1965): Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, Methods Carbohydrate Chemistry 5, pp. 83-91.

Whitton, B. A. and Potts, M. (2000), Whitton, B. A. and Potts, M., The ecology of cyanobacteria pp. 1-11, Kluwer, Dordrecht.

Wiedner, C.; Visser, P. M.; Fastner, J.; Metcalf, J. S.; Codd, G. A. and Mur, L. R. (2003): Effects of light on the microcystin content of *Microcystis* strain PCC 7806, Applied and Environmental Microbiology 69 [3], pp. 1475-1481.

Wigglesworth-Cooksey, B. and Cooksey, K. E. (2005): Use of fluorophore-conjugated lectins to study cell-cell interactions in model marine biofilms, Applied and Environmental Microbiology 71 [1], pp. 428-435.

Wright, J. R. (2005): Immunoregulatory functions of surfactant proteins, Nature Reviews. Immunology 5 [1], pp. 58-68.

Wu, H.; Zeng, M. and Fives-Taylor, P. (2007): The glycan moieties and the N-terminal polypeptide backbone of a fimbria-associated adhesin, Fap1, play distinct roles in the biofilm development of *Streptococcus parasanguinis*, Infection and Immunity 75 [5], pp. 2181-2188.

Yamaguchi, M.; Jimbo, M.; Sakai, R.; Muramoto, K. and Kamiya, H. (1998): Purification and characterization of *Microcystis aeruginosa* (freshwater cyanobacterium) lectin, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 119 [3], pp. 593-597.

Yamaguchi, M.; Ogawa, T.; Muramoto, K.; Jimbo, M. and Kamiya, H. (2000): Effects of culture conditions on the expression level of lectin in *Microcystis aeruginosa* (freshwater cyanobacterium), Fisheries Science 66 [4], pp. 665-669.

Yamaguchi, M.; Ogawa, T.; Muramoto, K.; Kamio, Y.; Jimbo, M. and Kamiya, H. (1999): Isolation and characterization of a mannan-binding lectin from the freshwater cyanobacterium (blue-green algae) *Microcystis viridis*, Biochemical and Biophysical Research Communications 265 [3], pp. 703-708.

Yang, F.; Bewley, C. A.; Louis, J. M.; Gustafson, K. R.; Boyd, M. R.; Gronenborn, A. M.; Clore, G. M. and Wlodawer, A. (1999): Crystal structure of cyanovirin-N, a potent HIV-inactivating protein, shows unexpected domain swapping, Journal of Molecular Biology 288 [3], pp. 403-412.

Young, F. M.; Thomson, C.; Metcalf, J. S.; Lucocq, J. M. and Codd, G. A. (2005): Immunogold localisation of microcystins in cryosectioned cells of *Microcystis*, Journal of Structural Biology 151 [2], pp. 208-214.

Zegers, I.; Deswarte, J. and Wyns, L. (1999): Trimeric domain-swapped barnase, Proceedings of the National Academy of Sciences 96 [3], pp. 818-822.

Zhang, M.; Kong, F.; Tan, X.; Yang, Z.; Cao, H. and Xing, P. (2007): Biochemical, morphological, and genetic variations in *Microcystis aeruginosa* due to colony disaggregation, World Journal of Microbiology and Biotechnology 23 [5], pp. 663-670.

Zhaxybayeva, O.; Gogarten, J. P.; Charlebois, R. L.; Doolittle, W. F. and Papke, R. T. (2006): Phylogenetic analyses of cyanobacterial genomes: quantification of horizontal gene transfer events, Genome Research 16 [9], pp. 1099-1108.

Zilliges, Y. (2007): Molekulare Funktionsanalyse von Microcystin in *Microcystis aeruginosa* PCC 7806, Institut für Biologie, Molekulare Ökologie, Humboldt-Universität, Berlin.

Zilliges, Y.; Kehr, J. C.; Mikkat, S.; Bouchier, C.; Tandeau de Marsac, N.; Börner, T. and Dittmann, E. (2008): An extracellular glycoprotein is implicated in cell-cell contacts in the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806, Journal of Bacteriology 190 [8], pp. 2871-2879.

Ziółkowska, N. E. and Wlodawer, A. (2006): Structural studies of algal lectins with anti-HIV activity, Acta Biochimica Polonica 53 [4], pp. 617-626.

Znachor, P.; Jurczak, T.; Komarkova, J.; Jezberova, J.; Mankiewicz, J.; Kascarontovska, K. and Zapomelova, E. (2006): Summer changes in cyanobacterial bloom composition and microcystin concentration in eutrophic Czech reservoirs, Environmental Toxicology 21 [3], pp. 236-243.

SUPPLEMENT

Alignment of CV-N family sequence used to calculate the phylogenetic tree in chapter 3.4.4. Additional C- and N-terminal domains present in some members were not included in the alignment.

ABBREVIATIONS

aa	amino acid
ACP	acyl carrier protein
Adda	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid
Amp	ampicilin
AMT	aminotransferase
APS	ammonium persulphate
AT	acyltransferase
bp	base pair(s)
BSA	bovine serum albumin
CM	C-methyltransferase
Cm	chloramphenicol
DH	dehydratase
DTT	1,4-dithiothreitole
DNA	deoxyribonucleic acid
dNTP	any desoxyribonucleotide
EDTA	ethylene diamine tetra-acetic acid
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
FTICR MS	Fourier transform ion cyclotron resonance mass spectrometry
HEPES	[4(2-hydroxyethyl)-1-piperazino]-ethanesulphonic acid
HGT	horizontal gene transfer
HPLC	high performance liquid chromatography
IFM	immunofluorescence microscopy
IPTG	isopropyl-thio-galactoside
JTT	Jones-Taylor-Thornton model for evolutionary rates
kb	kilo base pair(s)
kDa	kilo Dalton
KR	ketoacyl reductase

KS	ketoacyl synthase
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption ionisation
mc	microcystin
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	The National Center for Biotechnology Information
NIES	National Institute for Environmental Studies
NJ	neighbor joining algorithm
NRPS	non-ribosomal peptide synthase
nt	nucleotide(s)
OD	optical density
OM	O-methyltransferase
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
PMSF	phenyl-methyl-sulphonyl-fluoride
RNA	ribonucleic acid
rpm	round per minute
RT	room temperature
SAXS	small-angle X-ray scattering
SDS	sodium dodecyl sulphate
TBE	tris-borate-EDTA buffer
TEMED	N',N',N',N'-tetramethyl-ethylene-diamine
TOF	time of flight
Tris-HCl	tris-(hydroxymethyl)-aminomethane-hydrochloride
UV	ultraviolet light
UWOCC	University of Wisconsin at Oshkosh Culture Collection

WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PUBLICATIONS

PAPERS

J. C. Kehr, Y. Zilliges, S. Meissner, S. Mikkat, T. Börner, E. Dittmann
Two faces of a toxin: The cyanobacterial hepatotoxin microcystin is implicated in the postranslational modification of proteins
In preparation

L. Frangeul, P. Quillardet, A.M. Castets, J.F. Humbert, H.C.P. Matthijs, D. Cortez, A. Tolonen, C.C. Zhang, S. Gribaldo, J.C. Kehr, Y. Zilliges, N. Ziemert, S. Becker, E. Talla, A. Latifi, A. Billault, A. Lepelletier, E. Dittmann, C. Bouchier and N. Tandeau de Marsac
Highly plastic genome of *Microcystis aeruginosa* PCC7806, a ubiquitous toxic freshwater cyanobacterium
BMC Genomics 2008 8: 274

Y. Zilliges, J.C. Kehr, S. Mikkat, C. Bouchier, N. Tandeau de Marsac, T. Börner, E. Dittmann
An extracellular glycoprotein is implicated in cell-cell contacts in the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806
Journal of Bacteriology 2008 190: 8 2871-2879

J.C. Kehr, Y. Zilliges, A. Springer, M. D. Disney, D. D. Ratner, C. Bouchier, P. H. Seeberger, N. Tandeau de Marsac, E. Dittmann
A mannan binding lectin is involved in cell-cell attachment in a toxic strain of *Microcystis aeruginosa*
Molecular Microbiology 2006 59: 3 893-906

TALKS

Two extracellular proteins are implicated in cell-cell contacts in the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806
ESF-EMBO Symposium
Molecular Bioenergetics of Cyanobacteria: Towards Systems Biology Level of Understanding
April 29th–May 3rd, 2008, Sant Feliu de Guixols, Spain

The mannan-binding lectin Microvirin binds microcystin and is involved in cell-cell attachment in a toxic strain of *Microcystis aeruginosa*
VAAM Annual Meeting 2007
April 1st–4th, 2007, Osnabrück, Germany

A mannan-binding lectin is involved in cell-cell attachment in a toxic strain of *Microcystis aeruginosa*
International Workshop "Biology of Bacteria Producing Natural Products"
October 7th–9th, 2005, Dresden, Germany

POSTERS

A mannan-binding lectin is involved in cell-cell attachment in a toxic strain of *Microcystis aeruginosa*
VAAM Annual Meeting 2006
March 19th–22th, 2006, Jena

The mannan-binding lectin Microvirin binds microcystin and is involved in cell-cell attachment in a toxic strain of *Microcystis aeruginosa*
4th ESF Conference on the Molecular Bioenergetics of Cyanobacteria
May 21st–26th, 2005, Sant Feliu de Guixols, Spain

Microvirin, a putative mannose-binding lectin from *Microcystis aeruginosa* PCC7806
6th International Conference on Toxic Cyanobacteria
June 21st–26th, 2004, Bergen, Norway

AWARDS

3rd Price, Talk
A mannan binding lectin is involved in cell-cell attachment in *Microcystis aeruginosa* PCC 7806
3. Studententag der Lebenswissenschaften (3rd Student Meeting of Life Sciences)
June 24th, 2005, Berlin, Germany

SELBSTÄNDIGKEITSERKLÄRUNG

gemäß §6 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Humboldt-Universität zu Berlin

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den angegebenen Hilfsmitteln und Quellen dazu verwendet und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.