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Microbial Degradation of Microcystins

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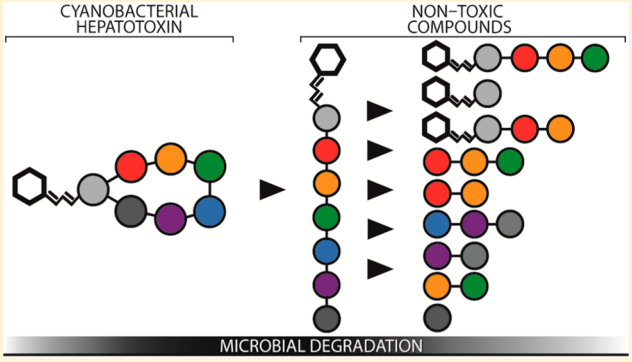
ABSTRACT: Hepatotoxic microcystins that are produced by freshwater cyanobacteria pose a risk to public health. These compounds may be eliminated by enzymatic degradation. Here, we review the enzymatic pathways for the degradation of these hepatotoxins, some of which are newly discovered processes. The efficiencies of microcystin biodegradation pathways are documented in several papers and are compared here. Additionally, a comprehensive description of the microcystin enzymatic degradation scheme has been supplemented with a proposal for a new biodegradation pathway. Critical comments on less documented hypotheses are also included. The genetic aspects of biodegradation activity are discussed in detail. We also describe some methods that are useful for studying the biological decomposition of microcystins, including screening for microcystin degraders and detecting microcystin degradation products, with an emphasis on mass spectrometric methodology.



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1. INTRODUCTION

The increased level of eutrophication of surface water results in frequent occurrences of toxic cyanobacterial blooms, and certain species of cyanobacteria are capable of producing several different toxins with a broad range of targets, predominantly the liver. Cyclic peptide toxins include microcystins (MCs) and nodularins (NODs), which are stable and resistant to chemical hydrolysis or oxidation, even at high temperatures[1] or low pH values.[2] The persistence and detoxification of these hepatotoxins in aquatic environments are important issues for public health, and the promotion of liver cancer at low doses of these toxins is well-known.[3,4] MCs are produced by freshwater cyanobacterial genera such as *Microcystis*, *Anabaena*, *Nostoc*, and *Planktothrix*. The most common representative of these toxins, microcystin-LR (MC-LR), has a cyclo-D-alanine-L-leucine-*erythro*-β-methyl-D-aspartic acid (iso)-L-arginine-Adda-D-glutamic acid (iso)-*N*-methyldehydroalanine structure, where Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Scheme 1a). MC variants contain different amino acid residues at positions 2 and 4, and one-letter symbols are used to define the variants. Other differences are the presence or absence of methyl groups on the methyl-aspartic acid or the methyl-dehydroalanine, the estrification of methyl-aspartic acid or iso-glutamic acid, and other minor modifications. Pentapeptide NODs have a cyclic structure and contain Adda; therefore, the molecular mechanisms of MC and NOD toxicity are the same and are based on the inhibition of protein phosphatases PP1, PP2A, PPP4, and PPP5.[5,6] The inactivation of these proteins changes

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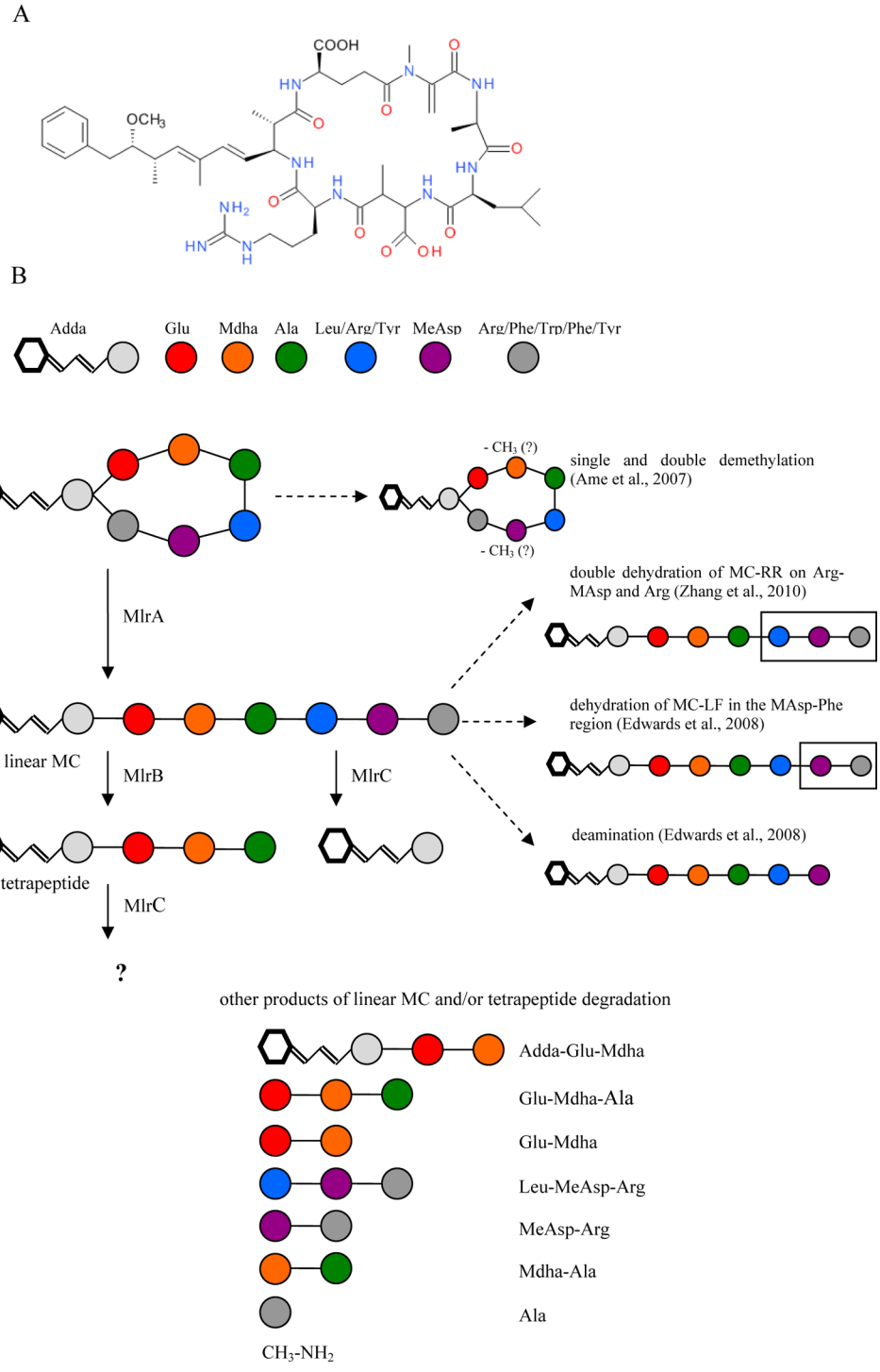
Scheme 1. Structure of MC-LR (A) and a Simplified Scheme of MC Biodegradation Modified According to New Discoveries (B)[a]



[a]Well-documented and hypothesized pathways of MC biotransformation are summarized. The colored circles indicate amino acids that form the structure of MCs. Solid lines indicate the confirmed pathways and known products released by Mlr enzymes, whereas dashed lines indicate incompletely documented reactions that have not been verified. The products at the bottom are not presented in the order of formation.

the homeostatic balance and can cause uncontrolled cell proliferation and cancer development. Liver damage caused by MCs leads to hepatic blood pooling and hypovolemic shock and is eventually lethal in mammals.[7,8] The World Health Organization has supplied a provisional guideline value of 1 μg/L as the highest acceptable concentration of microcystin equivalents in drinking water.[9]

Risk management strategies for cyanobacterial cells and toxins were proposed by Codd et al.[10] However, conventional water treatment methods are inefficient at removing cyanotoxins from raw water,[11] and protection from cyanobacterial toxins should not be limited to the chemical or physical treatment of drinking water. Furthermore, the accumulation of toxins in aquatic organisms, which has been well documented in a range

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Table 1. Summary of the Information about Classes of Microorganisms Involved in MC Utilization, Estimated Efficiencies, and Methods Used To Indicate the MC Biodegradation Capability of Selected Strains

microorganism	methodology	amount of degradation[a]	degradation rate ($\mu g\ L^{-1}\ day^{-1}$)	refs
Confirmed Presence of an *mlrA* Homologue				
Sphingomonas sp. ACM-3962 (AF401172), α-proteobacteria	HPLC and MS, degradation products	80% of MC (100 μg/L) degraded after 6 days	12100[b]	18, 23
Sphingomonas sp. Y2 (AB084247), α-proteobacteria	HPLC, level of substrate	degradation rates of MC-RR and -LR, 13000 and 5400 $\mu g\ L^{-1}\ day^{-1}$, respectively (initial concentration of 20000 μg/L), 6 days for total removal	5400−13000	25
Sphingomonas sp. MD-1 (AB110635), α-proteobacteria	HPLC, level of substrate	–	–	31
Sphingopyxis sp. LH21 (DQ112242)	HPLC, level of substrate	MCs completely degraded within 2 days (3 μg/L), 4 days (10 μg/L), and 12 days (25 μg/L)	1.5−2.5	36
Sphingopyxis sp. C-1, α-proteobacteria	HPLC and MS, detection of the products	–	–	37
Bacillus sp. AMRI-03 (GU294753), bacilli	ELISA and PP inhibition assay	degradation of MC-RR (10 mg/L) fully completed within 5 days after a lag phase of 2 days	2000	45
sand filters	HPLC with SPE and PP2A, level of substrate	MC- LR completely removed within 4 days (6 μg/L) and 3 days (20 μg/L)	1.5−6.7	80
Stenotrophomonas sp, EMS (FJ712028), γ-proteobacteria	HPLC, two unknown products detected	MCs (∼1200 μg/L) completely consumed within 1 day	1200	47
Sphingopyxis sp. USTB-05 (EF607053), α-proteobacteria	HPLC, degradation products	MC-RR (42300 μg/L) completely degraded within 10 h by cell extract	101520	38, 39, 83[c]
Novosphingobium sp. THN1 (HQ664117), α-proteobacteria	HPLC, level of substrate	91% of MC-LR (1400 μg/L) eliminated over period of 12 h, not detected after 2.5 days	2548	75
Sphingopyxis sp. TT25 (JQ398614), α-proteobacteria	HPLC with SPE, level of substrate	MC (10 μg/L) completely removed within 4 h	60	86
Close Homology to *Sphingomonas* but Unknown Presence of an *mlrA* Homologue				
Sphingomonas 7CY (AB076083), α-proteobacteria	HPLC, level of substrate	MCs (6000 μg/L) completely degraded after 4 days	1500	27
Sphingomonas sp. B9 (AB159609), α-proteobacteria	HPLC and MS, degradation products	–	–	28, 65[d]
Sphingomonas sp. CBA4 (AY920497), α-proteobacteria	HPLC, level of substrate and peaks of products detected; MS	almost complete degradation of MC-RR (200 μg/L) within 36 h; calculated half-life of MC, 18 h	120	70
Sphingomonas sp. MDB2 (AB219940) and MDB3 (AB219941), α-proteobacteria	–	–	–	87
Other Species/Samples				
P. aeruginosa, γ-proteobacteria	HPLC, level of substrate	95% of MC-LR (50000 μg/L) degraded in 3 weeks	2381	22
aggregated microorganisms consisting of biofilm	HPLC, level of substrate	MC-LR (1000 μg/L) degraded by indigenous aquatic bacteria on biofilm over 5 days	200	88
Ralstonia solanacearum, β-proteobacteria	HPLC, level of substrate	MC-RR (52500 μg/L) and LR (29500 μg/L) decreased to 2000 and 0 μg/L in 1 day, respectively	29500−50500	42, 43
biofilms dominated by *Oocystis lacustris* and *Protoderma* sp., chlorophyta	HPLC, level of substrate	half-times of MC (160 μg/L) removal ($t_{1/2}$), 20 h	96	55
Paucibacter toxinivorans, β-proteobacteria	PP inhibition assay	maximal rate of MC degradation, 4−16 $\mu g\ L^{-1}\ h^{-1}$ (2−30 $fg\ cell^{-1}\ h^{-1}$)	96−384	40
Poterioochromonas sp., Synurophyceae	ELISA	level of MC-LR decreased from 1050 to 20−30 μg/L in 5 days	204−206	63, 64
bacterial species not identified	HPLC and MS, degradation products	degradation of MC (1000 μg/L), half-lives from 4 to 18 days	28−125	69
Burkholderia sp. DQ459360, β-proteobacteria	HPLC and ELISA, level of substrate	the highest average degradation rate of MCs, 50 $\mu g\ L^{-1}\ day^{-1}$	50	41
Methylobacillus sp. J10 (FJ418599), β-proteobacteria	HPLC, level of substrate	MC-LR (3500 μg/L) and MC-RR (4500 μg/L) completely degraded within 17 h	4941−6353	46
Arthrobacter spp., *Brevibacterium* sp., *Rhodococcus* sp., actinobacteria	Biolog MT2 assay	total degradation of MC-LR (5000 μg/L) within 3 days	1667	44, 89[e]
isolates related to *M. morganii*	HPLC and ELISA, level of substrate	35% of MC (100 μg/L) degraded within 6 days	5300	49
Microbacterium sp., actinobacteria; *R. gallicum* (AY972457), α-proteobacteria	HPLC, level of substrate	up to 74% of MC (250 μg/L) degraded within 20 days (consortium consisting of *Microbacterium* sp. and *R. gallicum*)	9.4	48
Trichaptum abietinum 1302BG	HPLC and toxicity test using *Vicia faba* root tip micronucleus assay	MC-LR (40 μg/L) removed completely within 12 h	80	56

[a]Among different results from cited works, the most representative and/or the highest value of MC degradation is indicated (not for direct comparison). [b]Degradation rate of this strain calculated by Eleuterio and Batista.[49] [c]Detected *mlrA* in the investigated strain. [d]Degradation products detected by HPLC. [e]Presence of *mlrA* analyzed but not detected (confirmed lack of *mlrA*).

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of aquatic macrophytes and fauna,[12,13] presents the potential risk of transference of toxins to humans through the food chain.[14] The bioelimination of toxic cyanopeptides is an option for conventional water treatment.[15] Thus, investigating MC biodegraders is crucially important.[15−17]

2. MICROCYSTIN-DEGRADING ORGANISMS

MCs are resistant to enzymatic hydrolysis by common proteases.[18−20] However, several MC degraders have been identified in bacteria, primarily *Proteobacteria*, in environmental samples, and isolates from phylogenetically distinct branches of the bacterial kingdom can degrade MCs. Suspected degraders must be isolated and identified and have their activity confirmed. Amplifying the gene encoding 16S rRNA using the polymerase chain reaction (PCR) method, sequencing the amplicon, and comparing the obtained sequence with dedicated databases facilitate identification of the genus. Species identification is possible when there is sequence agreement with the sequence of an already known representative of the species. This identification is of special importance because MC degraders are not phylogenetically consistent.

Jones and Orr[18] identified the first known MC-degrading bacterium. Subsequently, Jones et al.[21] isolated a bacterium from surface water and documented that it was able to degrade MC-LR and MC-RR, but not NOD. The isolated strain was a Gram-negative rod, which appeared to be a *Pseudomonas* sp., although standard taxonomic tests gave inconclusive results. MC degradation was found to occur primarily intracellularly and began 2−8 days after the addition of toxin. No lag phase was observed when MC-LR was added to the water again. In 1996, Takenaka et al.[22] isolated a *Pseudomonas aeruginosa* strain that was able to degrade MC in a 3 week experiment. After MC had been incubated in *P. aeruginosa* culture medium, demethylated Adda was observed to be a product of MC decomposition. In an essential study by Bourne and collaborators,[23] the MC-degrading bacterium described by Jones et al.[21] was reclassified as a new *Sphingomonas* species (ACM-3962). The authors suggested that MC degradation was an enzymatic process of at least three steps[23] and identified the gene cluster involved in MC degradation (*mlrA*, *mlrB*, *mlrC*, and *mlrD*).[24]

A number of other *Sphingomonas* strains have been isolated from water reservoirs. The Y2 strain[25] was shown to degrade MCs (i.e., the MC-RR, MC-YR, and MC-LR variants) more efficiently than the ACM-3962 strain; additionally, no lag phase was observed. *Sphingomonas* strain 7CY[26,27] was documented to be capable of degrading several other MC variants (i.e., MC-LR, MC-LY, MC-LW, and MC-LF). Moreover, Harada et al.[28] reported that the *Sphingomonas* B9 strain degraded MC in a manner described previously[23,24] and that the Adda moiety was detected among the products. Further studies of the B9 strain[29] described the degradation pathways of a variety of peptide cyanotoxins.

Several strains, including Y2 and MD-1, were investigated with respect to the distribution of the *mlrA* gene, which encodes the most important enzyme for MC degradation,[30,31] and the authors showed that the ability to degrade MCs is related to the presence of this gene. An *mlrA* homologue was found in *Bacillus* spp.[32] and in other microorganisms.[33,34] It was concluded that *mlrA* was conserved in at least three different bacterial species and that it was unique to MC degraders but not to the genus *Sphingomonas*.[31]

Ho et al.[35,36] isolated and identified a bacterium capable of degrading MC-LR and MC-LA. Phylogenetic analysis of the 16S rRNA gene sequence revealed that the isolated LH21 organism was classified in the genus *Sphingopyxis* and carried the gene homologues previously associated with *Sphingomonas* strains. Okano et al. isolated and characterized an alkali-tolerant MC-degrading bacterium (C-1) that also belonged to the genus *Sphingopyxis*.[37] Further research identified yet another *Sphingopyxis* sp. strain (USTB-05) with relatively strong MC degrading ability.[38,39]

Some recognized MC degraders from the family Sphingomonadaceae (α-proteobacteria) are closely related and possess homologues of *mlrA*. However, other bacteria identified as MC degraders have been isolated from many water reservoirs, particularly those containing toxic cyanobacterial blooms. In a study by Rapala et al., 13 bacterial strains that were capable of degrading cyanobacterial hepatotoxins (MCs and NODs) were isolated from lake sediment.[40] Genomic characterization of these strains indicated that they were a single microdiverse genospecies most closely related to *Roseateles depolymerans*, and on the basis of the phylogenetic and phenotypic features, a novel genus and species (named *Paucibacter toxinivorans* gen. nov., sp. nov.) was proposed. A bacterium isolated from estuarine and coastal water samples from the Patos Lagoon region in Brazil[41] was 96% homologous with the *Burkholderia* genus, which belongs to the β-subdivision of proteobacteria. This was the first report indicating that bacteria from the genus *Burkholderia* were cyanobacterial toxin degraders, and different β-proteobacteria (*Ralstonia solanacearum*) were also recently reported as MC degraders.[42,43]

Other bacterial species were shown to be capable of MC degradation, including *Arthrobacter* spp., *Brevibacterium* sp., and *Rhodococcus* sp. (belonging to Actinobacteria),[44] the strain AMRI-03 closely related to the genus *Bacillus*,[45] and strain J10 isolated from Lake Taihu, which was tentatively identified as *Methylobacillus* sp.[46] Chen et al. described an EMS strain similar to *Stenotrophomonas maltophilia*, which was the first report of MC-degrading bacteria in the genus of the γ-division of proteobacteria that carry the *mlrA* gene.[47] Recently, two isolates that are capable of MC degradation, i.e., DC7 and DC8 from Lake Okeechobee, Florida, were classified as *Rhizobium gallicum* and *Microbacterium* sp., respectively.[48] Other examples of bacteria with this ability are highly similar to *Morganella morganii* and *Pseudomonas* sp.[49]

Interestingly, probiotic bacteria have been proposed as alternative microorganisms that are capable of removing cyanotoxins,[50−53] of which the strains *Lactobacillus rhamnosus* GG and LC-705 and *Bifidobacterium longum* 46 were shown to be the most effective. However, these strains were unable to completely remove MCs, as opposed to many of the Sphingomonadaceae, which degraded MCs to concentrations below detection limits. A recent study has provided evidence that cell wall-associated proteinases could be involved in the removal of MC.[54] Although the mechanism remains unknown, these experiments indicated that a significantly different biological process of cyanotoxin removal may exist in nature, which suggests another pathway of MC elimination.

Furthermore, both heterotrophs and organisms from river-originating phototrophic biofilms have been shown to be effective in MC degradation,[55] especially those with previous contact with cyanobacteria. Similarly, *Trichaptum abietinum*, a white rot fungus, was suggested as an effective MC degrader in

Table 2. Summarized List of MS Data of Mother Ions of MC Variants and Their Biotransformation Products

MC variant	m/z ion	refs
MC-LR	995.4 $[M + H]^+$	23
	135.0 $[PhCH_2CHOMe + H]^+$	
acyclo MC-LR Adda-Glu-Mdha-Ala-Leu-Masp-Arg-OH	1013.7 $[M + H_2O + H]^+$	23, 29, 37, 65, 69, 71, 90
	862.5 $[M - NH_2 - PhCH_2CHOMe + H]^+$	
	135.0 $[PhCH_2CHOMe + H]^+$	
	507 $[M + H_2O + 2H]^{2+}$	
	571.2 $[Mdha\text{-}Ala\text{-}Leu\text{-}Masp\text{-}Arg + H]^+$	
tetrapeptide Adda-Glu-Mdha-Ala-OH	615.3 $[M + H]^+$	23, 29, 65, 68
	598 $[M - NH_2 + H]^+$	
	135.0 $[PhCH_2CHOMe + H]^+$	
Adda	663.4 $[2M + H]^+$	28, 29, 67, 68
	332.3 $[M + H]^+$	
	315.2 $[M - NH_3 + H]^+$	
MC-RR	1038.7 $[M + H]^+$	39, 65, 70, 71, 83, 90
	905.6 $[M - NH_2 - PhCH_2CHOMe + H]^+$	
	519.9 $[M + 2H]^{2+}$	
	453.5 $[M - 133 + 2H]^{2+}$	
acyclo MC-RR Adda-Glu-Mdha-Ala-Arg-Masp-Arg-OH	1056.5 $[M + 18 + H]^+$	39, 65, 83
	529.1 $[M + 18 + 2H]^{2+}$	
acyclo dmMC-RR	528.9 $[M + 2H]^{2+}$	71
	453.5 $[M - NH_2 - PhCH_2CHOMe + 2H]^{2+}$	
acyclo MC-LW Adda-Glu-Mdha-Ala-Arg-Masp-Trp-OH	1043.6 $[M + H]^+$	71
	892.4 $[M - NH_2 - PhCH_2CHOMe + H]^+$	
MC-LF	986 $[M + H]^+$	69
	968 $[M - H_2O + H]^+$	
acyclo MC-LF Adda-Glu-Mdha-Ala-Arg-Masp-Phe-OH	1004.6 $[M + H]^+$	65, 69, 71
	853.5 $[M - NH_2 - PhCH_2CHOMe + H]^+$	
	856 $[NH_2\text{-}Adda\text{-}Glu\text{-}Mdha\text{-}Ala\text{-}Leu\text{-}MeAsp\text{-}OH + H]^+$	
MC-YR	135.0 $[PhCH_2CHOMe + H]^+$	90

the algal culture of *Microcystis aeruginosa*,[56] but no biochemical pathway of such fungal activity has been proposed.

Finally, we may assume that the growing number of isolates with MC biodegrading abilities does not significantly enhance our general understanding of the mechanisms and mutual relations between the strains involved. Table 1 presents the classification of known degraders and compares their reported degradation efficiencies. The real significance of the ability to utilize cyanotoxins is still unclear, both in the context of enhanced competitiveness of microorganisms and for our understanding of the impact on the final concentration of MCs in the environment.

3. HETEROTROPHIC MICROORGANISMS THAT COEXIST WITH HEPATOTOXIC CYANOBACTERIA AS A POSSIBLE SOURCE OF MC DEGRADERS

Heterotrophs, which coexist with hepatotoxic cyanobacteria, may constitute other less-explored sources of MC-degrading microorganisms. Interactions between bacterial communities and algae or cyanobacteria are well-known and influence the compositions of both communities. The coexistence of phototrophic and heterotrophic species thus benefits each partner; e.g., CO_2 or N_2 fixation products are transferred from cyanobacteria to attached bacteria.[57] Metabolic relationships between *Microcystis* and the attached bacteria have been observed, for example, the phosphorus exchange between *Mi. aeruginosa* and *Pseudomonas*.[58] Hepatotoxic cyanobacteria, such as *Microcystis* spp., naturally coexist with certain species of bacteria by inhabiting their extracellular mucus zones. As suggested by Casamatta and Wickstrom,[59] bacterial species that coexist with *Mi. aeruginosa* are attracted by cyanobacterial exudates. MC-degrading bacteria were detected in mucilage of *Microcystis*.[60] A comparison of several studies[25,41,61] revealed that the same group of bacteria were found to be closely associated with *Microcystis* spp. and are able to degrade toxic secondary metabolites produced by this group of cyanobacteria. The bacteria closely associated with *Microsystis* spp. are also present in *Nodularia* blooms from the Baltic Sea,[62] which are known producers of NOD, a hepatotoxin that is similar to MCs. These studies indicate that *Microcystis* spp. may use nutrients delivered by bacteria, whereas heterotrophic organisms may utilize the organic carbon produced by phototrophic cyanobacteria and MCs as an organic substrate.

Other mechanisms of MC utilization have been observed in species that are *Microcystis* grazers. Ou et al.[63] and Zhang et al.[64] described such an ability in a eukaryotic *Poterioochromonas* sp. that was isolated from *Microcystis* cultures and belongs to the Chrysophyceae. The growth of *Poterioochromonas* sp. was stimulated in the presence of MCs. The strain was able to degrade MCs, which was the first evidence of MC degradation by algae. However, the detoxification mechanisms were not identified.

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4. ENZYMES INVOLVED IN MC DEGRADATION

The first proposal of MC biodegradation mechanisms was described by Bourne et al.[23] On the basis of an MS analysis of MC-LR that was incubated with an MC-degrading bacterial species, a linear MC-LR (protonated molecular ion at m/z 1013) and a tetrapeptide (protonated molecular ion at m/z 615) were identified as degradation products (Table 2). The MC degradation pathway was described as a sequential, three-step process. The authors suggested that the linearization reaction, as catalyzed by MlrA, was related to the cleavage of the Adda−Arg peptide bond (in the case of the studied MC-LR). Subsequent tetrapeptide formation results from the hydrolysis of the Ala−Leu peptide bond in the acyclic MC-LR molecule, and this reaction is catalyzed by MlrB. The resulting tetrapeptide is then further decomposed by MlrC; however, the products of this reaction have not been identified. The proposed pathway, which involves the sequential action of three enzymes, has been confirmed by several authors.[15,28,65,66] However, as recently indicated by Dziga et al.[67] and independently confirmed by Shimizu et al.,[68] MlrC is able to hydrolyze not only the tetrapeptide but also the linear MC. The process described above may occur with no previous processing of acyclic MC by MlrB, and Adda is the main product of such MlrC activity. Other products of MC degradation were documented by Imanishi et al.,[65] Edwards et al.,[69] and Hashimoto et al.,[66] but the overall fate of MC derivatives remains unknown. MlrB seems to be specific for the Ala−Leu bond of linear MCs because the tetrapeptide was identified as a hydrolysis product of only those MC variants, which have Leu at position 2. Conversely, it may be hypothesized that MlrC has a relatively low specificity and may hydrolyze several peptide bonds of linear MC molecules.

It was suggested that enzymes other than proteases are involved in MC degradation,[69] and the typical reactions, other than proteolysis, that microbes utilize to degrade organic pollutants, such as decarboxylation and demethylation reactions, were proposed. Indeed, the unique mechanism of the *Sphingomonas* sp. CBA4 strain for demethylating MC-RR was proposed by Ame et al.[70] However, this hypothetical process has never been experimentally confirmed. New possible reactions involved in MC-RR degradation were proposed by Zhang et al.[39] using the bacterial species *Sphingopyxis* sp. USTB-05.[39] In this study, the authors concluded that the peaks observed via MS were from the modified MC-RR that lost two H_2O molecules as a result of internal cyclization, which occurred with the participation of two arginine residues. This assumption, however, needs further experimental verification. Similarly, a single dehydration in the Phe-MeAsp region of MC-LF was proposed by Edwards et al.[69] On the basis of the new discoveries described above, the previous MC degradation pathway should be verified; however, several of the recent proposals must be better documented (Scheme 1b).

On the basis of the sequence similarity, the enzymes involved in the canonical degradation pathway were recognized as metalloproteases (MlrA and MlrC) and a serine protease for MlrB. This finding was initially verified using the dedicated protease inhibitors and was later confirmed.[23,24] The proposed active site of MlrA, which could be a variant of the zinc-binding motif (HEXXH), was found in a number of metalloproteases. The postulated active center, $H^{260}AIH^{263}NE^{265}$,[24] was positively verified by site-directed mutagenesis.[71] The constructed point mutants, MlrAH260A and MlrAE265A, were inactive toward MC-LR,[71] and the inhibition patterns of EDTA and *o*-phenanthroline (typical inhibitors of metalloproteases) were confirmed using purified recombinant MlrA.[71] MlrA is a neutral protease, with optimal function at pH 7.6 and a range of activity from pH 6.5 to 9.5, which exhibits positive cooperativity with MC-LR, and the half-saturation constant ($K_{0.5}$) is in the micromolar range.[71] It was suggested that MlrA might function as a dimer under experimental conditions.[71] The activity of MlrC was abolished by EDTA and *o*-phenanthroline; however, there were no conserved metalloproteinase motifs identified in the MlrC sequence. MlrB bears a strong sequence similarity to members of the penicillin-recognizing enzyme family with the conserved sequence Ser-Xaa-Xaa-Lys and to a number of β-lactamases;[24,68] however, such activity of MlrB has not been documented.

There is no clear answer regarding the location of MlrA in the cells. The presence of a 26-amino acid signal sequence directing the protein to the periplasmic space suggests that this enzyme is a periplasmic protein,[24] whereas a homology analysis (using BLAST) indicates a similarity to the members of the CAAX amino-terminal protease family that are membrane proteins.[72] Computational studies using the DAS-TM filter server[73] predicted that the protein with six transmembrane α-helices is located in the membrane and that the enzyme active center is exposed to the periplasm.[71]

5. EFFICIENCY OF DEGRADATION

Because of the different MC variants and the concentrations used, the diverse physicochemical conditions, the various densities of the investigated bacterial culture, and the different methodologies used, it is difficult to compare the efficiencies of MC biodegradation among different strains. However, we may conclude that the rate of this process varies significantly among investigated strains and water samples and is relatively slow when compared to that of the chemical treatment. Several authors have estimated that the complete biodegradation of MCs (usually at concentrations close to those observed during cyanobacterial blooms) would take at least a few hours, and usually more than 24 h (Table 1).

It would be preferable to propose a uniform and simple algorithm for calculating the biodegradation ability. To compare the efficiency of different strains, the most useful information is the rate of forming linear MC or any other primary product, considering the initial toxin concentration and the density of the culture or inoculum. Conversely, in the context of application studies, a more suitable demonstration would be one that showed the amount of degraded MC per volume per time (e.g., micrograms of MC per liter per day), which could be easily compared to the efficiency of chlorination or other chemical treatment procedures.[49] A comparison of the biodegradation rate of *Sphingomonas* ACM-3962 and *M. morganii* was made in this manner.[49] Additionally, the differences between the biodegradation capability and/or rates of several MC variants[69,74] provide useful information in the context of the biochemical characterization of enzymes that are involved in the biodegradation process and their specificity against different variants of hepatotoxins, including nodularin.

6. GENETIC ASPECTS OF MC DEGRADATION

It has been documented that the process of removal of MC by the Sphingomonadaceae is driven by proteins that are encoded in a gene cluster designated as *mlrABCD*.[24] By cloning the total

genomic DNA of *Sphingomonas* sp., we found a 5.8 kb sequence responsible for proteolytic activity. Four genes are sequentially located on the cluster: *mlrC*, *mlrA*, *mlrD*, and *mlrB*. The two genes in the middle, *mlrA* and *mlrD*, are transcribed in the forward direction, and *mlrC* and *mlrB* are transcribed in the reverse direction. Genes *mlrA*, *mlrB*, and *mlrC* encode a metallopeptidase (responsible for the linearization of MCs), a serine protease, and a metalloprotease, respectively. The *mlrD* gene encodes a putative transporter protein that may allow for the active transport of MCs and/or its degradation products into or out of the cell. The putative amino acid *mlrA* sequence, containing 336 residues,[24] was deposited in GenBank (entry AF411068) and was amended by Dziga et al.[71]

Further studies have confirmed the existence of *mlr* cluster components in other MC-degrading bacteria. Ho et al.[36] identified homologues of four *mlr* genes in *Sphingopyxis* sp. LH21 and provided sequences of noncoding fragments between the open reading frames (ORFs). This gene cluster is present in *Novosphingobium* sp. THN1, but the *mlrB* gene has frameshift mutations and is transcriptionally silent.[75] Similarly, a homologous gene cluster was also detected in *Sphingopyxis* sp. C-1.[37] The sequence of the *mlrB* gene is identical to that of the former, suggesting that it is a pseudogene. However, the ORF for MlrB has recently been amended. Contrary to previous suggestions, using a heterologous expression system showed that the *mlrB* gene in *Sphingopyxis* sp. C-1 encodes a functional protein.[68] Moreover, the gene undergoes expression in the native host because a respective transcript has been detected.[68] Nonetheless, the inactivation of *mlrB* does not influence the ability of the strains to efficiently degrade MCs because MlrC has a similar function, which was described above.

Soon after the discovery of the MC degradation enzymatic pathway, it became evident that the product of the *mlrA* gene is a key protein that initiates the process and generates a virtually nontoxic linear semifinished product. Therefore, in many studies, the genetic basis of MC degradation has been limited to the detection of a genomic *mlrA* gene in the degrader. Such an approach was aided by the high degree of conservation of the *mlrA* gene sequence and the development of a method for detecting the gene using PCR.[31] Using this method, the presence of the *mlrA* gene has been confirmed in newly discovered MC-degrading strains (Table 1). Manage et al.[44] were unable to amplify *mlrA* from the genomes of *Arthrobacter* sp., *Brevibacterium* sp., and *Rhodococcus* sp., indicating either significant differences in the sequence of *mlrA* or a lack of the gene, which in turn might suggest an unknown mechanism of MC removal. Another conclusion may be drawn from a comparison of the occurrence of the *mlrA* gene in highly phylogenetically related species that either possess or lack the ability to remove MCs.[31] As might be expected, the former carry *mlrA*, whereas the latter do not; this finding indicates that the ability to degrade MCs is encoded in auxiliary genetic material rather than in the core genome. Moreover, though dendrograms built from the *mlrA* gene and the 16S rRNA gene sequences are mostly congruous, indicating that *mlrA* has co-evolved with the core genome,[75] there are apparent examples of the existence of *mlrA* homologues in phylogenetically distant bacteria, such as *Stenotrophomonas* sp. EMS47 and *Bacillus* sp. AMRI-03,[45] which indicates a recent gene transfer event. Unfortunately, the current lack of whole genomic sequences, or even of the flanking regions of the *mlr* gene cluster from a single MC-degrading strain, leaves open the question of whether the ability to degrade MCs is encoded on a mobile genetic element.

7. DEPENDENCE OF MLRA PRODUCTION ON A CARBON SOURCE

Ishii et al.[27] and Alamri et al.[45] reported that *Sphingomonas* 7CY and AMRI-03, respectively, can degrade MCs in both minimal and complete media containing a nitrogen and a carbon source and suggested that the bacterial enzymes involved in MC degradation are produced constitutively. Conversely, Christoffersen et al.[77] found a significant correlation between the decomposition of organic compounds and the degradation of MCs, which suggests that these processes are related. Several reports indicate that lag periods in MC biodegradation could be significantly reduced and even eliminated if bacteria were pre-exposed to MCs, as the degradation efficiency was found to be higher if the bacterial species originated from water with previous cyanobacterial blooms.[36,55,76−80] However, Edwards et al.[69] found that the ability to degrade MCs does not appear to be dependent on prior exposure, which suggests that different microbes with specific degradation capabilities exist in different habitats. It has been suggested that microorganisms require adaptation to the cyanobacterial metabolites that contain various organic compounds, including MCs, and that this adaptation increases the effectiveness of MC degradation.[77,79]

The different kinetics of removal of MC by bacteria have been evaluated in terms of the presence of MCs, their degradation products, and various nutrients in the environment. Unfortunately, only a few studies link such data with the expression of the gene encoding the enzyme that is involved in MC degradation. Jiang et al.[75] showed that the *mlrA* gene is upregulated upon being exposed to MC-LR in the *Novosphingobium* sp. THN1 strain. The addition of MC to the bacterial culture resulted in a 2−3-fold higher transcript level during the first 90 min, with the highest transcription level occurring at 45 min. Li et al. compared the level of *mlrA* and 16S rDNA gene expression in a complex bacterial consortium that forms a biofilm following exposure to various nutrients, such as nitrate, ammonium, and glucose.[81] The authors observed that an enhanced inhibition in biodegradation correlated with the increased level of inhibition of *mlrA* expression as the nutrient concentration increased. Conversely, the stimulation of biodegradation at low nitrate concentrations correlated with a more rapid increase in the abundance of the *mlrA* gene. This finding, however, reflects changes in the relative abundance of the *mlrA* gene carrying strains in a bacterial population rather than changes in the expression of the gene itself.

Ho et al.[82] indicated that removing MC-LR from wastewater closely followed the abundance of the *mlrA* gene, suggesting that this process influences the number of MC-LR-degrading organisms. The presence of MC-LR resulted in an increase in the abundance of *mlrA* to more than 2500 copies/mL, whereas the *mlrA* gene reached a plateau at approximately 500 copies/mL in the absence of MC-LR. This result demonstrates that MC-LR may be a primary substrate for facilitating the proliferation of MC-degrading bacteria. Recently, the expression of *mlrA*, *mlrB*, and *mlrC* in response to the occurrence of MC-LR and its degradation products in the culture medium of *Sphingopyxis* sp. C-1 was quantified. The expression of all of the genes was upregulated with MC-LR, but only the expression of *mlrA* and *mlrB* was stimulated by linear MC-

LR, tetrapeptide, and Adda. Thus, *mlrC* regulation is distinct from that of the two former genes.[68]

Unfortunately, recent progress in our understanding of MC degradation pathways in bacterial strains carrying the *mlr* gene cluster stands in marked contrast to a virtual lack of data concerning the genetic basis of other possible mechanisms of removal of MC by strains that appear to lack such genes. Undoubtedly, it would be of interest to discover the factors involved in the degradation of MCs in these bacteria, such as *Arthrobacter* sp., *Brevibacterium* sp., *Rhodococcus* sp., and probiotic bacteria.

Until recently, experiments with MC-degrading enzymes have been conducted using the viable cells of isolated native strains or crude extracts of such cells, with only one exception, the work of Bourne et al.,[24] who used cell extracts of *Escherichia coli* transformed with variants of the *mlr* gene cluster of *Sphingomonas* sp. Apart from *mlrD*, whose product has not been identified and whose role remains speculative, the three remaining products of the *mlr* gene cluster have recently been recombinantly expressed and initially characterized.[67,68,71,83] Such an approach has several advantages, such as controlling particular steps of MC degradation, investigating the biochemistry of the analyzed proteins, more easily recognizing future products, and verifying the degradation pathways. Because of the low level of Mlr enzyme production in natural hosts, heterologous expression seems to be the best way to test the properties of these important proteins.

8. ASSAYS FOR HEPATOTOXIN-DEGRADING BACTERIA

Earlier studies that were based on traditional experimental protocols required the collection of water or sediment samples as a natural source of microorganisms. Isolated microbial strains were screened (most often by HPLC and MS methods) to determine their capacity to hydrolyze hepatotoxin bonds, and genetic characteristics of isolated strains were sometimes examined. After a relatively thorough characterization of *Sphingomanas* strains (including gene sequences), a promising alternative approach has been developed, i.e., searching environmental samples for *mlrA* homologues, whose presence in the genome of investigated bacterial strains indicates the ability to degrade MC variants.[31,36,84,85]

In 2003, Saito et al.[31] successfully designed a PCR assay for detecting *mlrA* homologues, which was primarily documented for the *Sphingomonas* sp. ACM-3962 strain. On the basis of this technique, the authors reported homologues of *mlrA* in two strains of *Sphingomonas* sp., MD-1 and Y2, which were previously isolated from Japanese lakes and were shown to be MC-degrading bacteria. Similarly, Bourne et al. applied PCR and fluorescent *in situ* hybridization techniques to detect *mlrA* in *Sphingomonas* strain MJ-PV.[84] These methods were reported to be useful for studying the distribution of MC-LR-degrading strain MJ-PV in seeded bioremediation trials. Several authors have recently developed and used PCR assays to screen for the presence of the genes involved in MC degradation and their homologues, particularly for *mlrA*, among naturally occurring strains.

In addition to the conventional PCR method, Hoefel et al. developed an *mlrA* gene-directed TaqMan PCR assay to identify variations among *mlrA* homologues and for the rapid quantitative assessment of MC-degrading bacteria based on the accurate quantification of *mlrA* abundance.[85] This modified assay, in combination with 16S rDNA-directed quantitative PCR and denaturing gradient gel electrophoresis, allows a correlation to be found between *mlrA* abundance and the efficiency of MC removal.

Manage et al. have proposed a new assay for investigating MC degradation ability using plates containing tetrazolium redox dye, which forms a purple product if the carbon source (here, MC) is oxidized (Biolog MT2 screen).[44] Another potential method for investigating MC biodegradation is the detection of CO_2, which is the final product of MC mineralization.[78,79] However, this method requires using a ^{14}C-labeled toxin and does not verify partial biodegradation, which practically eliminates toxicity.

Despite the existing simple protocols that employ HPLC and MS methods, some authors have used other, less suitable assays to determine the MC degrading capacity of newly investigated microorganisms that involve monitoring only the MC level, with no detection of degradation products (Table 1). It is extremely important to note that the real documentation of enzymatic hydrolysis is possible only if the degradation products are detected and recognized because the simple monitoring of MC levels does not exclude other, nonenzymatic interactions of hepatotoxins with cells or cell extracts. Therefore, in several cases, the enzymatic character of MC degradation may be controversial, especially when the incubation takes several days. In some reported studies, no degradation products were found, but other indirect evidence of biological and/or enzymatic action was demonstrated. Nybom et al. documented that only viable probiotic cells removed MCs efficiently.[51] The evidence of a correlation between the proteolytic activity and MC removal capacity, as well as the inhibition of MC removal in the presence of inhibitors, of investigated enzymes also supported the enzymatic character of MC degradation.[54] Oxygen limitation may also be used to verify the biological potency of an investigated strain.[46] In the case of water sample analysis, an important approach is using sterile controls to quantify the possible abiotic losses, which may be the consequence of physical and chemical degradation and/or absorption. Additionally, it is relatively easy to confirm the enzymatic hydrolysis of MCs by the detection of *mlrA* homologues,[37] but their presence should also be supported by evidence of MlrA activity.

9. MS-BASED METHODOLOGY

The main analytical technique used to study MC degradation is reversed phase (RP) C-18 HPLC with absorption and/or MS detection. The Adda residue is lypophilic and contains a diene chromophore with a characteristic maximum at approximately 238 nm, which allows the detection, via HPLC, of linearized MC and other fragments of the MC molecule containing Adda. Mass spectrometry allows the detection of degradation products based on the m/z ratio (Table 2). This method may be useful in the enzymatic hydrolysis of MCs and predicting the pathway of their degradation. Recently, Hashimoto et al.[66] proposed an advanced Marfey method for detecting MC degradation products without a diene as the chromophore, which requires the derivatization of amino acids or peptides. Subsequently, compounds with an added chromophore can be detected either spectrophotometrically or using the putative m/z value with separation under the usual reversed phase conditions. Additionally, to confirm the results, an index of hydrophobicity has been used to predict the retention time of the derivatives.[66]

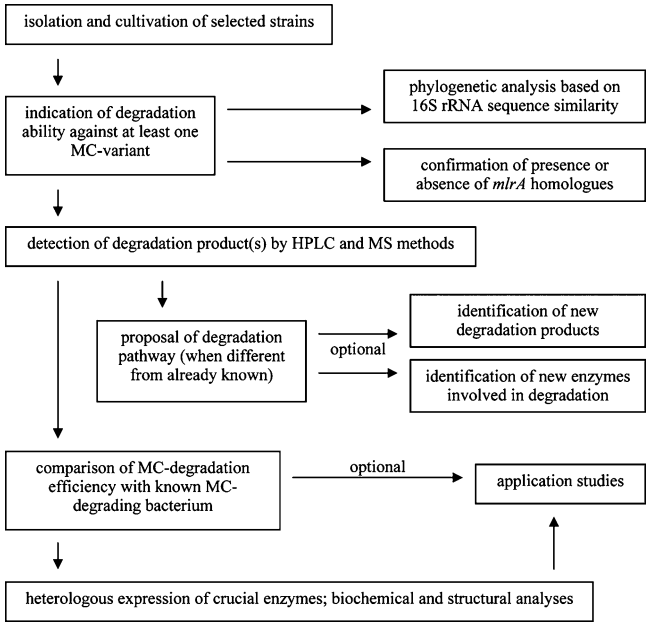
Linear forms of MCs are products of hydrolysis and have an m/z ratio that is increased by 18. However, Edwards et al.[69] and Dziga et al.[71] indicated that linear MCs, aside from peaks with expected m/z values, may exhibit m/z peaks that are reduced by 151 because of the loss of a portion of the Adda and amino groups. These peaks may have higher intensities than the mother ions. For example, the MS spectrum of MC-LR hydrolyzed by MlrA contains an additional peak at m/z 862.5. This ion was first reported by Bourne et al. as one of the MS/MS daughter ions (MC-LR + H − NH_2 − $PhCH_2CHOMe$) of linear MC-LR in MS/MS fragmentation.[23] The ionized residue $PhCH_2CHOMe$ can be observed with an ion at m/z 135 and is often used to track any Adda-containing compounds (Table 2).

The fragmentation patterns of different MC byproducts have been presented by Imanishi et al.[65] Unusual and unexpected m/z values may be identified in this way, including the m/z 663 ion, which was recognized as a dimeric ion of the Adda molecule ($[2M + H]^+$).[65,67] In addition, ions that are products of the removal of different groups from MC (e.g., ammonia, methyl group, water, or even amino acids) should be verified by MS fragmentation to confirm their origin from biodegradation byproducts.[65,69] Only a few studies have presented MS data on further degradation products that originated from linear MC hydrolysis. These studies provide information about the fragmentation pattern of Adda,[65] MS spectra of known intermediates,[28,68] or several new intermediate peptides as other degradation products.[37,69] However, the direct formation of such intermediates during enzymatic reaction must be confirmed using a method other than MS. For example, the demethylation described by Ame et al.[70] revealed novel intermediate degradation products of MC-RR without the detection of respective peaks by HPLC. Additionally, describing demethylation as part of biodegradation is questionable, especially because such a small change does not reduce the toxicity of the hypothesized product. Similarly, Zhang et al.[39] suggested that the dehydration of MC is an enzymatic process that triggers biodegradation. The MS detection of such new products is insufficient for documenting novel biodegradation intermediates because the identified ions may be a result of ionization processes or other nonbiological modifications that occur during an MS analysis. The proposal of a new degradation pathway, especially one that does not involve the sequential hydrolysis of peptide bonds, requires confirmation by HPLC that indicates specific peaks on the chromatogram and, in the next step, identifies the enzymes that catalyze such hypothetical reactions.

10. CONCLUSIONS

The main exposure route of humans to MCs is thought to be oral, and removing MCs from drinking water is thus of great importance. However, conventional water treatment methods are not efficient in cyanotoxin removal, and protection from cyanobacterial toxins should not be limited only to the chemical or physical treatment of drinking water. Therefore, an alternative approach should be developed to provide additional options for MC removal. In the past few years, several new publications have enhanced our knowledge regarding the biodegradation of MCs. As this review indicates, it is evident that many of the cyanobacterial metabolites are susceptible to biodegradation in water supplies. New groups of microorganisms that have different MC utilization capabilities have been discovered. Some of these microorganisms act through proteolytic hydrolysis, which is initiated by the linearization of MC, but the overall fate of degradation products is still unknown. The genetic background and enzymatic character of MC decomposition processes are also relatively unknown. Conversely, several newly discovered strains with MC removal potential require future research to confirm known degradation pathways and/or to propose and document alternative mechanisms.

Scheme 2. Proposed Scheme for Essential Research That Should Be Conducted To Broaden Our Knowledge of Known and New MC-Degrading Bacteria



Methodologies vary significantly among the cited papers, and further investigation of some proposed pathways of degradation is needed. Confirming the enzymatic character of MC biodegradation requires the recognition of degradation products and the characterization of the enzymes involved in the catalysis of subsequent reactions, as these facts are crucial for obtaining reliable documentation as a basis for further studies. A plan for subsequent experiments is proposed in Scheme 2, but this procedure is not exhaustive. New proposals of original methods and ideas are welcome; in particular, experiments that compare the MC degradation efficiencies of different microorganisms would be useful in the context of biotechnological applications and the construction of MC removal systems that are based on microorganisms.

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ABBREVIATIONS

MC, microcystin; Mdha, methyldehydroalanine; NOD, nodularin; ORF, open reading frame; PCR, polymerase chain reaction

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