

A mannan binding lectin is involved in cell–cell attachment in a toxic strain of *Microcystis aeruginosa*

Jan-Christoph Kehr,¹ Yvonne Zilliges,¹ Andreas Springer,² Matthew D. Disney,³ Daniel D. Ratner,⁴ Christiane Bouchier,⁵ Peter H. Seeberger,^{3,4} Nicole Tandeau de Marsac⁶ and Elke Dittmann^{1*}

¹Humboldt University, Institute of Biology, Department of Molecular Ecology, Chausseestr., 117, 10115 Berlin, Germany.

²Humboldt University, Institute of Chemistry, Analytical and Environmental Chemistry, Brook-Taylor-Str. 2, 12489 Berlin, Germany.

³Laboratory of Organic Chemistry, Swiss Federal Institute of Technology (ETH) Zurich, Wolfgang-Pauli-Str. 10, 8093 Zuerich, Switzerland.

⁴Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

⁵Plate-forme Génomique – Pasteur Genopole® Ile de France, Institut Pasteur 25-28 rue du Docteur Roux, 75724 Paris Cedex 15, France.

⁶Unité des Cyanobactéries (URA-CNRS 2172), Département de Microbiologie fondamentale et médicale, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France.

Summary

Microcystin, a hepatotoxin that represents a serious health risk for humans and livestock, is produced by the bloom-forming cyanobacterium *Microcystis aeruginosa* in freshwater bodies worldwide. Here we describe the discovery of a lectin, microvirin (MVN), in *M. aeruginosa* PCC7806 that shares 33% identity with the potent anti-HIV protein cyanovirin-N from *Nostoc ellipsosporum*. Carbohydrate microarrays were employed to demonstrate the high specificity of the protein for high-mannose structures containing $\alpha(1\rightarrow2)$ linked mannose residues. Lectin binding analyses and phenotypic characterizations of MVN-deficient mutants suggest that MVN is involved in cell–cell recognition and cell–cell attachment of *Microcystis*. A binding partner of MVN was identified in the lipopolysaccharide fraction of *M. aeruginosa*

PCC7806. MVN is differentially expressed in mutants lacking the hepatotoxin microcystin. Additionally, MVN-deficient mutants contain much lower amounts of microcystin than the wild-type cells. We discuss a possible functional correlation between microcystin and the lectin and possible implications on *Microcystis* morphotype formation. This study provides the first experimental evidence that microcystins may have an impact on *Microcystis* colony formation that is highly important for the competitive advantage of *Microcystis* over other phytoplankton species.

Introduction

Cyanobacteria of the genus *Microcystis* frequently form dense water blooms in freshwater lakes. While blooms are unpleasant both visually and due to released odour and taste factors, toxic metabolites produced by these cyanobacterial species pose a more serious problem for humans and livestock. Microcystins, the most widespread cyanobacterial toxins, are a family of cyclic heptapeptides that specifically inhibit eukaryotic type protein phosphatases of types 1 and 2A (Runnegar *et al.*, 1993). They were frequently implicated in human illness or even death (Jochimsen *et al.*, 1998; Kuiper-Goodman *et al.*, 1999). Microcystins are synthesized on a large enzyme complex comprising non-ribosomal peptide synthetases, polyketide synthases and tailoring enzymes (Tillett *et al.*, 2000). In the field, *Microcystis* occurs in form of characteristic colonies that can be classified into different morphotypes (Komárek and Anagnostidis, 1999). Colony formation and the presence of gas vesicles are crucial for the ability of *Microcystis* cells to regulate their buoyancy. These properties enable a fast vertical migration in lakes, a phenomenon that is widely discussed as the major competitive advantage over other phytoplankton species (Bonnet and Poulin, 2002). *Microcystis* colonies differ in shape and size, but also in the appearance of their mucilage (Via-Ordorika *et al.*, 2004). A number of studies has reported a relationship between colony type and the presence of specific oligopeptides. Different isoforms of microcystin were most frequently detected in *M. aeruginosa* colonies (Fastner *et al.*, 2001; Kurmayer *et al.*, 2003; Via-Ordorika *et al.*, 2004), whereas *M. ichthyoblabe* and *M. wesenbergii* colonies were found to contain non-toxic oligopeptides (Fastner *et al.*, 2001).

Accepted 11 November, 2005. *For correspondence. E-mail elke.dittmann@rz.hu-berlin.de; Tel. (+49) 30 2093 8145; Fax (+49) 30 2093 8141.

Recently, different mannan binding lectins were isolated and biochemically characterized from two different *Microcystis* morphospecies, the *M. viridis* lectin MVL (Yamaguchi *et al.*, 1999; Bewley *et al.*, 2004) and the *M. aeruginosa* lectin MAL (Jimbo *et al.*, 2000). These two proteins, which do not share sequence homologies, provided a first evidence that *Microcystis* morphospecies might not only differ in their toxin content, but also in their cell surface properties. Lectins are a family of mono- and oligosaccharide binding proteins found in a wide range of organisms, including plants, animals and microorganisms (Sharon and Lis, 1972). They usually lack any kind of enzymatic activity. In most cases one lectin molecule possesses two or more carbohydrate binding sites enabling the cross-linking of cells by binding sugar residues on their surfaces (Sharon, 1987). A few lectins of prokaryotic origin have been analysed in more detail. Among the best studied are the lectins PA-IL and PA-IIL from the opportunistic human pathogen *Pseudomonas aeruginosa* that constitutes a serious threat for patients suffering from cystic fibrosis (Imberty *et al.*, 2004). Due to their ability to network polysaccharides *P. aeruginosa* lectins are suggested to be involved in host recognition and biofilm formation (Loris *et al.*, 2003; Imberty *et al.*, 2004; Tielker *et al.*, 2005). Other lectins are implicated in symbioses, where they mediate the recognition of symbiosis partners (Ho *et al.*, 1994). Lectin-carbohydrate interactions were also shown to be important for the social development of bacteria, like the myxobacterial fruiting body formation (Cumsky and Zusman, 1981).

The highly specific sugar binding properties of lectins make them particularly interesting for biotechnological and medical applications. Lectins can prevent virus-host cell interactions by blocking specific sugar residues involved in the fusion process. Interestingly, two of the most promising anti-human immunodeficiency virus (HIV) preventive drug leads were isolated from cyanobacteria: cyanovirin-N (CV-N) from *Nostoc ellipsosporum* (Boyd *et al.*, 1997) and scytovirin from *Scytonema varium* (Bokešch *et al.* 2003). The ability of carbohydrate binding proteins to interact preferentially with specific cell types has also led to attempts to use them as drug carrier (Sharma *et al.*, 2004) and for blood typing (Khan *et al.*, 2002). Important further applications include the diagnostic use of lectins for the identification of microorganisms (Hynes *et al.*, 2002) and the use of lectins for the visualization of extracellular matrices to determine the carbohydrate composition of, e.g. biofilms (Neu *et al.*, 2001).

While cyanobacterial lectins have been extensively investigated on the structural and biochemical levels (Shenoy *et al.*, 2002; Bewley *et al.*, 2004), little is known about the function of cyanobacterial lectins for the producing strain. Here we report the discovery of a homologue of the oligomannan binding lectin CV-N in the toxic cyano-

bacterium *M. aeruginosa* PCC7806. A carbohydrate array technology was applied to determine the oligosaccharide specificity of the protein. Lectin binding analyses and phenotypic investigations of mutants revealed that the lectin specifically binds to a sugar moiety present on the surface of *M. aeruginosa* PCC7806 and might be involved in cell-cell attachment within a *Microcystis* colony. Furthermore, different lines of evidence indicate a functional correlation between the presence of lectin and that of the potent cyanobacterial toxin microcystin. This study provides the first molecular indication that the cyanobacterial toxin microcystin may not only play a role in grazing deterrence but may also have an impact on cell-cell recognition and morphotype formation of *M. aeruginosa*.

Results

Besides the authentic *mcy* promoter region of the microcystin biosynthesis operon a 84 nt sequence (*mcy*-box) was detected in the genome of *M. aeruginosa* PCC7806 (see *Experimental procedures*) that displayed 84% identity to a nucleotide region that overlaps one of the transcriptional start points of the *mcyA* gene (Kaebnick *et al.*, 2002). The sequence of the *mcy*-box and the position in relation to the transcription and translation start points of the adjacent genes is shown in Fig. 1. A second *mcy*-box is located upstream of an open reading frame with 33% identity to the mannan binding lectin CV-N from *N. ellipsosporum*. The *Microcystis* homologue was designated microvirin (MVN). The nucleotide sequence of the *mvn* gene was deposited in the EMBL database under the accession number AM041066.

Microvirin sequence analyses

Cyanovirin-N consists of two domains exhibiting 32% identity among each other. Likewise, the 108 aa protein encoded in the *Microcystis* genome is composed of two similar domains. An amino acid alignment of the individual domains of the two proteins was performed and revealed an overall low similarity (Fig. 2A). A detailed comparison of the amino acid sequences of MVN with that of CV-N revealed the presence of four conserved cysteine residues. These cysteine residues are known to be involved in internal disulphide bridge formation that is crucial for the structure and activity of CV-N (Gustafson *et al.*, 1997). MVN contains two additional cysteine residues in its C-terminal domain. CV-N comprises two carbohydrate binding sites, both with nanomolar affinity to Man(8) and Man(9) (Bewley and Otero-Quintero, 2001). From the amino acid residues that form part of the primary carbohydrate binding site of CV-N some are present or conservatively exchanged in MVN (N42, N53, E56, T57), others are absent (E41, P51, R76, Q78). Similarly, residues involved in the secondary carbohydrate binding site are

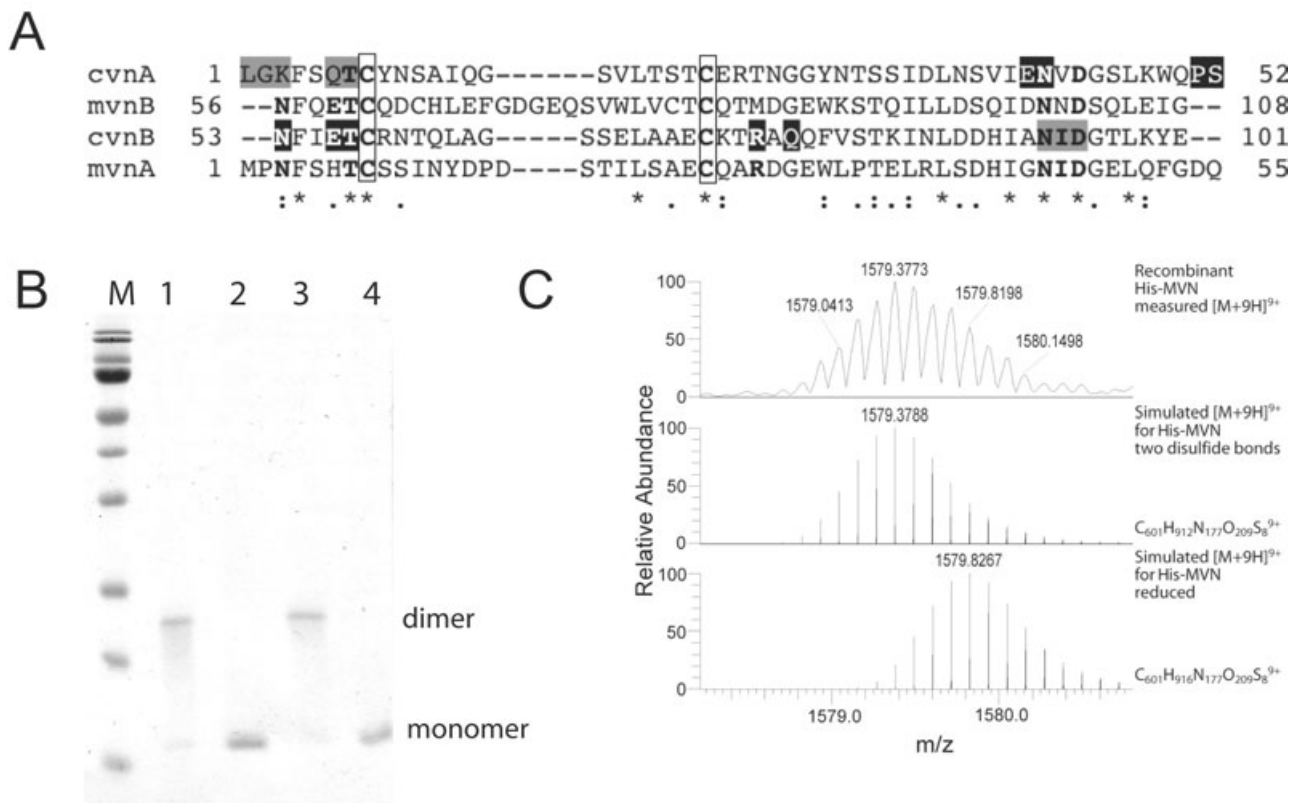


Fig. 2. A. Sequence alignment of the two domains of CV-N from *N. elliposporum* and MVN from *M. aeruginosa* PCC7806. Residues that are part of the high affinity carbohydrate binding site in CV-N are indicated as white letters on black backgrounds and residues that are part of the low affinity binding site in CV-N are underlined with grey backgrounds respectively. Carbohydrate binding residues that are identical in at least one domain of MVN and CV-N are indicated with bold letters. Conserved cysteines are shown as bold letters in rectangles.

B. SDS-PAGE of a single-step purification of the His₆-tagged MVN protein from *E. coli*. Lane 1 shows reduced His-MVN and lane 2 non-reduced His-MVN purified under native conditions. Lanes 3 shows reduced His-MVN and lane 4 non-reduced His-MVN purified under denaturing conditions.

C. 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.

the high-mannose carbohydrate that bind MVN can be determined. The results unambiguously show that MVN binds to carbohydrate and the highest signals are observed with structures that contain $\alpha(1\rightarrow2)$ linked mannose residues (Fig. 3). Specifically, the high mannose substructures **8** [Man(9)], **6** [D1 arm of Man(9)], and **4** [D3 arm of Man(9)] gave the highest signal for binding, whereas no signal is observed for binding to monosaccharides **1** (galactose, negative control) and **2** (mannose), and to oligosaccharides **3** and **5** [mannosides not containing $\alpha(1\rightarrow2)$ links] respectively (Fig. 3). Mannoside **7** gives a signal that is above background but with much lower intensity than for the other $\alpha(1\rightarrow2)$ linked mannosides. Previously, these high mannose arrays were used to probe the binding specificity of CV-N. Those results showed that CV-N preferentially bound to $\alpha(1\rightarrow2)$ linked mannose residues, like the type contained in the D1 and the D3 arms. Comparison of array results with MVN and CV-N show that both structures have similar carbohydrate binding specificities. Thus, both CV-N and MVN bind to

similar carbohydrate structures, which could be predicted based on the sequence homology.

Construction of a MVN mutant

To investigate the *in vivo* function of MVN, the gene was disrupted by homologous integrative transformation. The Δmvn disruption constructs were introduced into *M. aeruginosa* PCC7806 by both natural transformation and electroporation. Transformant clones were obtained after 4–6 weeks. Two clones from each disruption experiment were randomly selected and studied further. PCR amplification of *mvn* from the respective PCC7806 Cm^r mutant clones showed the stable chromosomal integration of the mutant constructs (Fig. 4A). The derived mutant cells seemed to grow as well as the wild-type cells. The wild-type strain has lost the ability to form cell aggregates under standard laboratory conditions as a result of long-term culturing under artificial conditions. However, the addition of the overexpressed MVN protein to the ΔMVN

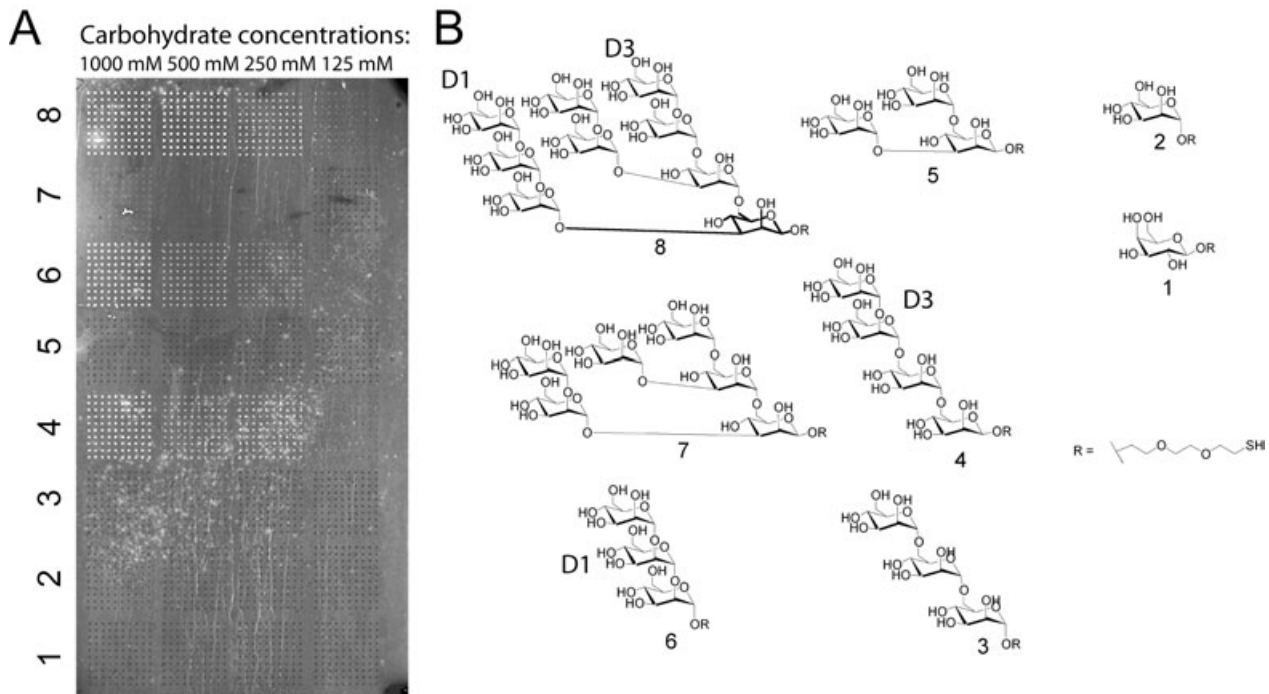


Fig. 3. Image of the carbohydrate microarray incubated with fluorescently labelled MVN (A). Synthetic mannans 2–8 and galactose 1 as negative control (B) were spotted on the glass slide at concentrations of 1000, 500, 250 and 125 mM. The D1 and D3 arms of mannoside 8 that are separately represented by mannosides 4 and 6 are indicated. Carbohydrates were synthesized as described in Ratner *et al.* (2002).

mutant strain resulted in a strong phenotype. The cells immediately started to aggregate into large cell clumps (Fig. 4B). The wild type only showed slow and weak aggregation in the same experiment.

Immunodetection of MVN in *M. aeruginosa* PCC7806 and $\Delta mcyB$ mutants

The anti-MVN antibody raised during this study was used to test the expression of MVN in *M. aeruginosa* PCC7806. The immunoblot analysis yielded two specific bands with an apparent M_r of 50 kDa and 54 kDa under reducing conditions (Fig. 5A). Both bands were present in samples

taken at mid growth phase (OD_{750} : 0.5) and at late growth phase (OD_{750} : 1.0). However, the intensity of both signals significantly increased at higher cell density. No differences in the amount of the 54 kDa band were observed under the different light quantities and qualities applied. However, the 50 kDa band was very weak at OD 0.5 under dark, blue and high light conditions. The size of both detected bands did not correspond to the monomeric or dimeric form of MVN. Southern blot experiment revealed no further homologue of the *mvn* gene in PCC7806 (data not shown). Therefore, the same immunoblot experiment was conducted with the Δmvn mutant. No specific band could be detected under any of the conditions tested

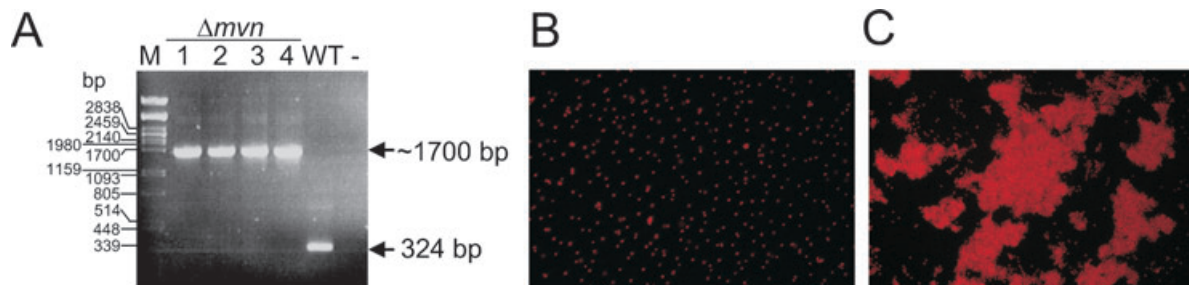


Fig. 4. A. PCR amplification with DNA from the Δmvn mutants of *M. aeruginosa* PCC7806 (lane 1–4) wild type (+) and a water control (–). A λ PstI marker (M) shows the sizes in kilobases. The amplicon of the homozygous mutants is 1.4 kb larger than the amplicon of the wild-type DNA as a result of the insertion of a Cm-resistance cartridge.

B. Δmvn mutant cells prior to the addition of *in vitro* expressed His-MVN.

C. Cell aggregates after addition of the *in vitro* expressed MVN protein to Δmvn mutant cells.

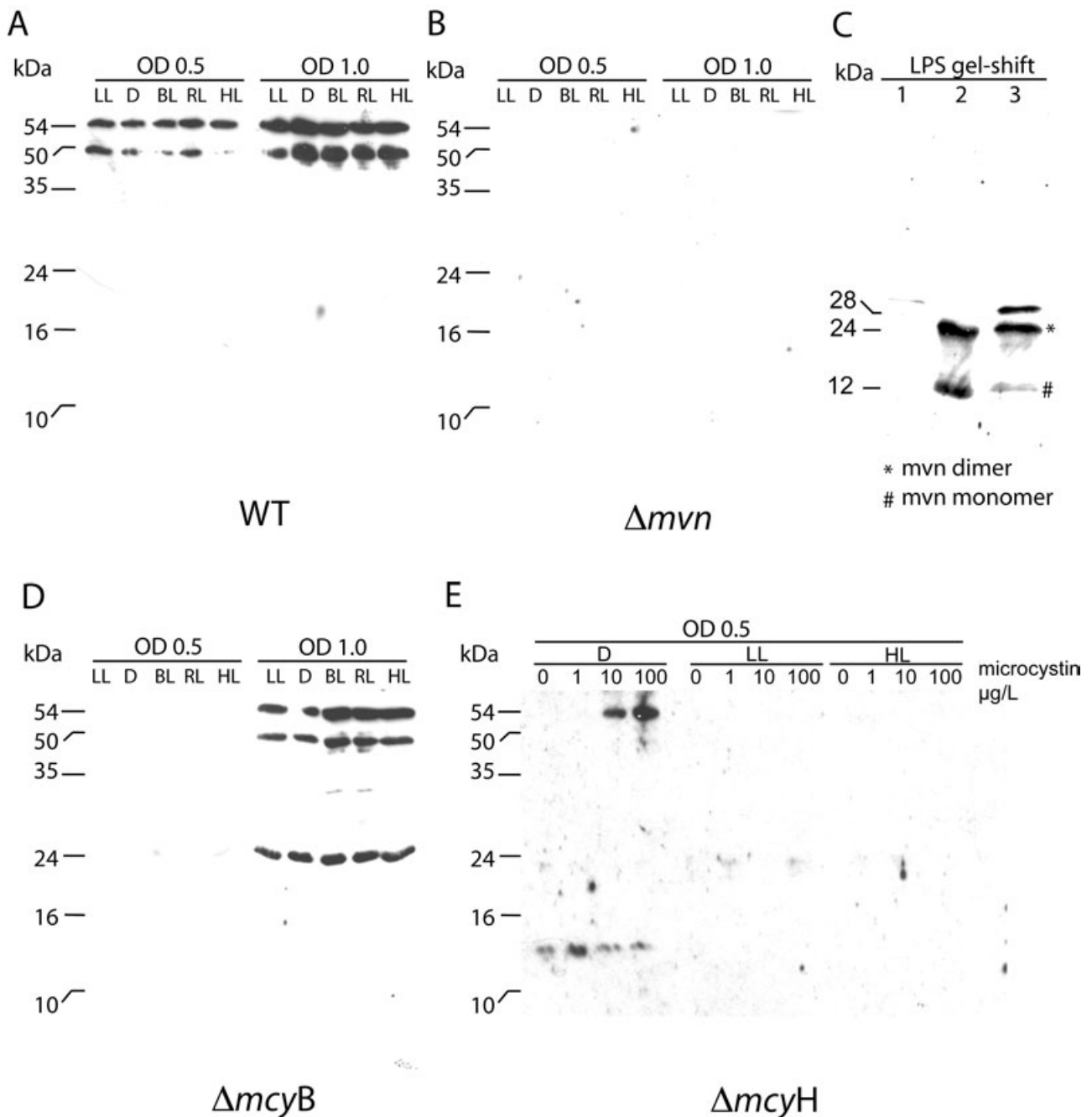


Fig. 5. Immunoblot analyses of *Microcystis* cell extracts using an anti-MVN antibody. Samples were separated by SDS-PAGE under reducing conditions.

A. Expression of MVN in wild-type PCC7806.

B. Verification of the lack of MVN in the Δmyn mutant.

C. Immunodetection of MVN in a LPS fraction of PCC7806. Lane 1 shows the crude LPS fraction, lane 2 the *in vitro* expressed His-MVN protein purified from *E. coli* and lane 3 the LPS fraction after addition of His-MVN.

D. Expression of MVN in the soluble fraction of the microcystin-deficient $\Delta mcyB$ mutant.

E. Expression of MVN in the $\Delta mcyH$ mutant at mid growth phase supplemented with 0, 1, 10 and 100 $\mu\text{g l}^{-1}$ microcystin-LR. Cultures were generally grown to mid and late growth phase respectively, and subsequently exposed for 2 h to different light conditions. LL, low light; D, dark; BL, blue light; RL, red light; HL, high light.

(Fig. 5B). These data allowed the conclusion that the two bands correspond to stable homo- or heteromultimeric forms of MVN in *M. aeruginosa* PCC7806 and do not result from unspecific cross-reactions. A high molecular weight band of approximately 50 kDa was also seen under non-reducing conditions (data not shown). The lower band could represent a stable homotetramer. The upper band presumably contains a heterologous binding partner. In order to test the hypothesis that a lipopolysaccharide (LPS) binding partner binds to MVN *in vivo*, the LPS fraction of PCC7806 was extracted using a hot phenol method (for details see *Experimental procedures*) and incubated with the *in vitro* expressed His-MVN. A Western blot analysis of this fraction under reducing conditions revealed a decrease in the amount of the monomeric His-MVN protein and the new appearance of a band of about 28 kDa (Fig. 5C). However, no signal was observed at 50 kDa or 54 kDa as in the wild-type protein extracts. These data suggest an interaction between MVN and LPS. CV-N possesses two carbohydrate binding sites and the same might be true for MVN. Possibly, a second binding partner capable of binding the second binding site was absent in the LPS extracts. A Western blot analysis was also performed with the $\Delta mcyB$ mutant strain that is deficient in microcystin production. No specific signal could be obtained for samples taken at mid-log phase (Fig. 5D). However, at higher cell density, both the 50 kDa band and the 54 kDa band were detected. In addition, a protein with an apparent M_r of 24 kDa reacted with the antibody. This protein size is in good agreement with the dimeric form of MVN. Similar results were obtained in further independent experiments. The free dimeric form was repeatedly detected in the microcystin-free $\Delta mcyB$ mutant, but never seen in the wild-type strain. The multimeric MVN protein form could not be detected in the microcystin-free $\Delta mcyH$ mutant that lacks the putative microcystin transporter McyH (Pearson *et al.*, 2004) in a series of experiments, whatever the cell density of the culture. Only small amounts of the MVN monomer could be detected under dark conditions (Fig. 5E). Thus, MVN seems to be more affected in the mutant lacking microcystin and the putative microcystin transporter than in the mutant lacking microcystin only. The addition of 10 or 100 ng ml⁻¹ microcystin to the $\Delta mcyH$ mutant cultures resulted in an increased abundance of a MVN multimer. However, this effect was only observed under dark conditions (Fig. 5E).

Lectin binding assay

To verify if the specific oligosaccharide recognized by MVN is present in the sheath of *M. aeruginosa* PCC7806, FITC-labelled His-MVN was used in a lectin binding assay (LBA). The fluorescent protein was added to aliquots of culture samples of *M. aeruginosa* PCC7806 and the cells

were subsequently analysed under a fluorescence microscope (for details see *Experimental procedures*). The labelled lectin was bound at the surface of *M. aeruginosa* PCC7806 cells resulting in a bright green fluorescence. The red chlorophyll fluorescence of the cyanobacterial cells served as a control verifying the number and localization of viable cells under the microscope (Fig. 6B and D). The fluorescently labelled lectin was further added to the Δmvn mutant strain. In contrast to the wild type, no fluorescence could be observed at the surface of the *Microcystis* cells, but only in the surrounding sheath. In the blue channel cyanobacterial cells appeared with a slight orange colour arising from the autofluorescence. Cells showed a bright autofluorescence in the green channel of the microscope (Fig. 6C and D). Thus, the oligosaccharide recognized by the lectin is not attached to the cell surface of Δmvn mutant cells while the sugar is still present in the sheath. This may indicate that the MVN protein links the specific sugar to the *Microcystis* cell surface in the wild type. To ensure the specificity of the binding, two other cyanobacterial strains were tested, namely *Synechocystis* PCC6803 and *Nostoc punctiforme* ATCC29133. In both cases no green fluorescence was observed for the cells or the sheath (data not shown). Thus, the oligosaccharide recognized by MVN is not present at the surface or sheath of these other cyanobacteria (data not shown). The specificity was further tested with mixed samples of *M. aeruginosa* and *Nostoc* and *Synechocystis* respectively. The lectin was able to differentiate between the different cell types (Fig. 6E and F). Similar experiments were conducted with the non-toxic *Microcystis* strain Nies 98. No specific fluorescence was detected on the surface of the cells or the sheath (data not shown).

Microcystin content of Δmvn mutant cells

To further test the hypothesis that the presence of MVN and microcystin are functionally correlated, microcystin content of PCC7806 wild type and Δmvn mutant cells was compared by high-performance liquid chromatography (HPLC). Methanolic extracts were prepared from 10 ml aliquots of triplicate cultures at an OD₇₅₀ 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. Analysis of the ΔMVN mutant extracts revealed only 44% and 39% of the wild-type microcystin content at an OD₇₅₀ of 0.5 and 1.0 respectively (Fig. 7). The microcystin content was also reduced in the supernatant fraction of ΔMVN mutant cells, but to a lower extent as observed in the cell-bound fraction (data not shown).

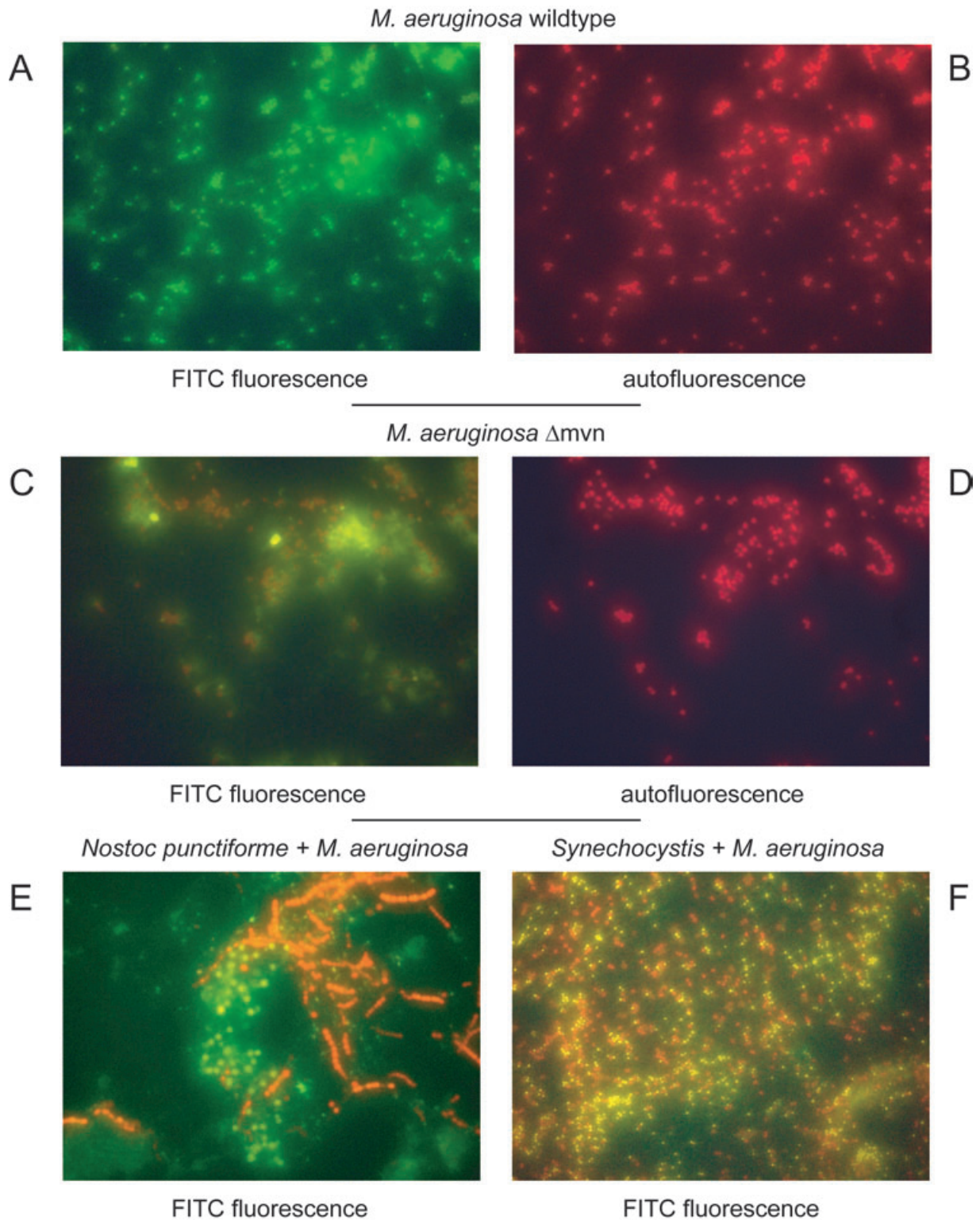


Fig. 6. Selective binding of MVN to the cell surface of *M. aeruginosa* PCC7806. Cells were treated with fluorescently labelled FITC-MVN in order to detect the oligosaccharide that is recognized by the lectin *in situ*.

A and B. PCC7806 wild-type cells in the blue and green channel of the microscope showing the FITC fluorescence and the chlorophyll autofluorescence respectively.

C and D. Δmvn mutant cells in the blue and green channel respectively.

E and F. Mixtures of *M. aeruginosa* PCC7806 and *N. punctiforme* ATCC29133 or *Synechocystis* sp. strain PCC6803 cells in the blue channel of the microscope.

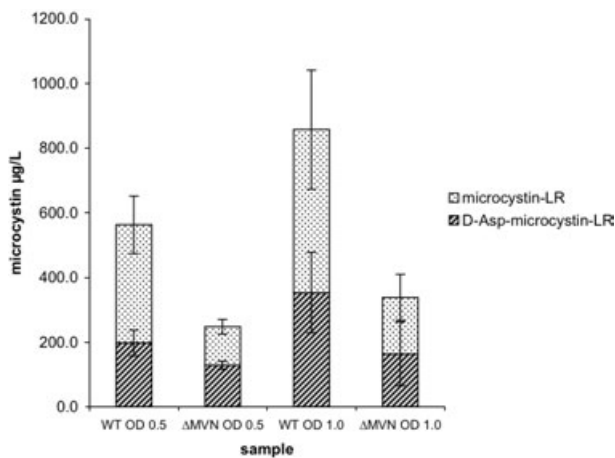


Fig. 7. Relative amount of microcystin in wild-type *M. aeruginosa* PCC7806 and the Δmvn mutant in the mid and late growth phase. Averaged values from triplicate cultures were determined by HPLC and are shown as microcystin-LR equivalents. Dotted and striped areas indicate the amounts of the two isoforms of microcystin, microcystin-LR and D-Asp³-microcystin-LR respectively.

Discussion

It has become widely accepted that carbohydrate structures are not merely structural components of the molecules but are, in many instances, a source of information to be decoded by biological systems. Lectins can specifically recognize a carbohydrate partner and can thus mediate attachment among macromolecules and cells. Recent research findings have significantly increased our knowledge about the physiological roles of bacterial lectins in cell–cell interactions. However, most of this research focused on inter-species interactions between pathogenic bacteria and their host cells or between bacterial symbionts and their host plants (Imberty *et al.*, 2004; Sudakevitz *et al.*, 2004). Little is known about the role of lectins for self-recognition of bacteria and about the impact of Lectin–carbohydrate interactions for the structure of bacterial communities. Here we describe the discovery of MVN, a protein with similarity to the well characterized anti-HIV protein CV-N from *N. elliposporum* in the toxin-producing freshwater cyanobacterium *M. aeruginosa* PCC7806.

Biochemical properties of MVN

The resolution of the crystal structure and NMR studies have unravelled the molecular basis of the specificity and affinity of CV-N to its carbohydrate ligands. It is discussed, that unique structural properties lead to an extraordinary high specificity of CV-N for Man(8) and (9). The two extensive carbohydrate binding pockets are specific for $\alpha(1\rightarrow2)$ linked mannose disaccharide, which represents the termini of the accessible ends of Man(8) and Man(9) that are part of the gp120 protein forming the envelope of HIV.

Under low pH conditions, a high percentage of CV-N forms a domain-swapped dimer (Yang *et al.*, 1999). Kelley *et al.* (2002) have engineered an obligate domain-swapped dimer of CV-N that represents a tetravalent carbohydrate binding protein. The stable dimer was a more potent inhibitor of HIV-1 fusion than the wild-type CV-N monomer (Kelley *et al.*, 2002). The homologous MVN protein from *M. aeruginosa* PCC7806 was found to form an extremely stable dimer when overexpressed in *E. coli* under non-reducing conditions (Fig. 2B). A dimer of MVN was also detected in the microcystin-deficient cyanobacterial cells (Fig. 5D). Whether this phenomenon indicates that MVN monomers undergo domain-swapping under physiological conditions has to be analysed more systematically in future experiments. Despite significant differences between CV-N and MVN on the primary sequence level (Fig. 2A), binding of the fluorescently labelled recombinant protein on an oligosaccharide microarray suggested a carbohydrate specificity similar to that observed for CV-N and scytovirin (Shenoy *et al.*, 2002; Fig. 3). The dimeric nature of the protein should give rise to a tetravalent carbohydrate binding protein in *Microcystis*.

Possible impact of MVN on cell–cell recognition and colony formation

The multivalent carbohydrate binding properties of MVN dimers enable these proteins to network different glycosylated partners within *Microcystis*. Indeed, immunodetection of MVN in *M. aeruginosa* PCC7806 revealed that it specifically binds to at least one, possibly two different binding partners. As seen for the MVN dimer in *E. coli*, these high molecular weight multimers are stable even under denaturing SDS-PAGE conditions.

Lectin binding analyses and the shift observed in the LPS binding study indicate that at least one of these binding partners is located in the outer cell membrane of *Microcystis* and possibly represents the O-antigen of a LPS. Lectin binding analyses further indicate that the second binding partner may be situated in the sheath of *Microcystis*. The carbohydrate specificity of MVN revealed by the carbohydrate microarray strongly suggests that these binding partners possess carbohydrate residues containing $\alpha(1\rightarrow2)$ linked mannose units. Lectin binding analysis indicates that a similar carbohydrate is recognized *in vivo* (Fig. 6). Localization and binding properties of MVN would thus enable the protein to network cells with the surrounding *Microcystis* sheath and in that way also to network cells within a colony. At the same time the carbohydrate binding character would allow a specific recognition of the same cell type (as determined by the carbohydrate composition of the cell sheath, see Fig. 6E and F) in a given mixture of cells. This would allow an aggregation of single *Microcystis* cells to colonies most

probably also in field situations. The laboratory strain PCC7806 has lost its ability to form colonies in culture, although MVN was detectable in the cell surface fraction after subcellular fractionation (data not shown). Addition of the MVN protein to MVN-deficient *Microcystis* cells led to the formation of large aggregates of cells (Fig. 4C). We can therefore assume that the intraspecific cell–cell attachment of *Microcystis* cells is an *in vivo* function of MVN. Whether MVN is one of the major discriminating factors between different *Microcystis* morphotypes and possibly specific for *M. aeruginosa* has to be tested in field situations in future experiments. Even though MVN is most likely not the only factor that is important for *Microcystis* colony formation, several lines of evidence suggest that it fulfils all preconditions for a protein specifically linking *Microcystis* cells.

Relationship between MVN and microcystin

The basis of this study was the identification of a conserved nucleotide box upstream of the translational start points of the microcystin biosynthesis genes and the *mvn* gene (Fig. 1). Therefore, the aim of this study was to test whether this common feature has any functional consequences for the two putative partners and indicates a functional association of microcystin and MVN. The lack of microcystin seems to have different types of consequences on the expression and oligomerization of MVN, but also on the binding or expression of its heterologous binding partner(s). First, at low cell densities no MVN could be detected in the $\Delta mcyB$ mutant cells (Fig. 5D). Second, under a number of conditions, free dimeric forms and in some cases even monomeric forms of MVN were detected in the microcystin-free $\Delta mcyB$ and $\Delta mcyH$ mutants that were never seen in the wild-type strain PCC7806 (Fig. 5D and E). Third, external addition of microcystin could partly restore a high molecular weight multimer of MVN in the microcystin-free *mcyH* mutant under dark conditions (Fig. 5E). Nevertheless, the two oligomeric forms present in the wild type were also seen in the microcystin-free $\Delta mcyB$ mutant, in particular at high cell densities. These data allow the conclusion that microcystin has an impact on the expression of MVN and its binding partners.

Microcystin is not essential, however, for the MVN expression, therefore, other factors might act synergistically on the protein. The nature of the correlation between microcystin and MVN and the role of the putative microcystin exporter *McyH* remain elusive and need more thorough investigation in the future. One possibility of an interaction is that microcystin is accepted as a signal and in that way influences the expression of both MVN and its binding partners. This hypothesis is supported by the increased expression of a multimeric MVN form observed

after addition of microcystin to the $\Delta mcyH$ mutant cells. However, this effect was only seen under dark conditions (Fig. 5E). Lectins are characteristic targets of intercellular signals in bacteria that grow in biofilms on solid surfaces. The *Pseudomonas* lectins PA-IL and PA-IIL are controlled by two types of acyl-homoserine lactones (AHLs) (Winzer *et al.*, 2000). Another possibility would be a direct structural interaction of MVN and microcystin. Microcystins could well be an integral part of a network between LPS and lectins on the cell surface. Together, MVN and microcystin could determine the properties of the outer cell membrane of *Microcystis*. Both factors may be involved in self- and non-self-recognition of *Microcystis* cells. These different possibilities of interactions do not necessarily exclude each other. In any case, microcystin would have an impact on *Microcystis* colony formation and would significantly contribute to the success of *Microcystis* in the field.

Experimental procedures

Cyanobacterial strains and culturing

The microcystin-producing strain *M. aeruginosa* PCC7806 was kindly provided by R. Rippka (Institut Pasteur, Paris, France). Mutants of this strain unable to produce microcystins were obtained by natural transformation and insertion of a chloramphenicol (Cm)-resistance cartridge into the peptide synthetase gene *mcyB* (Dittmann *et al.*, 1997). Wild type and mutant cell lines were grown at 23°C in batch cultures with Z8 medium (Rippka, 1988). Growth of the cyanobacteria was followed by monitoring the OD₇₅₀ with a Uvikon 930 spectrometer (Contron Instruments, Schlieren, Germany). Cells were grown under 16 $\mu\text{Em}^{-2}\text{s}^{-1}$ white light (low light, LL) to mid and late growth phase and the exposed to different light conditions for 2 h. The following light conditions were applied: 16 $\mu\text{Em}^{-2}\text{s}^{-1}$ (low light, LL), 68 $\mu\text{Em}^{-2}\text{s}^{-1}$ (high light, HL), blue light (BL), red light (RL) and darkness (D). Light intensities were measured using a Li-Cor LI250 light meter (Walz, Effeltrich, Germany). Different light qualities were obtained by the use of blue and red plastic cut-off filters [Lee Filters, Undover, Great Britain; Dark Blue (HT)* 119 and Light Red 182 respectively]. The blue light filter had a bandwidth of 400–510 nm with a peak at 472 nm and the red filter opened from 620 nm with $\tau_{1/2}$ at 666 nm. In both cases, a light intensity of 16 $\mu\text{Em}^{-2}\text{s}^{-1}$ was applied. Purified microcystin-LR was added to parallel aliquots of the cultures to give final concentrations of 1, 10 and 100 $\mu\text{g l}^{-1}$ for 2 h.

Gene identification

The bidirectional promoter region of the microcystin biosynthesis operon of *M. aeruginosa* PCC7806 was used as a query sequence for a BLASTN analysis of a database containing the partial genome sequence of this cyanobacterium. This non-public database forms part of a current sequencing genome project at the Génopole-Ile de France, Institut Pasteur, France, and is the preliminary assembly of shot-gun

sequencing of 700 bp fragments of genomic DNA cloned in the pcDNA-2.1 vector (Invitrogen life technologies, Carlsbad, CA, USA).

Amplification and cloning of the MVN gene

The specific primer pair *cvhexfw* (5'-CAT ATG CCT AAT TTT TCG CAC AC-3') and *cvhexrv* (5'-GGA TTC CTA TCC AAT TTC CAG TTG-3') was used to amplify the *mvn* gene from DNA of the strain *M. aeruginosa* PCC7806. In order to facilitate a directed cloning, restriction sites for *NdeI* (*cvhexfw*) and *BamHI* (*cvhexrv*) were incorporated into the primers. The amplification was performed with purified genomic DNA from *M. aeruginosa* PCC7806 and *Qiataq* polymerase as described by the manufacturer (*Qiagen*, Hilden, Germany). The programme was conducted at 95°C for 5 min followed by 35 cycles at 95°C 1 min, 55°C 30 s and 72°C 30 s and a final step of 72°C 10 min. The PCR product was purified using the *Quiaquick* kit (*Qiagen*, Hilden, Germany) and ligated into the *pGEM-T* vector (*Promega*, Mannheim, Germany). Plasmids were isolated using the *Roche High Pure Plasmid Isolation kit* (*Roche*, Mannheim, Germany). For cloning into the expression vector *pET-15b* (*Novagen*, Madison, WI, USA) the insert was subsequently cut out with the restriction endonucleases *NdeI* and *BamHI* and gel purified (*Genomed GmbH*, Bad Oeyenhausen Germany). Afterwards, the *mvn* gene fragment was ligated into the *pET-15b* vector, previously linearized with *NdeI* and *BamHI* and purified (*Qiagen*, Hilden, Germany). Plasmids were isolated as described above and sequenced (*Automated Sequencer Model 373*, *Applied Biosystems*, Weiterstadt, Germany). One of the plasmids containing the correct insert was transformed into *E. coli* BL21 (DE3) (*Novagen*, Madison, WI, USA).

Expression and purification of his-MVN

Expression was performed in 1 l LB medium containing 100 µg ml⁻¹ ampicillin, inoculated with 20 ml of an overnight culture and incubated at 37°C on a gyratory shaker running at 220 rpm. When the culture reached an OD₆₀₀ of 0.6, IPTG was added to a final concentration of 1 mM and the culture was further incubated for 3 h as above. The cells were harvested by centrifugation [4000 *g*, 20 min, room temperature (RT)] and the pellet was resuspended in 16 ml of buffer A (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) containing 10 mM imidazol. The cells were lysed by sonication (6 × 10 s with 10 s breaks, on ice). After centrifugation (10 000 *g*, 20 min, 4°C), the supernatant was mixed with 4 ml of 50% Ni²⁺ NTA (*Qiagen*, Hilden, Germany) and incubated with shaking for 1 h at 4°C. The suspension was applied onto a column (*Qiagen*), washed with washing buffer (buffer A with 30 mM imidazol) until the flowthrough reached an OD₂₈₀ of 0.01. The recombinant protein was eluted with four times 0.5 ml elution buffer (buffer A with 250 mM imidazol). The purity of the samples was evaluated by SDS-PAGE and immunoblotting and samples were stored at -20°C.

Mass spectrometry

The FTICR MS used is a *Finnigan LTQ FTMS* (*Thermo*

Electron, Bremen, Germany). To assign the sequence, a tryptic digest of MVN was reduced by DTT (DL-Dithiothreitol, 10 µmol l⁻¹ in solution) for 2 h at 50°C. For separation, the same mass spectrometer was coupled to an *Agilent 1100 Nano LC system* (*Agilent Technologies*, Darmstadt, Germany). To obtain information about the occurrence of S-S bonds, a non-reduced tryptic digest was analysed as well. A 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).

Generation of an antibody against MVN

To obtain a specific antibody against MVN, His-MVN was purified under denaturing conditions. A cell pellet from 750 ml culture was resuspended in 24 ml of buffer B (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M Urea, pH 8.0) containing 10 mM imidazol and lysed by sonication. After centrifugation (see above) 6 ml of 50% Ni²⁺-NTA were added to the supernatant and the mix was incubated for 1 h at 4°C. Afterwards the mix was applied to a column, washed with 20 ml of buffer (buffer B with 30 mM imidazol) and eluted with 10 × 0.5 ml of elution buffer (buffer B with 250 mM imidazol). The purity of the fraction was estimated on SDS-PAGE. The purest fractions were combined and the protein concentration determined (*Bio-Rad Protein Assay*, *Bio-Rad*, Hercules, CA, USA). Two aliquots containing 250 µg protein were precipitated with TCA at a final concentration of 5% v/v on ice for 30 min. After centrifugation (30 min, 13 000 *g*, 4°C) the pellets were washed with 500 µl of acetone and dried on air. The purified protein was used to raise a polyclonal rabbit antibody (*Pineda-Antibody-Service*, Berlin, Germany). Serum samples were taken every 30 days and tested by immunoblotting with protein extracts from *M. aeruginosa* PCC7806, *E. coli* and purified His-MVN.

Oligosaccharide microarray

The carbohydrate arrays 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 synthesized as described (*Ratner et al.*, 2002; 2004a; *Adams et al.*, 2003). Arrays were printed onto amine-functionalized *Corning GAPS II* slides that were incubated overnight at RT in 45 ml anhydrous *N,N*-dimethylformamide (DMF, *Aldrich*), 10 mg succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, *Pierce Endogen*) and 880 µl *N,N*-diisopropylethylamine (*Aldrich*) to allow attachment of the thiol-reactive maleimide group to the slide surface. Slides were washed with 3 vols of methyl alcohol, dried under a stream of nitrogen, and stored in a desiccator prior to printing. Prior to spotting, thiol-containing oligosaccharides were incubated at RT with one equivalent tris-(carboxyethyl)phosphine hydrochloride (TCEP, *Pierce Endogen*) in 1× PBS for 1 h to

reduce the disulphide bond. Structures were printed at concentrations ranging from 0.1 mM to 2 mM on maleimide-derivatized GAPS slides using a MicroGrid TAS array printer (30% humidity, 120 µm spots with 300 µm spacing). Printed slides were incubated 12 h in a humidity chamber, washed twice with distilled H₂O, and then incubated for 1 h in 1 mM 2-[2-(2-mercaptoethoxy)ethoxy]ethanol in PBS (50 ml) to quench reactive maleimide groups. Slides were rinsed with distilled H₂O (3 × 50 ml), 95% ethanol (3 × 50 ml), and stored in a desiccator prior to use.

The FITC-labelled MVN was placed into a solution containing 50 mM Hepes buffer (pH 7.5), 0.1 M NaCl, 1% w/v BSA and 1 mM CaCl₂. 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 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.

Mutagenesis

A PCR fragment of 3.7 kb length containing the flanking region of the *mvn* gene was amplified using the Primer pair cyepo (5'-GATGACCGCGCTTTAGAA-3') and cyepR (5'-CTACCATTATTGGCGACATTG-3') and cloned into the pDrive vector (Qiagen, Hilden, Germany). The 1.4 kb fragment from pACYC184 containing the Cm-resistance cartridge was inserted into the NruI site within the *mvn* gene and positive clones were identified by Cm resistance. The vector carrying the final mutagenesis construct (pΔmvn::cm) was linearized with SacI and column purified. A 10 ml sample of log-phase *M. aeruginosa* PCC7806 culture was harvested and washed twice with 1 mM Hepes and then subjected to electroporation with 10 µg of linearized plasmid DNA (1.5 kV, 25 µF, 200 Ω). Alternatively, *M. aeruginosa* was transformed taking advantage of its natural competence. Cells were sampled as described above and 10 µg of circular plasmid DNA was added and the cells were incubated for 2 h at low light conditions before plating. Positive clones were selected on BG-11 plates containing a Cm gradient (Dittmann *et al.*, 1997). These clones have been transferred to BG-11 plates with increasing Cm concentrations every 2–3 weeks to a final concentration of 5 µg ml⁻¹. Finally, cells were transferred into liquid Z8 medium with 5 µg ml⁻¹ Cm. The primers mvnfw (5'-ATG CCT AAT TTT TCG CAC AC-3') and mvnrv (5'-GGA TTC CTA TCC AAT TTC-3') were used to test for positive clones.

Immunoblots

Protein samples were diluted in 5× sample buffer containing 5 mM β-mercaptoethanol, heated for 10 min at 95°C and subsequently separated by SDS-PAGE on gels containing 12.5% acrylamide (Laemmli, 1970) and immobilized on nylon membranes (Amersham, Freiburg, Germany). 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. Either an anti-His-Tag or anti-MVN antibody was added at a dilution of 1:10 000. 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 horse-radish peroxidase conjugate, Sigma, Weiterstadt, Germany) 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 (Pearce, Rockford, IL, USA) and exposed to X-ray film (Amersham, Freiburg, Germany).

Fluorescence labelling of His-MVN and lectin binding analysis

Purified recombinant His-MVN was labelled with the FluoroTag FITC Conjugation Kit (Sigma, Weiterstadt, Germany) 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 was added to the labelled protein and the samples were kept at 4°C in the dark. The 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 20 µl of a FITC-MVN solution (0.1 µg µl⁻¹) and incubated at RT for 30 min. The solution was carefully removed with filter paper. The samples were washed three times with 100 µl of PBS. The remaining PBS buffer was carefully drawn off with filter paper. The cells were viewed under a fluorescence microscope (Zeiss Axioscope, Jena, Germany) using the filter set 13 (Zeiss, Jena, Germany) and pictures were taken with a digital microscope camera (Zeiss, Jena, Germany).

Isolation of LPS

Lipopolysaccharides were isolated from *M. aeruginosa* PCC7806 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.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Proteolysis with trypsin, without reduction.

Table S2. Proteolysis with trypsin after reduction (DTT).

This material is available as part of the online article from <http://www.blackwell-synergy.com>