Comparative Protein Expression in Different Strains of the Bloom-forming Cyanobacterium *Microcystis aeruginosa*[*][S]

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Toxin production in algal blooms presents a significant problem for the water industry. Of particular concern is microcystin, a potent hepatotoxin produced by the unicellular freshwater species *Microcystis aeruginosa.* In this study, the proteomes of six toxic and nontoxic strains of *M. aeruginosa* were analyzed to gain further knowledge in elucidating the role of microcystin production in this microorganism. This represents the first comparative proteomic study in a cyanobacterial species. A large diversity in the protein expression profiles of each strain was observed, with a significant proportion of the identified proteins appearing to be strain-specific. In total, 475 proteins were identified reproducibly and of these, 82 comprised the core proteome of *M. aeruginosa*. The expression of several hypothetical and unknown proteins, including four possible operons was confirmed. Surprisingly, no proteins were found to be produced only by toxic or nontoxic strains. Quantitative proteome analysis using the label-free normalized spectrum abundance factor approach revealed nine proteins that were differentially expressed between toxic and nontoxic strains. These proteins participate in carbon-nitrogen metabolism and redox balance maintenance and point to an involvement of the global nitrogen regulator NtcA in toxicity. In addition, the switching of a previously inactive toxin-producing strain to microcystin synthesis is reported.   *Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.003749, 1–16, 2011.*

Blooms of cyanobacteria occur worldwide in nutrient-rich waters and can pose a public health threat when toxin-producing species are involved. One of the most significant cyanotoxins is microcystin, produced by the genera *Microcystis, Anabaena, Anabaenopsis, Aphanizomenon, Fischerella, Planktothrix,* and the terrestrial *Hapalosiphon* (1–3). Microcystins are cyclic heptapeptides with the general structure cyclo-(-D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), where Adda is 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6,-decad-

ienoic acid, MeAsp is 3-methylaspartic acid, and Mdha is *N*-methyl-dehydroalanine (4). The most common isoform produced is microcystin-LR (MC-LR)[1] with a molecular weight of 995 Da. Incorporation of variable amino acids at the X and Z positions, together with differences in the peptide backbone, account for the generation of close to 90 microcystin isoforms that have been identified to date, each with differing polarity and toxicity (2, 5, 6).

Microcystins exert their hepatotoxicity in mammals by inhibiting protein phosphatases in the liver (PP1 and PP2a) (7, 8). Prolonged exposure to microcystin and the ensuing reactive oxygen species (ROS)-induced damage to DNA have been shown to be a powerful tumor promoter in rodents, but its impact as a carcinogen is yet to be established in humans (6, 8, 9). The effects of microcystin are not limited to mammals, with the toxin also able to cause oxidative stress damage, via the production of ROS, loss of mitochondrial potential, and apoptosis in macrophytes (10).

Numerous studies have been published on the putative role of microcystin, suggesting the possibility of the toxin to be a molecule involved in quorum-sensing, light sensitivity, or the acquisition of iron (11–14). However, the essential contribution of microcystin to the metabolism of cyanobacteria has been questioned by the existence of nontoxic strains within all species capable of microcystin production. In fact, under certain environmental conditions, nontoxic strains in both *Planktothrix* and *Microcystis* spp. are capable of dominating their environment and most blooms contain a mixture of toxic and nontoxic strains (5, 15–17).

In addition, the currently available nontoxic strains generated by insertional inactivation of *mcyS* genes show altered morphology, compared with the wild-type toxic strain *Microcystis aeruginosa* PCC 7806. Some of these changes are readily observable, such as the presence of gas vesicles, changes in pigmentation and thylakoid organization, reduced cell size, as well as the tendency of mutant cells to form aggregates (18, 19). Such changes have been suggested to

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[1] The abbreviations used are: MC-LR, microcystin-LR; Adda, 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-4,6,-decadienoic acid; nSAF, normalized spectrum abundance factor; PP2a, protein phosphatase 2a; qRT-PCR, quantitative real-time PCR; 2DE, two-dimensional electrophoresis; PRIDE, Proteomics Identifications Database.

TABLE I
Strains used in this study

Strain	Isolate location	Isolate date	Toxicity[a]	Reference
PCC 7806	Braakman Reservoir, The Netherlands	1972	+	[87]
UWOCC MRC	Malpas Dam, Armidale, Australia	1973	+	[88]
UWOCC MRD	Malpas Dam, Armidale, Australia	1973	+	[88]
UWOCC CBS	Lake Mendota, WI, USA	Pre 1983	−	
PCC 7005	Florida, USA	1948	−	[87]
HUB5.3	Lake Pehlitzsee, Germany	1977	−	[89]

[a] As determined by this study.

be influenced by the association of microcystin with the thylakoid membranes in toxic cells, observed by electron microscopy and possibly, the binding of microcystin to phycobilin proteins (20, 21). However, a study by Vela and colleagues (22) considered this association to be nonspecific.

Proteomic studies in several bacterial species have opened the possibility to assess the role of various metabolites and the effect of stress-inducing conditions on cellular function. On the other hand, proteomic research of toxic cyanobacteria has been limited because of the relative scarcity of sequenced genomes. The first proteome studies in cyanobacteria were performed in the model photosynthetic prokaryote *Synechocystis* sp. PCC 6803 following the sequencing of its genome (23). As the wealth of cyanobacterial genomic information increased, proteomic investigations into the response of cyanobacteria under various stress conditions, the composition of the thylakoid membranes, and the processes of heterocyst formation and nitrogen fixation, among others have been carried out (24–27). These initial studies reported optimized extraction and separation protocols for cyanobacterial proteomes, which contain large amounts of phycobilisomes that can mask less-abundant proteins, in particular when two-dimensional electrophoresis (2DE) is used (28). Although the majority of studies have used 2DE, several groups have also reported comparable results with gel-free shotgun approaches (29, 30).

In 2001, Dittmann *et al.* performed the only proteomic study in *M. aeruginosa* using 2DE to compare the wild-type strain PCC 7806 and the *mcyB*$^-$ mutant strain (11). Using the partially sequenced genome, three proteins, showing some homology to quorum-sensing and light-regulated proteins, were identified as differentially displayed and were assigned as microcystin-related proteins (MrpA-C) (11). However, these proteins were not present in the genome of *Microcystis aeruginosa* NIES-843, indicating that they may not be necessarily linked to toxicity, but rather represent a strain-specific feature (31).

The recent publication of two *Microcystis aeruginosa* genomes has opened the possibility of studying the proteome expression of this toxin-producing species in more detail (31, 32). Comparison of the two genomes has revealed that they are both highly plastic and contain regions that could be the result of recent horizontal gene transfer (31). It was found that

16% of the *M. aeruginosa* PCC 7806 genome and 28% of the NIES-843 genome coded for proteins not present in the other strain, showing many strain-specific differences, despite the high 16S rRNA gene sequence conservation reported for the *M. aeruginosa* species cluster (31). In the past, phylogenetic comparisons have led to the belief that the *Microcystis* genus should be unified and strains should be defined as ecotypes adapted to a particular environment, rather than be divided into species based on morphological characteristics (33). Although most comparative proteomic studies have been performed on the same organism grown in different conditions, there is a general lack of understanding of what constitutes the proteomic diversity within cyanobacterial species.

In this study, the proteome of the model microcystin-producing organism *M. aeruginosa* PCC 7806 and five other morphologically distinct strains of *M. aeruginosa* isolated from various geographic locations was determined under nutrient-replete conditions. The aim of these studies was to see if the genome plasticity characteristic of the *Microcystis* species is observed at the protein expression level. In addition, protein expression differences between toxic and nontoxic strains grown in nutrient-replete conditions may be the underlying reason for the reported differential response of these organisms to nutrient stress (14, 16, 17). To test this hypothesis, protein expression was quantified using the label-free normalized spectral abundance factor (nSAF) method in an attempt to expand the current knowledge on the putative metabolic role of microcystin in the cyanobacterial cells. Several proteins involved in carbon-nitrogen metabolism and redox balance were found to be differentially expressed in toxic and nontoxic strains suggesting a link between microcystin synthesis and metabolic control by the global nitrogen regulator NtcA.

EXPERIMENTAL PROCEDURES

Strains and Culturing—*M. aeruginosa* cultures were grown in BG-11 media (Fluka) without shaking under continuous light (25 μM photons m^{-2} s^{-1}) supplied by cool light fluorescent lamps at 28 °C. The strains used in this study are listed in Table I. Cells were filtered through a 3 μm pore-size membrane (Millipore) to remove contaminating bacteria and maintained in 100 μg/ml cyclohexamide to achieve axenic culture.

Toxicity Assays

Microcystin Extraction—To verify the toxicity of the strains chosen for proteomic studies, 2 ml of late exponential culture were pelleted by centrifugation, the cells were resuspended in 70% methanol and lysed by four 30-s cycles of bead-beating. Cell debris was removed by centrifugation. The cell lysate was dried completely under vacuum, resuspended, and mixed vigorously in 750 μl of chloroform and an equal volume of 20% methanol. After centrifugation at 13,400 $\times$ *g* for 20 min, the supernatant was collected and stored at $-20\,^{\circ}$C for further analysis.

Protein Phosphatase Inhibition Assay—Microcystin extracts were tested for inhibition of protein phosphatase type 2a (PP2a) (Promega, Madison, WI) as described in the study by Carmichael and An (34). Samples or standards (0–33 nM MCYST-LR in 20% methanol) were incubated for 5 min at 37 °C with 0.05 U PP2a diluted in 50 mM Tris pH 7.0, 2 mM MnCl$_2$, 1 mg/ml bovine serum albumin (BSA) and 2 mM dithiotreitol. A reaction mixture of 25 mM p-NPP substrate in 0.2 M Tris pH 8.1, 80 mM MgCl$_2$, 0.4 mM MnCl$_2$, 2 mg/ml BSA and 4 mM dithiotreitol was added and the reaction was allowed to proceed at 37 °C for a further 80 min. Sample absorbance was read in a BioRad microplate reader at 405 nm and the percent inhibition was calculated.

High Performance Liquid Chromatography (HPLC) Detection of Microcystins—Samples showing inhibition of PP2a were further tested by HPLC according to the procedure described by (35). Briefly, microcystin extracts were loaded on an Alltech 5 μm Nucleosil C18 column (250 $\times$ 4.6 mm) and separated using a linear gradient of 30–70% Solvent B (acetonitrile containing 0.05% trifluoroacetic acid) for 30 min. Solvent A was 0.05% trifluoroacetic acid in water with a flow rate of 1 ml/min. Fractions eluting between 10 and 30 min were collected, concentrated under vacuum to 50 μl and were tested again for PP2a inhibition as described above.

Electrospray Ioization Quadrupole Time-of-Flight Tandem MS (ESI Q-TOF MS/MS)—To distinguish the isoforms of the toxins produced by *M. aeruginosa* PCC 7806, UWOCC MRC and UWOCC MRD, fractions collected by HPLC were subjected to mass spectrometry in a Q-TOF Ultima (Micromass) fitted with nanospray needles prepared in-house at the Biological Mass Spectrometry Facility, UNSW. Tandem mass spectra were acquired using Ar as the collision gas at different collision energies (8–50 eV). Capillary voltage was set at 1.3 kV. Cone voltage was 50 V. Microcystin-LR (Sigma) was used as a standard.

Proteome Analysis

Protein Extraction—Before protein extraction, cells were grown to mid-exponential phase (OD$_{730\,nm}$ 0.75–0.85) and synchronized using the block-release method (48 h dark/72 h light) as described in Yoshida *et al.* (36). The cultures were grown for further 5 days with a 12:12 light:dark cycle and 100 ml of cells were harvested by centrifugation. The pellets were washed twice with sterile Milli-Q water before resuspending in a final volume of 500 μl MilliQ. The cells were partially lysed by four freeze-thaw cycles in liquid nitrogen and 37 °C in the presence of 15 μM PMSF (Sigma) and the lysates were treated with endonuclease (Sigma). The cells were resuspended with an equal volume of acid extraction buffer containing 7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), 2% sulfobetaine 3–10 (SB3–10) and 80 mM citric acid, pH 4 and proteins were extracted according to the method in Herbert and colleagues (37). The proteins were resuspended in 150 μl 2DE buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% SB3–10) and quantified by a Bradford assay (Sigma), as well as by serial dilutions of the sample using one-dimensional SDS-PAGE against known BSA standards. Proteome analysis for all strains was performed with biological triplicates.

One-dimensional SDS-PAGE—One hundred micrograms of protein were buffer-exchanged in one-dimensional SDS-PAGE buffer and boiled for 10 min. Samples were loaded on a 10–20% Criterion gel (BioRad) and electrophoresis was performed at 5 mA/gel for 15 min followed by 200 V until the dye front had reached the end of the gel. The gels were fixed in 40% methanol and 10% acetic acid and stained with Coomassie G250 overnight. The gels were destained with Milli-Q water before visualization. A standard containing 1 μg BSA (Sigma) was included in all electrophoresis runs.

In-gel Digestion—For nanoLC/MS-MS analysis, each lane of a Coomassie-stained gel was cut into 16 segments and these were sliced to 1 mm^3 pieces. Destaining was achieved by washing the gel pieces with 50% acetonitrile/50 mM NH$_4$HCO$_3$ until the stain was no longer visible. After dehydrating with 100% acetonitrile, and air-drying to remove residual acetonitrile, proteins were reduced in 10 mM dithiothreitol dissolved in 100 mM NH$_4$HCO$_3$ for 1 h at 55 °C and alkylated in 55 mM iodoacetamide in 100 mM NH$_4$HCO$_3$ for 45 min in the dark at room temperature. The washing steps described above were repeated twice and gel pieces were dehydrated with 100% acetonitrile.

The gel pieces were covered with 15 μg ml^{-1} sequencing-grade trypsin solution (Promega) and rehydrated at 4 °C for 30 min. Samples were then covered with an additional 25 μl NH$_4$HCO$_3$ buffer and digested overnight at 37 °C.

The digest supernatant was transferred to a clean tube and 30 μl of 50% acetonitrile/2% formic acid was added to the gel pieces, which were vortexed over 20 min to extract the remaining peptides. The supernatant was combined with the initial digest solution and the step was repeated to give a final volume of $\sim$60 μl. The volume of the extracted peptides was reduced to 10 μl under vacuum and the solution was desalted using C18 tips (Eppendorf). The extracted peptides were spun at 16,000 $\times$ *g* for 10 min to remove particulate matter and diluted to 10 μl with 1% formic acid if necessary.

nanoLC-Tandem Mass Spectrometry—The tryptic digest extracts from 1DE gel slices were analyzed by nanoLC-MS/MS using a LTQ-XL ion-trap mass spectrometer (Thermo) according to Hattrup and colleagues (38). Reversed phase columns were packed in-house to $\sim$7 cm (100 mm i.d.) using 100 Å, 5 mM Zorbax C18 resin (Agilent Technologies, Santa Clara, CA) in a fused silica capillary with an integrated electrospray tip. A 1.8 kV electrospray voltage was applied via a liquid junction up-stream of the C18 column. Samples were injected onto the C18 column using a Surveyor autosampler (Thermo). Each sample was loaded onto the C18 column followed by an initial wash step with buffer A (5% (v/v) acetonitrile, 0.1% (v/v) formic acid) for 10 min at 1 ml/min. Peptides were subsequently eluted from the C18 column with 0–50% Buffer B (95% (v/v) acetonitrile, 0.1% (v/v) formic acid) over 58 min at 500 nL min^{-1}, followed by 50–95% Buffer B over 5 min at 500 nL/min. The column eluate was directed into a nanospray ionization source of the mass spectrometer. Spectra were scanned over the range 400–1500 *m/z*. Automated peak recognition, dynamic exclusion, and tandem MS of the top six most intense precursor ions at 35% normalization collision energy were performed using the Xcalibur software (version 2.06) (Thermo).

Protein Identification—Raw files were converted to mzXML format and MS/MS spectra from all fractions were processed using the Global Proteome Machine Tandem software (version 2007.08.29) against the proteome of *M. aeruginosa* NIES-843 containing 6312 open reading frames (database derived from National Center for Biotechnology Information (NCBI)), and the common repository for abundant peptides (39, 40). This proteome was chosen so that only proteins universal to the *M. aeruginosa* species rather than strain-specific proteins were identified. Reverse database searching was used for estimating false discovery frequencies (41). To ensure the specificity of the database, mass spectra were also searched against

Primer name	Sequence (5′-3′)	Target
trxMF	TCAGGAACTGTTGCAATCCA	thioredoxin *trxM*
trxMR	AGAATCGGAGCCATCATTTG	
MAE27590F	AATCGAACCCGATAAACCCT	hypothetical protein MAE27590
MAE27590R	TCACAACCGACAACAGAAGC	
ccmKF	ACGGATCACGATTGTTGGTT	carboxysome shell subunit *ccmK3*
ccmKR	TCGGTTTTATTGACGGCTTC	
P$_{II}$F	AAGCGATTATCCGACCCTTT	nitrogen regulatory protein P$_{II}$ *glnB*
P$_{II}$R	ATTGACCTTTCTGACGACCG	
ccmLF	TCTGCTTTTGCAATTCATCG	carboxysome shell subunit *ccmL*
ccmLR	CAATAATCCCCACCACCATC	
MAE06820F	AGTGGTAGCCGAAAGCGATA	hypothetical protein MAE06820
MAE06820R	TCCCTTCCAAGAACAAATGG	
nrtAF	TGATGGTCGCAAAATTGAAA	nitrate transporter *nrtA*
nrtAR	GGAATATAGCCCCAACGGAT	
ndhKF	ACTACCCACAAAATGCAGGC	NADH dehydrogenase subunit K *ndhK*
ndhKR	CTCTTTCGGAGGTGCTTGAC	
MAE06360F	GCACGATCGGTTTTTGTTTT	carboxymethylenebutenolidase
MAE06360R	ATGCGTAGATTGTCCCCTTG	
rpoC1F	CCTCAGCGAAGATCAATGGT	RNA polymerase gamma subunit *rpoC1*
rpoC1R	CCGTTTTTGCCCCTTACTTT	
ntcArealF	CATTTCCGTTTGCAGAATCC	global nitrogen regulator *ntcA*
ntcArealR	TGTTTTTGGGGTGCTATCCT	

proteome databases of known bacterial contaminants in *M. aeruginosa* cultures, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but no significant matches were found. Peptide identification was determined using a 0.4 Da fragment ion tolerance and a parent ion tolerance of 1.4 Da. Carbamidomethyl was considered as a complete modification and partial modifications, including the oxidation of methionine and deamidation of asparagine and glutamine, were also considered. Up to one missed cleavage was permitted. Peptide matches and proteins identified by a single protein are listed in supplemental Data 1 and 2.

The mass spectrometry data and identification files were processed with PRIDE Converter and submitted to the Proteomics Identifications database (PRIDE).

Data Analysis—Protein function was assigned according to the categories used in CyanoBase (bacteria.kazusa.or.jp/cyanobase/). Hierarchical multivariate cluster analysis with the chi-square method of cluster distance calculation was performed in SYSTAT on proteins that were present in all biological replicates for each strain.

The nSAF quantitation method developed by Zybailov *et al.* (2006) and Chick *et al.* (2008) (42–43) was used to quantify changes in the expression levels of differentially displayed proteins between toxic and nontoxic strains, as well as pairwise comparison of proteins in UWOCC CBS, UWOCC MRC, and UWOCC MRD. For each protein k, the number of assigned spectral counts SpC, was corrected for the predicted molecular weight MW of the protein in kDa. The (SpC/MW) values were divided by the sum of (SpC/MW) for all proteins to give the nSAF values used for further analysis. A spectral fraction of 0.5 was added to all nSAF values to achieve normal distribution of the data. To determine differential protein expression in toxic and nontoxic *M. aeruginosa*, ANOVA with no multiple adjustments was performed on the log-transformed data using the statistical package R (44). Only proteins present in at least eight of the total nine replicates for the toxic or nontoxic strain group were considered for this analysis.

The full log nSAF abundance data (242 proteins × 18 samples, supplemental Data 5) was also examined for patterns in the overall protein abundance profile that could be associated with the synthesis of particular toxin isoforms. To this end, the Pearson correlation

matrix for all samples was generated and then visualized as an image using basic functionality from the R stats package. Also a principal component analysis was carried out, and the different samples were plotted in the space of the first three principal components. All computations were carried out using the R statistical programming environment (44).

Transcription Analysis

RNA Extraction—Parallel to protein extraction, RNA was extracted from the same cultures of *M. aeruginosa*. Fifty milliliters of cells were pelleted by centrifugation, washed with Milli-Q water, and resuspended in 1 ml of Trizol reagent (Invitrogen). The cells were snap-frozen in liquid nitrogen and all subsequent steps were performed at 4 °C where possible. Cell lysis was achieved by vigorous pipetting and 400 μl chloroform was added to the lysate. After centrifugation at 13,400 × g for 15 min, the aqueous layer was collected and RNA was precipitated in ice-cold isopropanol. The extracts were centrifuged at 13,400 × g for 30 min and the pellets were washed twice with 75% ethanol, before being resuspended in DEPC-treated water (Invitrogen).

DNase Treatment—RNA extracts were treated with 3 U of Turbo DNase (Ambion) for 4 h at 37 °C. This was followed by a second extraction in Trizol as outlined above. The success of DNA removal was assessed by PCR targeting the 16S rDNA gene. The purity and quantity of RNA were estimated using a Nanodrop spectrophotometer (Nanodrop Technologies).

cDNA Synthesis—The Marligen random cDNA synthesis kit (Marligen Bioscience) was used for reverse transcription of 500 ng RNA following the manufacturer's instructions. The reaction conditions were 22 °C for 5 min, 42 °C for 90 min, and 85 °C for 5 min.

Quantitative Real-time PCR (qRT-PCR)—qRT-PCR was used to quantify transcription levels for genes that were found to be differentially expressed by the nSAF proteomic analysis. Primer sequences are listed in Table II. Both the 16S rRNA and the RNA polymerase subunit C (*rpoC1*) genes were considered as possible reference genes and *rpoC1* was chosen for further studies after validation.
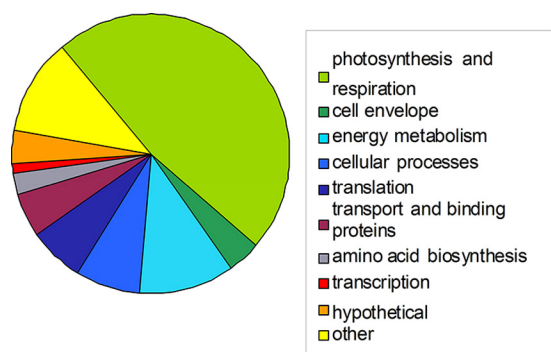
FIG. 1. **Functional class distribution in the core *M. aeruginosa* proteome.** Functional class categories were assigned according to CyanoBase (http://genome.kazusa.or.jp/cyanobase).

Transcript levels were quantified by qPCR using the Rotor-Gene 3000 system (Corbett). Reactions were performed in a total volume of 25 μl using 1 μg cDNA, 10 pmol of forward and reverse primer and the Platinum SYBR Green qPCR supermix UDG kit (Invitrogen). Two-step cycling was performed with an initial hold of 60 °C for 2 min and 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The efficiency of amplification for each primer set was determined using standard cDNA curves and calculated according to the equation E = 10 [-1/slope] (45). Transcript levels were normalized to *rpoC* transcription and calculated relative to values for toxic strains using the $2^{-\Delta\Delta Ct}$ method as described elsewhere (45). All analyses were performed using biological and technical triplicates.

Reverse-transcription PCR—To assess the cotranscription of *trxM* and the neighboring ORF MAE06820, 1 μg of RNA extracted from PCC 7806 was reverse-transcribed using the Marligen random cDNA synthesis kit as described above. A 470 bp fragment spanning both the *trxM* and *MAE06820* gene was amplified with the primers trxMF and MAE06820R and the successful amplification was visualized after electrophoresis in a 1% agarose gel.

RESULTS

The Core Proteome of M. aeruginosa Strains—Proteome analysis of the six strains in this study identified 475 proteins reproducible in biological triplicates, which were considered for further statistical analysis (supplemental Data 3). Of the compiled proteins, only 82 were found to be expressed in all strains (Fig. 1 and Table III), constituting between 29 and 43% of the identified proteins. This interstrain variability in identified proteins confirms the observed differences in SDS-PAGE migration patterns (supplemental Data 4).

Ten functional categories were represented in the core proteome, the highest fraction being comprised of proteins involved in photosynthesis and respiration. Seven hypothetical proteins were identified in all strains (Table III, supplemental Data 2), including MAE06270, which shows homology to *slr1623* and *cce 4330*, a novel NADH dehydrogenase subunit in *Synechocystis* sp. PCC 6803 and *Cyanothece* sp. ATCCC 51142, respectively. No unknown proteins were present in the *M. aeruginosa* core proteome.

Functional Categories of Proteins Expressed in M. aeruginosa Strains—The total number of proteins identified for each strain varied, with the most found in PCC 7005 (280 proteins)

and the least in UWOCC CBS (192 proteins). However, in the six strains proteins from all functional categories were identified (Table III). Similar to the results obtained for the core proteome, the photosynthetic, respiratory and hypothetical proteins, as well as proteins involved in diverse functions (the category designated "other" in CyanoBase) were predominant.

A total of 90 hypothetical proteins and nine unknown proteins were identified reproducibly, confirming the correct annotation of these open reading frames and the expression of these proteins in nutrient-replete conditions in the exponential growth phase. Two of the unknown proteins (MAE60260 and MAE60250) were in adjacent open reading frames and may form part of an operon, expressed only in strain PCC 7005. Similarly, two putatively cotranscribed clusters of hypothetical proteins were found in PCC 7005 - MAE36710 and MAE36720, and MAE45800 and MAE45790, the latter pair also being expressed in HUB5.3. The fact that these proteins were only expressed in a small number of the strains suggests that they may be involved in adaptation processes specific for a particular environmental niche.

Diversity of Protein Expression in M. aeruginosa Strains—Genome analysis of *M. aeruginosa* strains has revealed that these organisms are genetically highly plastic and contain a large number of transposases and loci that could have been the result of horizontal gene transfer (31, 32). We were interested to see whether this diversity was also present at the proteome level. For each strain, the larger part of the proteome consisted of proteins that were present in the *M. aeruginosa* core proteome (Fig. 2). Strain PCC 7005 was the most divergent, expressing 65 strain-specific proteins (23% of the strain proteome). The majority of the proteins expressed uniquely in this strain were involved in carbon and nitrogen metabolism, as well as cofactor synthesis (supplemental Data 3). In contrast, in HUB5.3 only seven strain-specific proteins were identified (3% of the proteome). When the number of proteins shared among strains was considered, it became evident that proteins that were present in a single strain (unique) comprised the largest category, followed by proteins in the core proteome (Fig. 2, Table IV). Most of the proteins expressed in a single strain were hypothetical or proteins in the other category. This suggests that the *M. aeruginosa* strains differ mostly in processes involved in adaptation to a particular environment or a growth condition, rather than in essential metabolic reactions. No protein was expressed uniquely in either the toxic or the nontoxic strains, which could serve as potential markers for toxicity.

Cluster analysis was also performed based on the distribution pattern of proteins expressed reproducibly in *M. aeruginosa* strains, and all strains were found to be closely related with the largest distance between them being only 10% (Fig. 3). Two large clades were observed, each containing both toxic and nontoxic strains. Interestingly, UWOCC MRD and UWOCC MRC, which are identical in 16S rRNA gene phylogeny and two intergenic spacer region sequences, studied so

TABLE III

Functional categories of proteins in M. aeruginosa strains. The proteins universally expressed for all strains are shown in the first column (M. aeruginosa). The number of proteins identified is in black and the % of the proteome is below in italics. Functional categories were assigned according to CyanoBase (http://genome.kazusa.or.jp/cyanobase)

Functional category	Protein %						
	M. aeruginosa	PCC 7806	UWOCC MRC	UWOCC MRD	UWOCC CBS	HUB5.3	PCC 7005
Amino acid biosynthesis	4	20	13	11	12	11	17
	4.8	*7.6*	*6.0*	*4.1*	*6.2*	*5.2*	*6.1*
Biosynthesis of cofactors, prosthetic groups and carriers	-	4	2	3	3	4	9
	-	*1.5*	*0.9*	*1.1*	*1.5*	*1.9*	*3.2*
Cell envelope	3	5	7	5	5	4	3
	3.6	*1.9*	*3.2*	*1.9*	*2.6*	*1.9*	*1.1*
Cellular processes	5	10	10	11	7	9	10
	6.1	*3.8*	*4.6*	*4.1*	*3.6*	*4.2*	*3.6*
Central intermediary metabolism	-	3	2	2	-	1	4
	-	*1.1*	*0.9*	*0.7*	-	*0.4*	*1.4*
Energy metabolism	5	18	15	15	12	12	22
	6.1	*6.9*	*6.9*	*5.7*	*6.2*	*5.7*	*7.8*
Fatty acid, phospholipids and sterol metabolism	-	-	1	1	1	1	2
	-	-	*0.4*	*0.3*	*0.5*	*0.4*	*0.7*
Photosynthesis and respiration	32	53	54	56	41	53	52
	39	*20.3*	*25*	*21.3*	*21.3*	*25.2*	*18.5*
Purines, pyrimidines, nucleosides and nucleotides	-	1	1	2	-	1	2
	-	*0.3*	*0.4*	*0.7*	-	*0.4*	*0.7*
Regulatory functions	-	2	-	5	2	2	4
	-	*0.7*	-	*1.9*	*1.0*	*0.9*	*1.4*
DNA replication, restriction, modification, recombination and repair	-	2	2	1	1	1	4
	-	*0.7*	*0.9*	*0.3*	*0.5*	*0.4*	*1.4*
Transcription	-	4	1	2	-	2	2
	-	*1.5*	*0.4*	*0.7*	-	*0.9*	*0.7*
Translation	7	27	17	17	10	17	40
	8.5	*10.3*	*7.9*	*6.4*	*5.2*	*8.1*	*14.3*
Transport and binding proteins	6	10	9	17	14	9	8
	7.3	*3.8*	*4.1*	*6.4*	*7.3*	*4.2*	*2.8*
Other	13	53	41	55	47	44	61
	15.8	*20.3*	*18.9*	*20.9*	*24.4*	*20.9*	*21.7*
Hypothetical	7	46	37	56	35	36	37
	8.5	*17.7*	*17.1*	*21.3*	*18.2*	*17.1*	*13.2*
Unknown	-	2	4	4	2	3	3
	-	*0.7*	*1.8*	*1.5*	*1.0*	*1.4*	*1.1*
Total proteins	82	260	216	263	192	210	280

far, were separated in different clades of the tree. UWOCC MRC, which has recently reverted to toxin production, was grouped with two nontoxic strains, whereas the protein expression profile of the toxic UWOCC MRD was closest to that of the model toxic strain PCC 7806.

Differentially Expressed Proteins in M. aeruginosa—The expression levels of nine proteins were found to differ significantly between toxic and nontoxic strains when ANOVA was performed on nSAF calculated for proteins in the whole proteome data set (Table V and Fig. 4, supplemental Data 5). Of these, two proteins were involved in nitrogen uptake and metabolism (P_{II} and NrtA), two comprised components of the carboxysome shell (CcmK3 and CcmL) and two are involved in maintaining the cellular redox balance (NdhK and TrxM). The two hypothetical proteins with a putative role in redox balance maintenance were also identified as differentially expressed. The majority of the proteins were down-regulated in the nontoxic strains, with the exception of P_{II}, NdhK and the carboxymethylenebutenolidase. Quantitative real-time PCR

was used to measure differences at the transcript level and the patterns predicted at the protein level were observed at the RNA level for *nrtA, ndhK*, and *ccmL* (Table V). In general, greater differences were observed at the protein expression level compared with gene transcription.

Because of the possible involvement of the transcriptional regulator NtcA in this differential display (see Discussion), the transcription of this gene was also measured and found to be 2.61-fold up-regulated in nontoxic strains.

Coexpression of MAE06820 and TrxM—The similar expression profiles of TrxM and the hypothetical protein MAE06820 observed in Fig. 4, prompted us to perform reverse transcription in order to evaluate whether the two open reading frames were cotranscribed. The amplification of a fragment of the expected size (470 bp) spanning both genes was successful, confirming that both genes can be transcribed from a single promoter (Fig. 5).

Toxicity Switching in M. aeruginosa UWOCC MRC—A toxicity screen of the cultures used in this study revealed that

UWOCC MRC, a strain that was expected to be nontoxic, was producing MCYST-LR and [DAsp³] MCYST-LR (Fig. 6 and Table VI, supplemental Data 6). These isoforms are found in *M. aeruginosa* PCC 7806 and are different from the microcystin species produced by the toxic strain UWOOC MRD, which produced several aromatic and homoamino acid isoforms of the toxin (Table VI). Contamination by other *Microcystis* spp. was ruled out by sequencing the 16S rRNA gene and PCR of the regions of the *mcy* cluster characteristic for MRC and MRD (46). Several subcultures were tested over a period of two years, and the toxicity of MRC appears to be a stable trait.

Effects of Toxin Isoform Synthesis on Protein Expression— The different isoforms of microcystin synthesized by the three toxic strains in this study, prompted us to consider the possibility that the toxin variant produced has an effect on the protein expression profile of the producing cells. However, significant separation of UWOCC MRD from UWOCC MRC

and PCC 7806 could not be achieved when the protein expression patterns in these strains were considered (supplemental Data 7). The protein phosphatase inhibition assay toxin analysis revealed that the microcystin concentration was similar in all toxic strains used here, excluding the possibility that quantitative differences in the intracellular microcystin content influence protein expression in *M. aeruginosa*. PCC 7806, UWOCC MRC, and UWOCC MRD contained 245.28 ± 0.20 nM, 245.94 ± 0.11 nM, and 245.75 ± 0.37 nM microcystin, respectively. Correlation analysis of the expressed proteins in all strains also did not achieve distinct separation between the toxic and nontoxic strain group (supplemental Data 7).

DISCUSSION

Diversity of Protein Expression in Strains of M. aeruginosa— The proteome studies presented here indicate that protein expression in strains of *M. aeruginosa* is highly variable, with only a third of the expressed proteins in each strain constituting the core proteome of the species. Such protein diversity was also observed when the protein samples were run on SDS-PAGE (supplemental Data 4). Such inter-strain variability in protein expression was unexpected, given the close 16S rRNA gene homology shared within strains of this species. The observed proteome diversity supports previous suggestions that *M. aeruginosa* strains should be considered as ecotypes adapted to survival in a particular environmental niche (33). It also highlights the dangers of using predominantly *M. aeruginosa* PCC 7806 as a model organism for the entire species. In particular, under stress-inducing conditions, such as nutrient starvation or changes in light quality and intensity, many strain-specific acclimation processes would be expected to take place and differences in protein expression are expected.

The use of a single model strain also seems an inappropriate approach to study toxicity and the effects of microcystin production on cyanobacterial metabolism because proteome diversity was high within the toxic and nontoxic strain groups studied here as well. This is evident from the cluster analysis (Fig. 3), which failed to separate the strains based on their ability to produce microcystin, geographical origin or period of culture in the laboratory. No proteins were expressed solely in toxic or nontoxic strains, including the McyS proteins involved in microcystin analysis. The mixed toxic and nontoxic strains
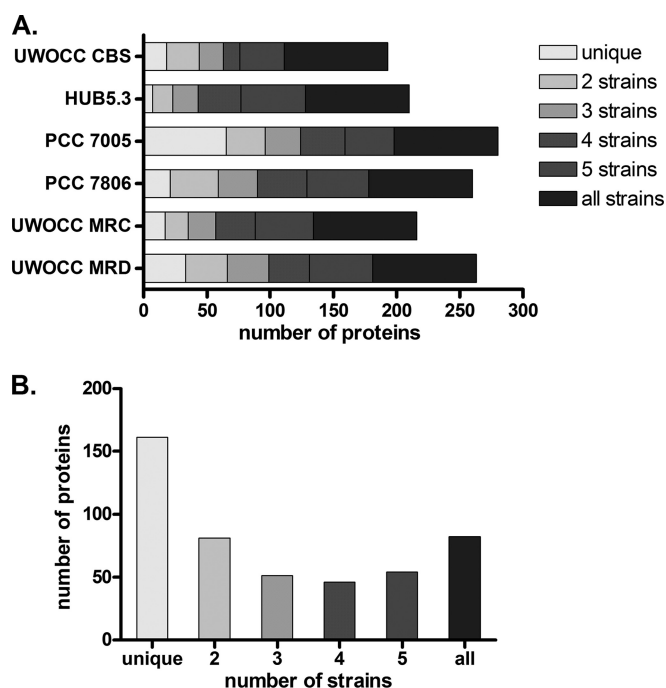

FIG. 2. **Protein distribution among the six strains in this study.** *A*, Distribution of unique and shared proteins for each strain *B*. Total number of proteins involved in each combination (1 being unique and 6 being present in all strains).

TABLE IV
Protein distribution in strains of M. aeruginosa. Combinations of proteins expressed by different strains

Strain	Number of proteins						
	Unique	2 Strains	3 Strains	4 Strains	5 Strains	All Strains	Grand Total
PCC 7005	65	31	28	35	39	82	280
PCC 7806	21	38	31	39	49	82	260
UWOCC CBS	18	26	19	13	35	82	192
HUB5.3	7	16	20	34	51	82	210
UWOCC MRC	17	18	22	31	46	82	216
UWOCC MRD	33	33	33	32	50	82	263
Total proteins	**161**	**81**	**51**	**46**	**54**	**82**	**475**

in the same clade of the cluster tree present a similar situation to what has been observed on a genetic level, where phylogeny-based methods have not been able to discriminate between toxic and nontoxic, but toxigenic strains. The existing proteome and genome diversity in *M. aeruginosa* should therefore be taken into consideration, in particular in the study of physiological responses to particular conditions. Ensuring that several strains are used for such comparative studies would avoid confusing conclusions as illustrated by the study of PCC 7806 and its knock-out mutant by Dittmann and colleagues in 2001 (11) and the more recent comparative genomic analysis of PCC 7806 and NIES-843 (31). The authors reported the identification of two light-sensitive proteins in the *mcyB-* mutant strain and proposed their use as a suitable marker for toxin production (11). However, these proteins were not found in the genome of the toxic NIES-843 and therefore are not universal for the *M. aeruginosa* genus.

An interesting observation was the unusually large number of proteins expressed solely in PCC 7005 (Table I). In the environment, strains from a dense culture in the late exponential growth stage such as the ones used here, are likely to be starved for micronutrients, such as iron. One possibility for

FIG. 3. **Cluster dendrogram of protein expression in strains of *M. aeruginosa*.** Hierarchical multivariate cluster analysis ($n = 3475$ proteins) with chi-square method of cluster distance calculation.

the increased expression of proteins in this strain, may be its ability to synthesize cofactors needed for the utilization and storage of the excess carbon and nitrogen provided in the laboratory media (supplemental Data 3). Because this strain has been maintained in laboratory culture for several decades longer than the other strains in this study, its proteome may reflect an ability to adapt to the nutrient-rich environment of laboratory media and make use of the excess macronutrients provided. Such changes have been observed previously in *M. aeruginosa* with the loss of gas vesicles because of transposition events and the dispersal of colonies after prolonged culturing (18, 47).

The *M. aeruginosa* strain PCC 7005 also contained most of the hypothetical and unknown proteins found in adjacent reading frames, the majority of which were absent in the remaining strains of the cohort. In fact, only nine unknown proteins, out of 1404 predicted, were identified in the proteome of the strains studied here and none of these were present in the core proteome. It is likely that the majority of proteins designated as unknown are involved in adaptation or growth-stage specific processes in *M. aeruginosa*.

The Core Proteome of M. aeruginosa—The majority of proteins shared among all strains of *M. aeruginosa* were involved in photosynthesis and respiration. This distribution of protein expression is typical for cyanobacteria, where phycobilisome components are regularly found in high abundance and obscure less-abundant proteins (48). The number of proteins that were identified, as well as the pI and molecular range covers are also similar to other gel-based studies of total cell extracts of cyanobacteria, such as the unicellular *Synechocystis* sp. PCC 6803 and the filamentous nitrogen-fixing *Anabaena variabilis* ATCC 29413 (49). It is likely that other proteins remained undetected because only one genomic database was used for identification and there may be proteins expressed in certain strains but not encoded in the NIES-843 genome. However, the aim of this study was to find proteins shared across *M. aeruginosa* species, rather than perform an exhaustive proteome study on one strain, for which sub-cellular protein fraction analysis and multiple analytical approaches would be required.
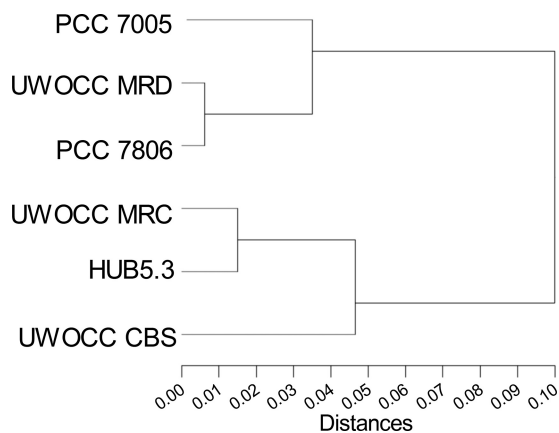
TABLE V

Proteins identified as significantly different between the M. aeruginosa toxic and nontoxic group after nSAF analysis and ANOVA. Expression patterns determined by nSAF and transcription values from qRT-PCR are shown for nontoxic strains relative to those for the toxic strains. Standard deviation values are in brackets and italicized. Accession numbers are shown as they appear in CyanoBase

| Protein GI	*p* value	Accession	Function	Expression	Transcription
gi 166085797	0.01	MAE06830	thioredoxin (*trxM*)	−2.79	1.47 *(1.12)*
gi 166087873	0.02	MAE27590	hypothetical protein	−1.76	1.02 *(1.98)*
gi 166090653	0.02	MAE55390	carbon concentrating mechanism protein (*ccmK3*)	−1.94	2.52 *(1.08)*
gi 166090860	0.02	MAE57460	nitrogen regulatory protein P_{II} (*glnB*)	2.87	−1.85 *(0.95)*
gi 166089906	0.02	MAE47920	carbon concentrating mechanism protein (*ccmL*)	−2.02	−1.94 *(0.92)*
gi 166085796	0.03	MAE06820	hypothetical protein	−2.14	3.65 *(1.36)*
gi 166086594	0.03	MAE14800	nitrogen transport protein (*nrtA*)	−1.71	−2.58 *(0.93)*
gi 166086291	0.04	MAE11770	NADH dehydrogenase subunit (*ndhK*)	1.67	1.95 *(1.31)*
gi 166085750	0.05	MAE06360	carboxymethylenebutenolidase	3.84	−1.02 *(1.71)*
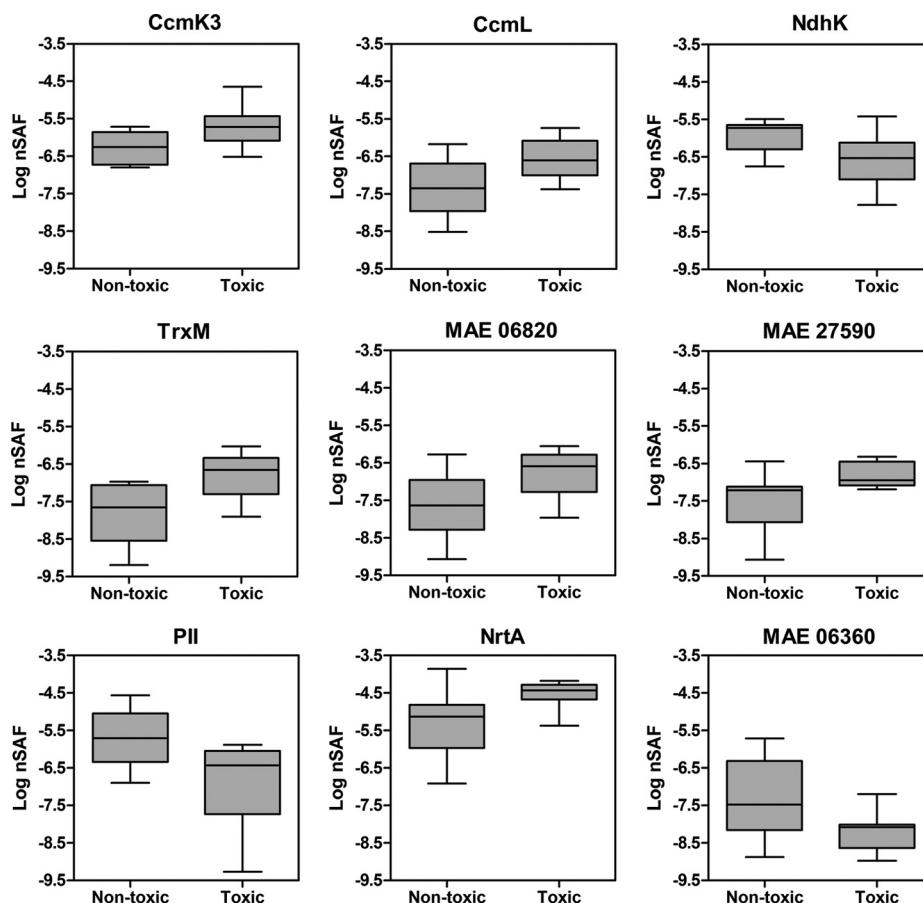
FIG. 4. **Box-plots of nSAF values for the proteins identified by ANOVA as significantly different ($p < 0.05$) between the toxic (PCC7806, UWOCC MRC, UWOCC MRD) and nontoxic (PCC7005, HUB5–3, UWOCC CBS) strain group.** nSAF values were calculated according to Zybailov *et al.* (42).
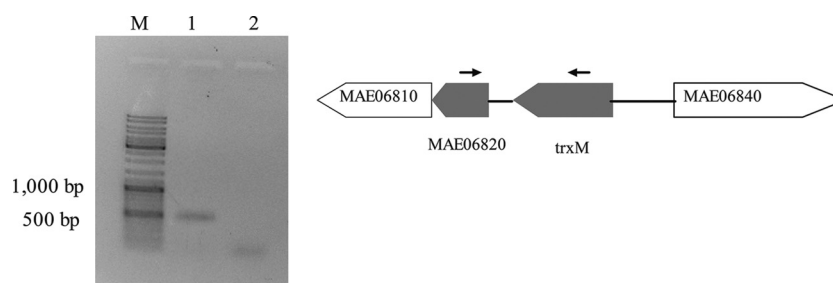

FIG. 5. **Reverse-transcription of *trxM* and *MAE06820*.** After amplification with primers spanning a 470 bp region of the two open reading frames shown in the *right* panel, amplicons were electrophoresed in a 1% agarose gel. M, Gene Ruler DNA ladder (MBI Fermentas, Burlington Canada); 1, reverse-transcribed PCC 7806 RNA; 2, negative control. The binding sites of the primers used in the reverse transcription reaction are marked with black arrows and the cotranscribed region is in *gray*. The open reading frames are shown as their accession numbers in CyanoBase: MAE06810, phosphopantheine adenyltransferase; MAE06820, hypothetical protein; trxM, thioredoxin M; MAE06840, methionine aminopeptidase.

The large differences in protein expression among strains of *M. aeruginosa*, although unexpected, are not unique for bacteria. A comparative analysis of two strains of *E. coli* found that only 38% of the secreted proteins were conserved and a similar fraction were strain-specific (50). Similarly, a study of strains of the marine cyanobacteria *Prochlorococcus* spp. found that the elemental composition of their proteomes was strongly influenced by their site of isolation and the nutritional availability at that site (51). However, most proteome studies comparing bacterial strains have regularly found the core proteome to consist of 70 to 90% of the identified proteins (52–55).

It is important to note that the proteomes presented here reflect the state of the cells during nutrient-replete, exponential growth. Although no protein with the potential to be used as a biomarker for toxicity was detected, the possibility that one would be expressed during other growth stages or under different environmental conditions cannot be excluded.

Differential Display Between Toxic and Nontoxic Strains of M. aeruginosa—The nSAF approach was used as a label-free
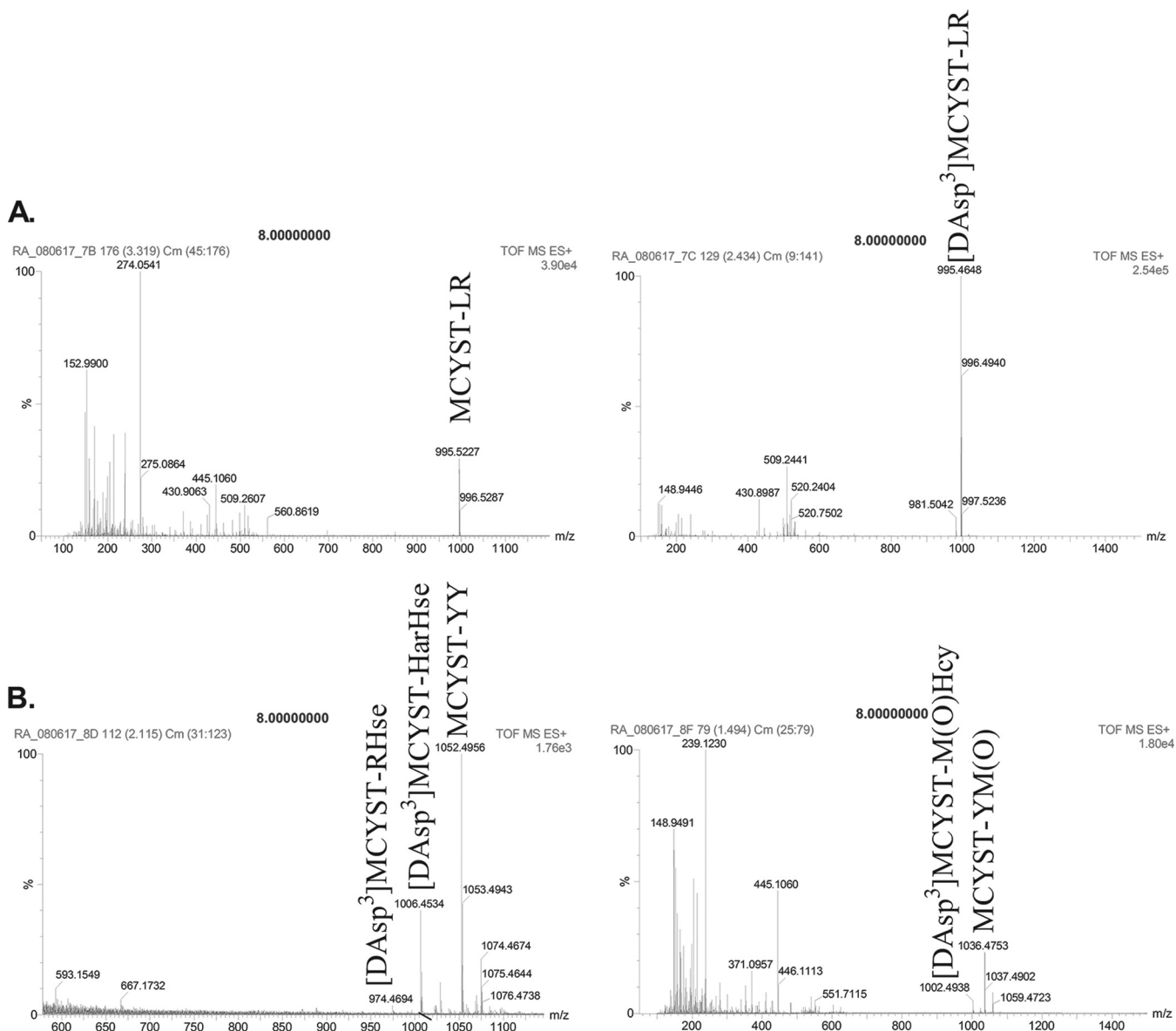
FIG. 6. **MS spectra showing microcystin production by** *M. aeruginosa* **UWOCC MRC (*A*) and UWOCC MRD (*B*).** Labeled peaks were identified by MS/MS spectra as microcystin isoforms.

method to quantify expression of proteins in the toxic and nontoxic subsets of *M. aeruginosa* strains. Differences between toxic and nontoxic strains were revealed in proteins involved in maintaining the cellular redox balance and integration of carbon-nitrogen (C-N) metabolism, as well as two hypothetical proteins MAE06820 and MAE27590 (Table V and Fig. 4).

Suitability of the nSAF Approach for Comparison of Bacterial Strains—The nSAF approach was initially used to compare the expression profiles of a single organism grown under different conditions (42). In this study, this label-free quantitation method was applied to analyze protein expression in several strains of a bacterial species. The *M. aeruginosa* strains studied here showed large diversity in their proteomes.

The analysis of similarly diverse proteomes is not possible with image-based methods such as 2DE, which has been recently attempted unsuccessfully in cylindrospermopsin-producing cyanobacteria (28). However, the reciprocal expression profiles of P$_{II}$ and NrtA, the similar expression of the CcmL/CcmK3 and TrxM/MAE06820 pairs, as well as the up-regulation of the gas vesicle and phycocyanin proteins in UWOCC MRC, that were seen here, all suggest that biologically meaningful data can be extracted from even highly diverse datasets using nSAF.

Nitrogen Uptake and Metabolism Proteins—The expression of the signal transduction protein, P$_{II}$, encoded by *glnB*, was altered to a significant extent by the presence of microcystin in *M. aeruginosa* cells (Table V). This protein is central to

TABLE VI

Microcystin isoforms identified by Q-TOF MS/MS. Purified MCYST-LR was used as a standard for both HPLC and MS studies

Strain	Toxin isoforms[a]	Molecular weight (Da)	HPLC elution time (min)	Reference
PCC 7806	MCYST-LR	995.45	19.4–20.4	Phelan and Downing,
	[DAsp[3]]MCYST-LR	995.5	18.7–19.4	2007 [90]
UWOCC MRD	[DAsp[3]] MCYST-RHse	974	25.0–25.6	This study
	[DAsp[3]] MCYST-M(O)Hcy	1002	30.1–30.7	
	[DAsp[3]] MCYST-HarHse	1006	25.0–25.6	
	MCYST-YY	1052.3	25.0–25.6	
	MCYST-YM(O)	1036.5	30.1–30.7	
UWOCC MRC	MCYST-LR	995.5	17.2–19.7	This study
	[DAsp[3]]MCYST-LR	995.5	18.4–19.7	

[a] Har, homoarginine; Hse, homoserine; Hcy, homocysteine; M(O), methionine-S-oxide.

FIG. 7. **Proposed model for the involvement of microcystin and NtcA regulation in the differential expression of proteins in *M. aeruginosa*.** Boxes in *gray* have a putative function in this proposed model and have not been shown to be differentially expressed experimentally.
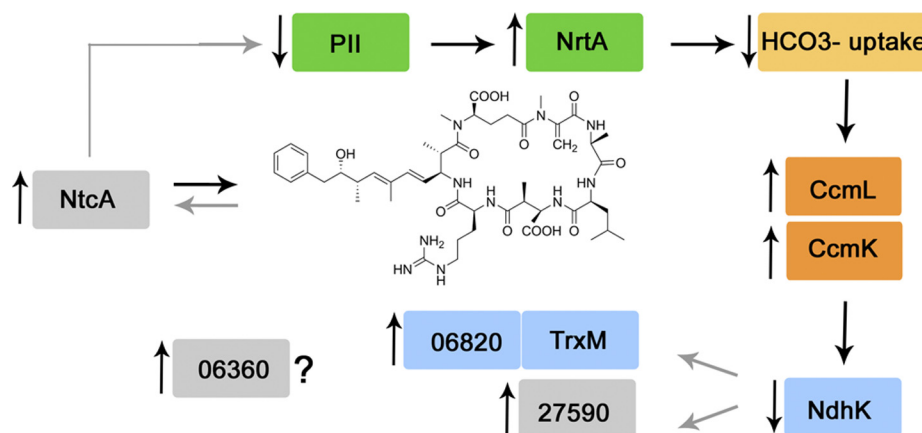
coordinating photosynthetic activity, carbon metabolism and nitrogen assimilation, because of its dependence on the availability of ATP, 2-oxoglutarate (2OG) levels and competition with the global nitrogen regulator NtcA for binding to the P_{II} interaction protein X (PipX) (56, 57) (Fig. 7). This complex post-transcriptional regulation of P_{II} may be the reason for the lack of correlation between the expression and transcription data for *glnB* observed here (Table V). Nevertheless, its decreased abundance in the toxic *M. aeruginosa* was consistent with the observed up-regulation of the nitrate transporter NrtA in the same strains (Table V, Fig. 7). Such reciprocal relationship between P_{II} and NrtA is the result of the regulation of both the *glnB* and *nrtA* genes by NtcA, and has been reported previously (58). The differential expression of P_{II} between toxic and nontoxic strains is interesting, given the fact that the *mcy* gene cluster also appears to be regulated by *ntcA* (59), and may suggest a regulatory process of microcystin synthesis, involving the P_{II}-NtcA couple. The observed expression patterns of P_{II} and NrtA are indicative of a high 2OG level in the cell as a result of a high C:N metabolic ratio, and the following activation of NtcA and up-regulation of nitrogen assimilation (60). Unfortunately, NtcA expression was at a level below the detection limit of the proteomic approach used here and the levels of the NtcA protein could not be determined directly, but *ntcA* transcription did not change significantly. Thus, the differential regulation of these proteins may be the result of

increased NtcA activity, rather than levels of expression, being modulated by microcystin (Fig. 7).

The nitrate ABC transporter, of which NrtA is a subunit has an unusual structure that is shared with members if the cyanobacterial cyanate (Cyn) and bicarbonate (Cmp) transporters (61). Both NrtA and CmpA are inhibited by high levels of nitrate, providing one of the links between carbon and nitrogen metabolism (61). In addition, P_{II} has been shown to regulate carbon transport and respond to the carbon fixation rate in the model cyanobacterium *Synechocystis* sp. PCC 6803 (62, 63). In this microorganism, P_{II} control results in immediate decrease of nitrate transport, when CO_2 fixation is inhibited or CO_2 supply is limited (62). However, a ΔP_{II} mutant expressed high-affinity carbon transporters, even in the presence of inorganic carbon (63). Thus, the decreased abundance of the P_{II} protein in the toxic strains studied here, is expected to affect both nitrate and carbon metabolism, resulting in enhanced carbon fixation in microcystin-producing cells.

Carboxysome Proteins—Carbon fixation in cyanobacteria occurs in specialized compartments, the carboxysomes. In β-cyanobacteria these are formed by a multifaceted protein shell, comprised of repeated hexamers of the homologous *carbon concentrating mechanism proteins CcmK1–4, as well as CcmL, a pentamer that forms pores at the vertices of the icosahedral shell and allows flux of metabolites in and out of the carboxysome (64, 65). The protein shell encases the en-

zymes Rubisco and carbonic anhydrase, which are involved in extraction of CO_2 from HCO_3^-, and thus, the final stages of carbon fixation (66). From the data presented here, it appears that the nontoxic strains down-regulated the expression of two shell subunits, CcmK3 and CcmL. It is likely that this was the result of an increase in carboxysome numbers in toxic strains, rather than a change in the carboxysome size (in which case the expression level of CcmL would remain constant). However, this was not reflected by a change in expression of the internal components of the carboxysome, such as Rubisco and carbonic anhydrase, suggesting that at the time of harvesting the cells, the toxic strains contained several empty carboxysomes.

In cyanobacteria, such anomalies in carboxysome structure have been observed previously by electron microscopy in high-carbon requiring mutants (67). This hypothesis is consistent with the observed down-regulation of P_{II}, causing an increase in NrtA, and nitrate uptake, with subsequent inhibition of bicarbonate uptake in microcystin producers. Interestingly, of the four CcmK homologs, which were all expressed in the strains studied here, only CcmK3 was differentially regulated based on toxicity. The contribution of CcmK3 to the structure of the shell has not been studied. However, changes in the carboxysome shell composition have been proposed to influence the permeability of this compartment to metabolites and could be dependent on the carbon status of the cell (68). At the transcript level, *ccmL* was down-regulated in nontoxic strains, consistent with expression data, but unexpectedly, *ccmK* transcription appeared to be up-regulated. Such discrepancies in the transcription and expression of carboxysome genes have been reported previously in carbon-limited cells of *Synechocystis* sp. PCC 6803, and have been accounted to a yet unknown mechanism of post-transcriptional regulation (68). It is possible that NtcA is involved in the expression of both *ccmK* and *ccmL* genes to some extent (69).

In terms of the relationship between carbon-fixation and microcystin synthesis, immunogold labeling experiments have shown that the toxin is sometimes localized in proximity to carboxysomes and the possibility of the molecule acting as a inhibitor for Rubisco in carbon-limiting conditions has been discussed (20). However, the relationship between carboxysomes and microcystin is not exclusive, as the toxin is also found close to the thylakoid membranes, polyphosphate bodies and in the nucleoplasm (20, 70). The association of microcystin with the thylakoids, in particular under different light regimes, as well as its proposed ability to bind divalent metal ions have lead to hypotheses that it could be involved in the general oxidative stress response of *M. aeruginosa* (20, 71).

Proteins Involved in Redox Balance Maintenance—NdhK is an essential subunit of the NADH dehydrogenase complex, similar in function to the NAD(P)H: quinine oxidoreductase (NDH-1) in mitochondria and eubacteria. This enzyme is involved in accepting electrons from reduced plastoquinone,

respiration and CO_2 uptake (72). This multitude of functions is possible because of the presence of several forms of the complex containing alternative D and F subunits, as well as an accessory carbon uptake (CUP) domain (73, 74). The substrate specificity of the cyanobacterial NDH-1 complex is still unclear, with NADH, NADPH, and reduced ferredoxin being considered as possibilities (72). The NdhK subunit is a soluble protein involved in connecting the membrane and peripheral domains of all NDH complexes and is present both in the thylakoid and plasma membranes (75). It is unclear how the changed expression of this particular NDH subunit would influence the metabolism of *M. aeruginosa*, but an n*dhK* knock-out strain of *Synechocystis* sp. PCC 6803 demonstrated a decreased ability of cells to accumulate inorganic carbon (76). Such a response, where NdhK expression is low in toxic strains, but CcmK and CcmL proteins are highly expressed is consistent with the expected high-carbon requiring phenotype of microcystin-producers discussed above.

Thioredoxin M expression was found to be elevated in toxic cells and further supports a difference in the mechanism for coping with alteration in the redox status of microcystin-producing and nontoxic cells. Thioredoxins are small redox-active proteins, which in cyanobacteria receive electrons from the photosynthetic chain via ferredoxin and ferredoxin-dependent thioredoxin reductase. In high light conditions, NADPH levels increase, leading to an increase in reduced thioredoxin and oxidized target proteins, which are then deactivated and thus protected from oxidative stress (77). In *Synechocystis* sp. PCC 6803 thioredoxins interact with and modulate the activity of proteins involved in CO_2 fixation, glycolysis, nitrogen metabolism and the oxidative stress response (78, 79). These processes are all tightly linked to photosynthetic activity, and accordingly, thioredoxin is placed under transcriptional regulation in response to light, where dark or photoinhibitory light intensities inhibit thioredoxin transcription (80).

The hypothetical protein MAE06820 encoded by the open reading frame adjacent to thioredoxin M was found to follow the TrxM expression pattern. The two open reading frames are separated by a conserved region of 34 nucleotides in both published genomes of *M. aeruginosa* PCC 7806 and NIES-843 and are cotranscribed, as shown on Fig. 4. This apparent coregulation at both the transcriptional and expression level suggests a thioredoxin or redox-related function of MAE06820. Interestingly, none of the homologs of MAE06820 in other cyanobacteria are encoded in open reading frames proximal to thioredoxin, suggesting that this protein may have a more divergent function in *M. aeruginosa*. Another hypothetical protein (MAE27590) was also found to be down regulated in nontoxic strains. The role of this protein has not been established yet, although it contains a putative flavin mononucleotide-binding domain and as such, may also be involved in redox balance or photosynthesis. The differential expression of TrxM and the TrxM-associated hypothetical protein in

the toxic and nontoxic strains studied here may be the result of altered NdhK activity and thus, changes in photosynthetic electron flow. The increase in TrxM levels in toxic strains would lead to oxidation of its target proteins and protection in oxidative stress, which may explain why toxic strains seem to be better competitors in iron limitation or high light, processes that lead to the formation of reactive oxygen species.

NtcA Regulation and Microcystin Synthesis—Five of the proteins that were differentially expressed, have been identified as targets of the global nitrogen regulator NtcA in cyanobacteria. These include NrtA, P_{II}, CcmK, CcmL, and TrxM (69). The expression of MAE06820 is also likely to be influenced by NtcA activity when it is cotranscribed with TrxM. In conjunction with P_{II}, NtcA is able to either activate or suppress transcription and links photosynthesis and nitrogen metabolism in cyanobacteria (69). The general response of the toxic strains observed here, with an increase in nitrate transporter expression because of lower levels of P_{II}, increased carboxysome shell subunits, low NdhK expression and higher thioredoxin/MAE06820 expression suggest that toxic strains would have higher carbon requirements and increased NtcA activity (Fig. 7).

NtcA generally acts on its own promoter as a positive regulator, hence it was surprising to find that *ntcA* transcription was lower in toxic strains, relative to nontoxic *M. aeruginosa*. This may be explained by interaction between NtcA with P_{II}, 2-OG levels in the cell and PipX availability. The lower levels of P_{II} in the microcystin-producing cells would make more PipX available for binding to NtcA-DNA complexes, stimulating NtcA activity on promoters recognized by the global nitrogen regulator. On the other hand, the higher NrtA levels and putatively, nitrate uptake, would lead to low levels of 2-OG, inhibiting *ntcA* transcription. In fact, high 2-OG during nitrogen starvation has been shown to stimulate binding of pre-existing low levels of NtcA to one of its own promoters, P_1 in the nontoxic *Anabaena* sp. PCC 7120 (81).

As discussed, the NtcA-binding sites present in the microcystin promoter and the differential expression of several NtcA-regulated genes in toxic and nontoxic strains of *M. aeruginosa* strongly suggests an interaction of the toxin molecule with NtcA. The *mcyS* gene cluster promoter contains binding sites for two other transcription factors, the ferric uptake regulator Fur and the light-responsive Rca. However, the results presented here did not include genes regulated by either Fur or Rca, although interaction and cross-regulation of all three transcription factors with the toxin cluster cannot be excluded. Instead, the data points to an involvement of microcystin in processes that ultimately influence the integration of photosynthesis with carbon and nitrogen metabolism. These could explain previous observations of toxic and nontoxic strains behaving differently under varying light intensities or in nutrient starvation (11, 16, 82, 83). It is important to consider that the results from this study reflect protein expression in the exponential growth phase and in nutrient-replete conditions. It is highly likely that many more proteins would be differentially expressed under different nutrient and light regimes, in addition to proteins that could contribute to strain-specific adaptation in a particular environment regardless of the ability to synthesize microcystin.

Induction of Toxicity in an Inactive Microcystin Producing Strain—Two of the strains used in this analysis, UWOCC MRC and UWOCC MRD were chosen because of their 16S rRNA gene sequence identity and the reported inability of UWOCC MRC to produce microcystin (84). However, during this study UWOCC MRC was found to have reverted to toxicity and was able to produce the commonly identified MCYST-LR and [DAsp³]MCYST-LR not found in its parent strain UWOCC MRD (Fig. 6 and Table VI). The rare microcystin isoforms produced by UWOCC MRD (Table VI), do not appear to correlate with the protein expression profile in *M. aeruginosa* strains (supplemental Data 7). In addition, pair-wise nSAF analysis of UWOCC MRC and UWOCC MRD did not reveal differentially expressed proteins that could account for the reversal to toxin synthesis in UWOCC MRC (supplemental Data 8). These findings support previous reports that the toxin isoform is genetically predetermined by the relaxed specificity of the microcystin synthetase aminoacyl adenylation domains, and the activity and presence of the various tailoring enzymes. Nevertheless, a post-transcriptional regulatory process of microcystin synthesis, cannot be excluded in the case of inactive toxigenic strains such as UWOCC MRC, which represent ~3% of the bloom population in *Microcystis* and *Planktothrix* spp (85). This is suggested by the ability of UWOCC MRC to revert back to toxicity, without any associated mutations in the *mcy* cluster or other phenotypic changes. It is possible that point mutations within the cluster may cause premature stop codons. Indeed, several insertions in the *mcy* gene cluster have been reported in *M. aeruginosa* strains, including UWOCC MRC and may affect the transcription of toxin genes (46). These insertions, identified in the promoter region and *mcyB*, could be the result of past transposition events but do not seem to correlate with toxin production in the strain.

A study by Wilson and colleagues (86) speculated that solid media selects for nontoxic strains, whereas liquid media favors toxin production. Because our strains were maintained in liquid BG11, prolonged culturing under these conditions may have induced the toxicity in UWOCC MRC over time, although the actual event that has caused the reversal of UWOCC MRC to toxicity remains unclear. Unfortunately, we have not been able to acquire a nontoxic UWOCC MRC strain, which would enable us to compare its protein expression to its presently toxic counterpart in our laboratory and the parental UWOCC MRD. This would certainly shed light on the regulatory mechanisms governing toxin biosynthesis.

Proteome analysis of *M. aeruginosa* revealed that the strains studied here have highly variable protein expression profiles, which may be a result of the adaptation of these organisms to the particular environmental niche that they

occupy. No qualitative differences were found between toxic and nontoxic strains, and cluster analysis of protein expression did not group strains according to their toxicity. The literature reports that production of microcystin isoforms shifts with changing nutrient conditions (16), and toxic strains have an advantage in low-iron or high light environments. Thus, it can be expected that the culture conditions used here may not be optimal for the segregation of toxic and nontoxic strains based on their protein expression profile. However, differences in the expression of proteins involved in C-N metabolism and cellular redox status identified in the nSAF analysis, suggest a link between toxin production and cyanobacterial primary metabolism. The expression of several of these proteins, as well as microcystin, is regulated by the global nitrogen transcriptional regulator NtcA, and may reflect a cross-regulatory mechanism linking toxin production to NtcA-controlled metabolic processes under nutrient limitation.

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