Cloning and expression of first gene for biodegrading microcystins by *Sphingopyxis* sp. USTB-05

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Harmful cyanobacterial blooms (HCBs) in natural waters are a growing environmental problem worldwide[1] because microcystins (MCs) produced by cyanobacteria are potent hepatotoxins and tumor promoters[2]. MCs are resistant against physical and chemical factors[3,4]. Thus, biodegradation is the most efficient method for removing MCs, and a number of bacterial strains, especially genus *Sphingomonas*, have been isolated for biodegrading MCs[5–13]. Although the pathway, enzyme, and gene for biodegrading MCs by *Sphingomonas* sp. have been widely identified recently[14–18], no gene concerned with the biodegradation of MCs has been successfully cloned and expressed. In this study, we show that the first and most important gene of *mlrA*, containing 1,008 bp nucleotides in length, in the biodegradation pathway of MCs by *Sphingopyxis* sp. USTB-05, which encodes an enzyme MlrA containing 336 amino acid residues, is firstly cloned and expressed in *E. coli* DH5α, with a cloning vector of pGEM-T easy and an expression vector of pGEX-4T-1. The encoded and expressed enzyme MlrA is responsible for cleaving the target peptide bond between 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda) and Arg in the cyclic structure of microcystin-RR (MC-RR) and microcystin-LR (MC-LR), two typical and toxic types of MCs. Linear MC-RR and MC-LR are produced as the first products. These findings are important in constructing a new genetic bacterial strain for the efficient removal of MCs from the important water supplies and resolving the controversy on the biodegradation pathway of different types of MCs by genus *Sphingomonas*.

With increased wastewater discharge containing nitrogen and phosphorus to rivers and lakes, HCBs have become more frequent worldwide. This has led to the destruction of the natural ecological system and production of cyanobacterial toxins such as MCs. MCs produced by *Microcystis*, *Anabaena* and *Nostoc* are the most dangerous toxins to humans[19,20]. MCs are monocyclic heptapeptides with Adda

responsible for the hepatotoxicity of molecules. More than 70 microcystin isoforms are found in part due to the variable L-amino acids X and Z. The most frequent and studied variants are MC-LR and MC-RR. MCs have been studied extensively, not only for their ability to cause acute poisonings, but also due to their cancer promotion potential by chronic exposure at low concentrations in drinking water. Since the first report of animal death caused by drinking water containing cyanobacteria in 1878, the poisoning and death of aquatic animals, birds, and cattle have been frequently reported. A toxic incident leading to the deaths of over 50 people occurred in Brazil in 1996 due to MCs in the water used for hemodialysis[21]. In 1998, the World Health Organization established a guideline value of 1 μg/L as the maximum concentration of MC-LR in drinking water.

MCs are chemically stable compounds, thus, conventional drinking water treatments have limited efficacy in removing MCs[22, 23]. MCs can be readily biodegraded by a range of aquatic bacteria. In 1994, a microcystin-degrading bacterium identified as *Sphingomonas* sp. was isolated from Australian water bodies[6]. Afterwards, several bacterial strains of *Sphingomonas* sp.[12,13,15], *Pseudomonas aeruginosa*[24], *Stenotrophomonas* sp.[25], *Sphingosinicella* sp.[26], *Burkholderia* sp.[27], *Arthrobacter* spp[28]., *Brevibacterium* sp.[28], and *Rhodococcus* sp.[28] from different areas have been shown to biodegrade MCs. Only *Sphingomonas* sp. has been widely investigated and found to have a strong ability in biodegrading MCs. In 2004, we isolated a MCs-biodegrading bacterial strain tentatively identified as *Ralstonia solanacearum* from the sediment of eutrophicated Dianchi Lake in China. Both MC-RR and MC-LR could be rapidly removed by using carbon nanotubes embedded with *R. solanacearum*[5]. Furthermore, we isolated and identified another promising bacterial strain of *Sphingopyxis* sp., USTB-05, by the analysis of 16s rDNA (GenBank database under accession number: EF607053) for biodegrading MCs. Initial MC-RR of 42.3 mg/L was completely biodegraded by USTB-05 within 36 h, and its cell-free extract (CE) within 10 h. Presently, this is the highest reported biodegradation rate of MC-RR[13].

To identify the biodegradation pathway of MCs by *Sphingomonas* sp., three enzymes were found involved in sequentially biodegrading MC-LR[14,15]. The initial site of hydrolytic cleavage of MC-LR by microcystinase is at the Adda–Arg peptide bond, and linear MC-LR produced as a first product. The first enzyme, microcystinase, appears to be the most important because it opens the highly stable cyclic peptide, leading to a 160-fold reduction in the activity of parent MC-LR. The second enzyme of serine protease is responsible for the conversion of linearized MC-LR to tetrapeptide NH_2-Adda-Glu (iso)-Mdha-Ala-OH. The third enzyme of peptidase is responsible for dividing tetrapeptide into each amino acid. Previous studies have performed cloning and gene library screening of *Sphingomonas* sp. strain and detected the microcystin-degrading gene cluster, *mlrA*, B, C, and D. The enzyme encoded by the *mlrA* gene can cleave the Adda-Arg peptide bond in MC-LR. After opening of the cyclic structure, linear MC-LR is degraded by the peptidases encoded by *mlrB* and *mlrC*, and divided into each amino acid. The *mlrD* encodes the transporter protein that allows the uptake of MCs into the cell. Previous analysis of

the gene homologues and their deduced enzymes from the strain ACM-3962, Y2, and MD-1 [6,9,29] showed that the first step to degrading MC-LR is cleaving the Adda-Arg peptide bond. The biodegradation pathway of MC-RR has not been studied extensively, except for reports of demethylating MC-RR[10] as a first step and three products being derived from MC-RR catalyzed by the enzymes of USTB-05[13]. Although researchers have verified that the enzymes encoded by genes are very important in investigating the biodegradation pathway of MCs, the lack of knowledge in the heterologously expressed microcystinase hinders further studies. In order to determine the relationship among the gene, enzyme, and product of MCs biodegradation, we successfully cloned and expressed the first gene of USTB-05 for biodegrading MC-RR and MC-LR (Supplementary Table 1; Supplementary Method 1). Analysis shows that cDNA clone of *USTB-05-A* is 1,008 bp in length (GeneBank accession number: HM245411, Supplementary Figures 1, 2, and 3) and the similarity of nucleotide acid sequences between *USTB-05-A* and *mlrA* deposited in Genebank is 92.5% (Supplementary Figure 4). The deduced amino acid sequence encoded by *USTB-05-A* has 336 identical residues, which have the similarity of 80% identicals and 83% positives to the translated amino acid sequence encoded by *mlrA* (Supplementary Figure 5). The sites of alanine and leucine found at the 26thand 27th position of encoding protein in *USTB-05-A* are responsible for cleaving the cyclic peptides of MCs[14]. The first enzyme encoded by *USTB-05-A* has the original function and the fundamental structure for degrading MCs.

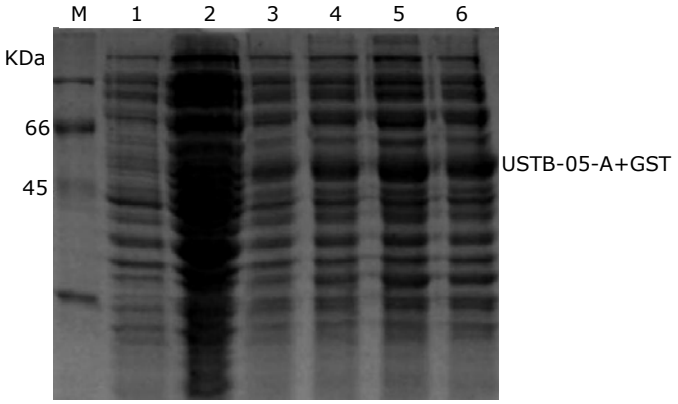

Figure 1| **SDS-PAGE analysis of protein encoded by *USTB-05-A***

Lane M—molecular weight standards (from the top): bovine serum albumin (66.0 KDa), ovalbumin (45.0 KDa); lane 1: positive control of total proteins before induction; lane 2: negative control of *E. coli* DH5α proteins; lane 3-6: four parallels of total proteins after induction;

We expressed *USTB-05-A* in prokaryotic *E.coli* DH5α (Supplementary Method 2). The target protein with a GST label has a molecular weight (MW) between 66 and 45 KDa (Figure 1). After deducting MW (25 KDa) of GST, the MW of protein encoded by *USTB-05-A* is approximately 36 KDa. The expressed target protein with a GST label has an enzymatic activity in biodegrading MCs (Figure 2).

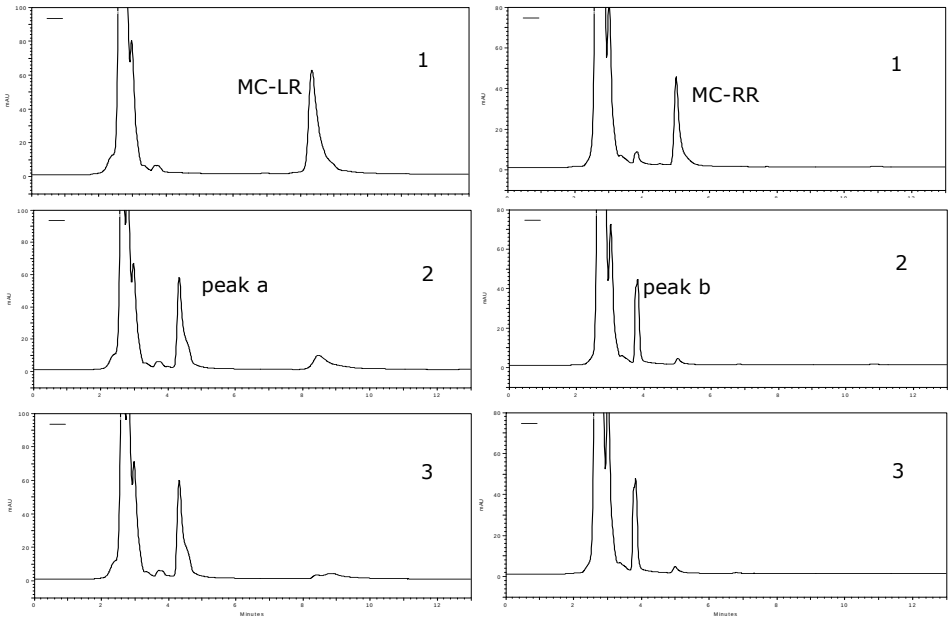

Figure 2 | HPLC profiles for the biodegradation of MCs by crude protease encoded by *USTB-05-A* with time course. (from top to down): (1) 0 min; (2) 10 min; (3) 60 min; lane left represents HPLC profiles of MC-LR biodegradation; lane right represents HPLC profiles of MC-RR biodegradation

To confirm the enzymatic activity of expressed protease, we prepared the cell-free extraction, including the expressed protease from *E. coli* DH5α containing recombinant *USTB-05-A*, to biodegrade MC-LR or MC-RR (Supplementary Method 3). The retention times of MC-RR and MC-LR peaks are at 5.00 min and 8.34 min, respectively (Figure 2). As for MC-LR, a new peak at retention time of 4.34 min appears and increases. In contrast, MC-LR peak decreases with time (Figure 2 left) indicating that the encoded protein by *USTB-05-A* has the enzymatic activity, and peak a is the first product of MC-LR. Compared to MC-LR, MC-RR showed similar characteristics, and peak b at a retention time of 3.81 min is the first product of MC-RR (Figure 2, right; Supplementary Figure 9, right). The expressed protease encoded by *USTB-05-A*, which has been initially constructed and expressed in *E.coli* DH5α, is capable of degrading both MC-LR and MC-RR.

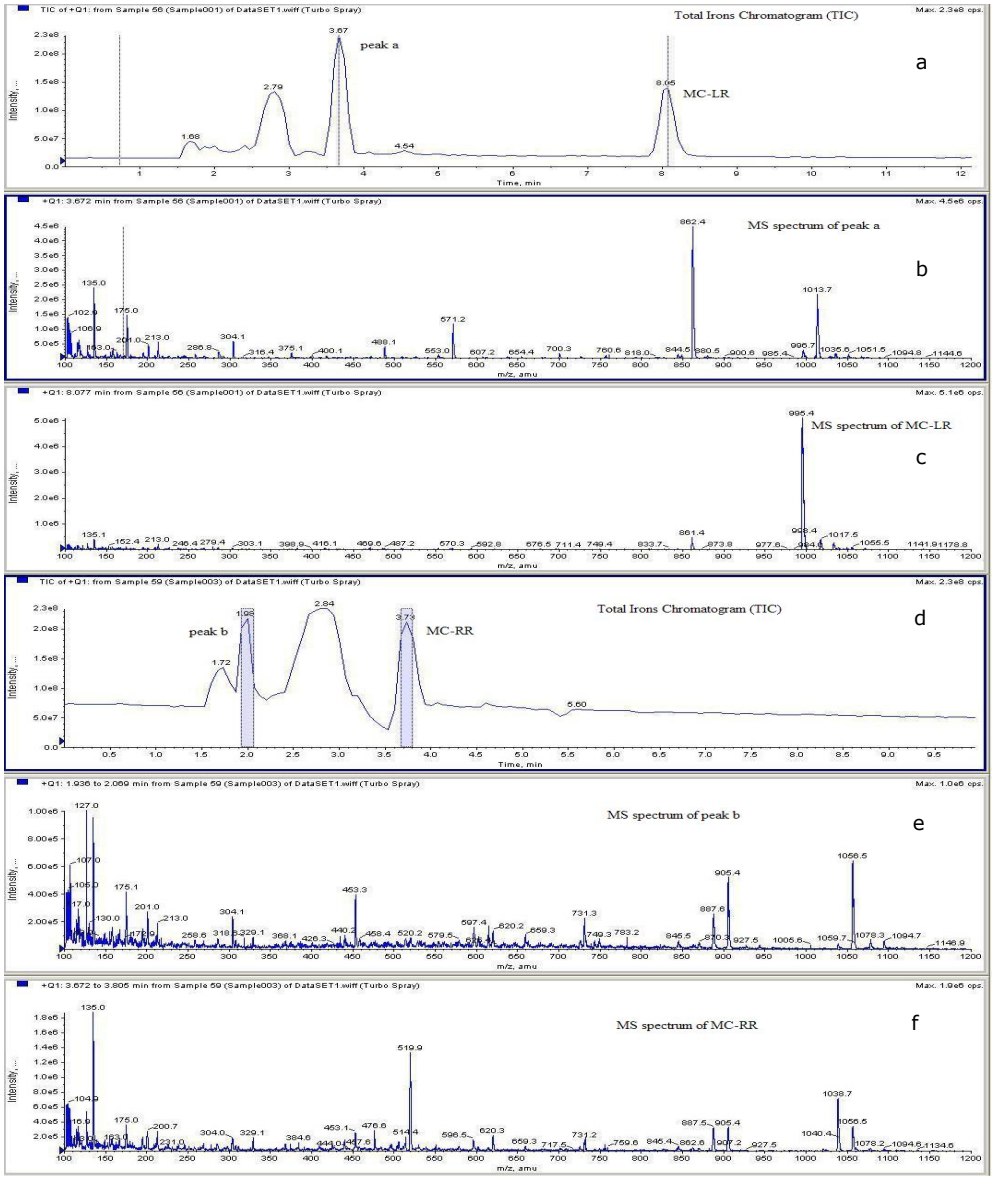

Figure 3 | LC/MS profiles of MCs and its biodegradation products.

(a) Total ion chromatogram (TIC) of MC-LR and its product; (b) MS spectrum for peak a; (c) MS spectrum for MC-LR; (d) total ion chromatogram (TIC) of MC-RR and its product; (e) MS spectrum for peak b; (f) MS spectrum for MC-RR.

To identify the biodegradation products of MCs, LC-MS was performed to measure mass charge ratios of both MCs and their products (Supplementary Method 4). Based on the retention time and UV chromatogram in HPLC profiles, the third and fourth peaks marked with vertical bar in the total ion chromatogram (TIC) correspond to MC-LR (retention time 8.05 min) and peak a (retention time 3.67 min) (Figure 3a). The MS spectrum of MC-LR reveals a protonated molecular ion at m/z 995.4, corresponding to the protonated molecular ion $[M+H]^+$ (Figure 3c). The MS spectrum for peak a (retention time 3.67 min) shows four ion peaks at *m/z* 135.0, 571.2, 862.4, and 1013.7 (Figure 3b). Based on a previous report[15], the peaks at *m/z* 135.0 and *m/z* 571.2 represent the $PhCH_2CHOMe+H^+$ and

Mdha-Ala-Leu-Masp-Arg+H^+, and a peak at *m/z* 862.0(M-151) corresponds to the loss of terminal phenylethyl-methoxy group from Adda (M-135). The presence of this ion is indicative of the linear microcystin product containing N-terminal Adda. A peak at *m/z* 1013.7($[M+18+H]^+$) is essential to the first step in the pathway of MC-LR biodegradation because this ion represents the first product derived from MC-LR.

For MC-RR, the second and fourth peaks marked in the column in TIC correspond to MC-RR (retention time 3.73 min) and peak b (retention time 1.96 min) (Figure 3d). The MS spectrum for MC-RR (Figure 3f) shows two major ion peaks at *m/z* 519.9 and *m/z* 1038.7, corresponding to protonated molecular ions $[M+2H]^{2+}$ and $[M+H]^+$, respectively. The MS spectrum for peak b reveals four ion peaks at *m/z* 1056.9, 905.6, 453.5, and 135.0 (Figure 3e). Due to the different degrees of protonation, ion peak at *m/z* 1056.9 represents the first biodegradation product, corresponding to the protonated molecular ion $[M+18+H]^+$. Other ion peaks at *m/z* 905.6 and 453.5 represent the protonated molecular ion $M+H^+$-NH_2-$PhCH_2CHOMe$ $[M-135+H]^+$ and $M+2H^+$-NH_2-$PhCH_2CHOMe$ $[M-135+2H]^{2+}$[15], respectively.

Both first products of MC-RR and MC-LR have higher MW of 18 compared with those of parents (Figure 3). This may have resulted from MC-RR and MC-LR opening the Adda-Arg bond (Figure 4a) and combining one hydrogen on the NH_2 group of Adda and one hydroxyl on the carboxyl group of Arginine. This proves that MC-RR and MC-LR have been converted to linear MC-RR and linear MC-LR (Figure 4b). Previous studies have revealed that Adda-Arg bond of MC-LR is easily attacked by enzymatic degradation with many bacteria[14–16]. However, the first biodegradation products of another important typical variant of MC-RR have not been previously identified. Here, we inferred that *Sphingopyxis* sp. USTB-05 has the same first step in the biodegradation of MCs by opening the Adda-Arg bond (Figure 4a) and combining one hydrogen on the NH_2 group of Adda and one hydroxyl on the carboxyl group of Arginine. Linear MCs are produced as their first products.

Biodegradation is one of the essential processes for the reduction of MCs in natural eutrophic lakes and reservoirs. Based on the successful isolation of a promising bacterial strain of *Sphingopyxis* sp. USTB-05, the first gene, which encodes an enzyme MlrA containing 336 amino acid residues, is successfully cloned and expressed in *E. coli* DH5α. The encoded and expressed enzyme MlrA is responsible for cleaving the target of peptide bond between Adda and Arg in MCs rings, and the linear MC-RR and linear MC-LR are produced as the first products. This study is very important in understanding the pathway and mechanism of the biodegradation of MCs. Presently, the cloning and expression of second and third genes for biodegrading MCs are being studied.

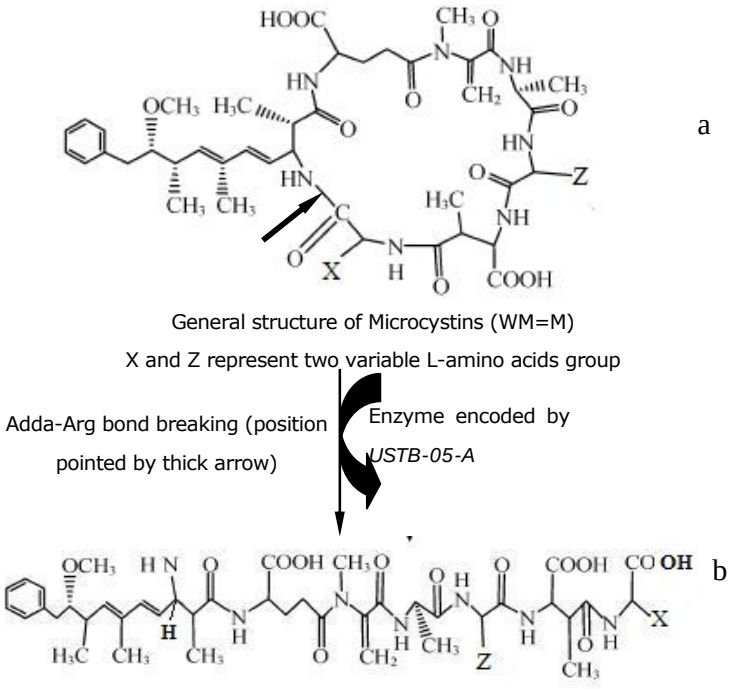

Figure 4□The first identified step on pathway of biodegrading MCs (tow typical variants: MC-LR and MC-RR) by the first enzyme encoded by *USTB-05-A*. The hydragen (OH) and hydroxyl (H) added when Adda-Arg bond breaking were boldfaced respectively.

METHODS SUMMARY

The upstream and downstream primers were designed and synthesized before PCR. The constructed cloning vector pGEM-T easy/*USTB-05-A* was transferred into the *E.coli* DH5α for copying. The positive clones were obtained to subsequence through the bio-information analysis software package Vector NTI 10.0. The genomic DNA and plasmid are extracted according to Genomic DNA extraction kit (Tiangen Biotech Co., Ltd.) and small plasmid extraction kit (Bio Basic, Inc.), respectively. The recombinant plasmid pGEX-4T-1/USTB-05-A was constructed and then transferred into *E.coli* DH5α to express target protease.

In order to determine the enzymatic activity of expressed protease, the cell-free extraction of recombinant *E.coli* DH5α was added in phosphate buffer solution (pH 7.0) at initial MC-RR or MC-LR of 40 mg/L and samples were taken at different reaction times for HPLC analysis. The enzymatic activity was based on the removal rate of MCs.

With the decrease of MCs peaks, the product peaks appeared and increased in HPLC profiles. The samples of products were collected and fully characterized using LC-MS.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgments We thank H-H.Zheng for technical assistance. This work is funded by National Nature Science Fundation of China (203777008), State Key Joint Laboratory of Environment Simulation and Pollution Control (09K08ESPCT), and Fundation of University of Science and Technology Beijing.

Authors contributions H.Y and W.W designed this study; J-F.W and J.C cloned, expressed and characterized the enzyme; J-F.W and J.C performed enzymatic activity, collected the data and performed bioinformatic analysis; J-F.W, J.C and H.W performed the analysis of LC-MS; H.Y, J-F.W and J.C analyzed the data and wrote the paper. All authors discussed the results and prepared the manuscript.

Authors information