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

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Abstract

Harmful cyanobacterial blooms are a growing environmental problem worldwide in natural waters, the biodegradation is found to be the most efficient method for removing microcystins (MCs) produced by harmful cyanobacteria. Based on the isolation of a promising bacterial strain of *Sphingopyxis* sp. USTB-05 for biodegrading MCs, we for the first time cloned and expressed a gene *USTB-05-A* (HM245411) that is responsible for the first step in the biodegradation of microcystin LR (MC-LR) in *E. coli* DH5α, with a cloning vector of pGEM-T easy and an expression vector of pGEX-4T-1, respectively. The cell-free extracts (CE) of recombinant *E. coli* DH5α containing *USTB-05-A* had high activity for biodegrading MC-LR. The initial MC-LR concentration of 40 mg/L was completely biodegraded within 1 hr in the presence of CE with a protein concentration of 0.35 mg/mL. Based on an analysis of the liquid chromatogram-mass spectrum (LC-MS), the enzyme encoded by gene *USTB-05-A* was found to be active in cleaving the target peptide bond between 3-amino-9-methoxy-2,6, 8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda) and arginine of MC-LR, and converting cyclic MC-LR to linear MC-LR as a first product that is much less toxic than parent MC-LR, which offered direct evidence for the first step on the pathway of MC-LR biodegradation by *Sphingopyxis* sp. USTB-05.

Key words: microcystin LR; *Sphingopyxis* sp. USTB-05; biodegradation; gene

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Introduction

With increased wastewater discharge containing nitrogen and phosphorus into rivers and lakes, harmful cyanobacterial blooms have become more frequent worldwide. This has led to the destruction of the natural ecological system and production of cyanobacterial toxins such as microcystins (MCs). MCs produced by *Microcystis*, *Anabaena* and *Nostoc*, which are the most dangerous of these toxins to humans (Figueiredo et al., 2004; Ibelings and Chorus, 2007), are monocyclic heptapeptides, with 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid (Adda) responsible for the hepatotoxicity of the 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 microcystin LR (MC-LR) and microcystin RR (MC-RR). MCs have been studied extensively, not only for their ability to cause acute poisonings (Ding et al., 2006; Xie, 2009), but also for their cancer promotion potential from chronic exposure at low concentrations in drinking water (Chen et al., 2002; Hu et al., 2002). 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 water used for hemodialysis (Azevedo et al., 2002). In 1998, the World Health Organization established a guideline value of 1 μg/L as the maximum concentration of MC-LR in drinking water.

Microcystins are chemically stable compounds and conventional drinking water treatments have limited efficacy in removing MCs (Lahti et al., 1997; Svrcek and Smith, 2004), while they can be readily biodegraded by a range of aquatic bacteria. In 1994, the first microcystin-biodegrading bacterium identified as *Sphingomonas* sp. was isolated from Australian water bodies (Jones et al., 1994). Afterwards, several bacterial strains of *Sphingomonas* sp. (Bourne et al., 1996; Wang et al., 2010), *Pseudomonas* sp. (Takenaka and Watanabe, 1997; Yuan et al., 2005), *Stenotrophomonas* sp. (Chen et al., 2010), *Burkholderia* sp. (Lemes et al., 2008), *Arthrobacter* sp. (Manage et al., 2009), *Brevibacterium* sp. (Manage et al., 2009), *Rhodococcus* sp. (Manage et al., 2009) and *Kurthia gibsonii* (Wu et al., 2011) from different areas have been shown to biodegrade MCs. In 2004, we isolated a MCs-biodegrading bacterial strain tentatively identified

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as *Delftic acidovorans* from the sediment of eutrophic Dianchi Lake in China (Zhou et al., 2006). 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). The initial MC-RR concentration of 42.3 mg/L was completely biodegraded within 36 and 10 hr by USTB-05 and its cell-free extracts (CE) containing 0.35 mg/mL protein, respectively (Wang et al., 2010), while an initial MC-LR content of 21 mg/L was completely biodegraded within 20 and 1 hr by USTB-05 and its CE containing 0.35 mg/mL protein, respectively (unpublished data).

In the research to identify the biodegradation pathway of MCs by *Sphingomonas* sp., three enzymes were found to be involved in sequentially biodegrading MC-LR (Bourne et al., 1996, 2001). The initial site of hydrolytic cleavage of MC-LR by microcystinase is at the Adda-Arg peptide bond, and linear MC-LR is 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 the parent MC-LR. The second enzyme, serine protease, is responsible for the conversion of linearized MC-LR to the tetrapeptide NH_2-Adda-Glu (iso)-Mdha-Ala-OH. The third enzyme, peptidase, is responsible for dividing the 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, *mlr*A, B, C, and D. The enzyme encoded by the *mlr*A gene can cleave the Adda-Arg peptide bond in MC-LR. After opening of the cyclic structure, linear MC-LR is biodegraded by the peptidases encoded by *mlr*B and *mlr*C, and divided into each amino acid. *mlr*D 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 (Jones et al., 1994; Park et al., 2001) showed that the first step to degrading MC-LR is cleaving the Adda-Arg peptide bond. 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.

The first gene (named *USTB-05-A*) of *Sphingopyxis* sp. USTB-05 involved in biodegradation of MC-LR was cloned and expressed successfully for the first time. The objective of this study was to obtain direct evidence of the first step on the biodegradation pathway of MC-LR by *Sphingopyxis* sp. USTB-05, and to lay a foundation for constructing a genetically engineered bacterium to remove MC-LR efficiently using the gene of *Sphingopyxis* sp. USTB-05 involved in biodegradation of MC-LR.

1 Materials and methods

1.1 Bacterial strains, cultural conditions and vectors

A strain of *Sphingomonas* sp. USTB-05, which was shown to biodegrade microcystin LR (Wang et al., 2010), was maintained in the medium ($MgSO_4 \cdot 7H_2O$ 1.0 g, KH_2PO_4 0.5 g, K_2HPO_4 4.0 g, NaCl 1.0 g, $CaCl_2$ 20.0 mg, $FeSO_4$ 5.0 mg, $ZnCl_2$ 5.0 mg, $MnCl_2 \cdot 4H_2O$ 5.0 mg, $CuCl_2$ 0.5 mg, glucose 15.0 g, yeast 1.5 g per 1000 mL at pH 7.2), and exhibited constitutive MCs biodegrading activity. The host strain *E. coli* DH5α was cultured in Luria-Bertani (LB) medium (tryptone 10 g, yeast extract 5 g, and NaCl 10 g per 1000 mL at pH 7.2) at the shaking rate of 200 r/min and its growing temperature was adjusted as required.

Standard MC-LR (Molecular formula: $C_{49}H_{74}N_{10}O_{12}$, MW: 995.2) with the purity of 95% was purchased from Sigma Chemical Co., Ltd., USA. The cloning vector pGEM-T easy and prokaryotic expression vector pGEX-4T-1 were purchased from Promega Corporation (USA) and Pharmacia Corporation (Sweden) respectively. The genomic DNA of *Sphingopyxis* sp. USTB-05 was extracted with a genomic DNA extraction kit (Tiangen Biotech Co., Ltd., China). The plasmids transformed into *E. coli* DH5α were obtained using a plasmid mini-preps kit (BS413, Bio Basic Inc., China). The target DNA bands were extracted from the gel using a DNA gel extraction kit (BS353, Bio Basic Inc., China). All other chemicals used in this study were analytical grade except as specified by the kits.

1.2 Cloning and expression of *USTB-05-A*

Primers designed on the conserved sequence of *mlr*A were used to obtain the partial sequence of *USTB-05-A* in *Sphingopyxis* sp. USTB-05 (Wang et al., 2010). Primer P_1: 5′-GGATCCATGCGGGAGTTTGTCAAAC-3′ and P_2: 5′-CTCGAGCGCGTTCGCGCCGGACTTG-3′ were synthesized by Sunbiotech Inc. China. The underlined sequences are *BamH* I and *Xho* I. restriction sites, respectively.

Polymerase chain reaction (PCR) was performed in a total volume of 100 μL with the following conditions: USTB-05 DNA template 5 μL, Taq plus buffer 10 μL, dNTP 2 μL, forward primer 1 μL, reverse primer 1 μL, Taq plus polymerase 1 μL, and ddH_2O 80 μL. Cycling parameters were as follows: an initial denaturation of 5 min at 94°C; 30 cycles, consisting of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C; and an extension for 10 min at 72°C. The PCR product was analyzed by electrophoresis on 1% agarose gel stained with Goldview (HGV-II SBS Genetech Co., Ltd., China). The target band was extracted from the gel, ligated into pGEM-T easy vector and then transferred into *E. coli* DH5α. which were cultured on solid LB media with initial ampicillin sodium salt concentration of 60 μg/mL at 37°C for 12 hr. Positive clones were screened out by the method of blue white spot screening, named pGEM-T/*USTB-05-A*, and then sent to Sunbiotech Inc., China, for sequencing. The nucleotide sequence of the inserted *USTB-05-A* was analyzed by the bio-information analysis software package: Vector NTI 10.0. The plasmid pGEM-T/*USTB-05-A* was digested by *BamH* I and *Xho* I, and the fragments of *USTB-05-A* were extracted from the gel using an agarose gel DNA extraction kit (Roche, Swissland), and were ligated with pGEX-4T-1 vector (after being digested with *BamH* I and *Xho* I) by T4 DNA ligase. The ligated products were transferred

into *E. coli* DH5α. The recombinant plasmids (pGEX-4T-1/*USTB-05-A*) were identified first by restriction analysis and then by being sequenced correctly.

1.3 Preparation of CE

The confirmed clone was grown in LB media at an initial ampicillin sodium salt concentration of 60 μg/mL for 3 hr (OD_{600}, 0.6) and then induced with isopropyl-beta-D-thiogalactopyranoside (IPTG, 0.1 mmol/L) at 30°C for 3 hr. A sample of 1 mL for each clone was harvested by centrifugation (15,000 r/min at 4°C for 20 min) and then rinsed by 50 mmol phosphate buffered solution (PBS) at pH 7.0 three times. Finally, soluble protein was prepared from the bacterial pellets by an ultrasonic disruptor with an output power of 400 W at 4°C for 36 min. The supernatant was collected and used as CE and its protein profile was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gel. The concentration of protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

1.4 Enzymatic activity of recombinant *USTB-05-A*

To detect the activity of recombinant pGEX-4T-1/*USTB-05-A*, expression assays were carried out with four parallels including one negative control and one positive control. All samples, mixed with 5× SDS buffer, were deactivated by boiling for 5 min before SDS-PAGE. The CE containing crude protein from confirmed recombinant cells induced by IPTG was added into the test tubes loaded with PBS containing MC-LR. In this reaction system, the initial MC-LR concentration of 40 mg/L was biodegraded by CE containing protein at 0.35 mg/mL. As the control, another test tube was filled with PBS containing MC-LR only at 30°C with the shaking rate of 200 r/min. The concentrated hydrochloric acid 2 μL was added into 200 μL samples from each tube to stop the reaction at various reaction time points. All samples were centrifuged at 15,000 r/min for 2 min, and the supernatant was taken and used to measure MC-LR using high performance liquid chromatography (HPLC).

Microcystin LR was measured using a HPLC system (Shimadzu LC-l0ATVP, Shimadzu Co., Ltd., Japan) with a ultraviolet (UV) Diode Array Detector at 239 nm using a Agilent TC-C18 column (4.6 mm × 250 mm) (1200 series, Agilent Co., Ltd., USA). The mobile phase was 35% (*V*/*V*) acetonitrile (chromatographic grade) water solution containing 0.05% (*V*/*V*) of trifluoroacetic acid; the flow-rate was 1.0 mL/min, and the injection amount was 20 μL. A calibration curve was established relating the peak areas and concentration of MC-LR and used to calculate the concentration of MC-LR in samples.

1.5 Analysis of MC-LR and its first degradation product

In order to identify the character of MC-LR and its first degradation product, 0.2 mL samples were taken at 10, 60, 120, 480 and 1440 min, respectively, and were concentrated using a C_{18} solid-phase extraction cartridge (OASIS™ HLB, Waters Corporation, USA). Methanol was used to elute the concentrated products with the rate of 1 mL/min. The elution was used to determine the molecular weight (MW) of the parent MC-LR and its first product on the liquid chromatogram-mass spectrum (LC-MS) (3200 Q TRAP, Applied Biosystems, USA).

Mass spectral (MS) analysis was performed in positive ion electrospray mode. An Agilent TC-C_{18} analytical column from Waters Corp. (USA) was used in the chromatographic measurement in LC-MS analysis. For MS detection, precursor ions for samples and internal standards were determined from MS obtained during infusion into the mass spectrometer (3200 Q TRAP, Applied Biosystems, USA). Using an electrospray ionization (ESI) source, the mass spectrometer was operated in the positive ionization mode with the collision gas off. ESI conditions in the positive ion mode were as follows: curtain gas = 15, ionspray voltage = 5000, temperature = 450, ion source gas 1 (N_2) = 70, ion source gas 2 (N_2) = 50, declustering potential = 100, entrance potential = 10.

2 Results

2.1 Cloning and expression of *USTB-05-A*

A proper DNA fragment of about 1 kb was obtained from PCR amplification of the total DNA of USTB-05. The fragment *USTB-05-A* was ligated into pGEM-T easy vector and sequenced. The fragment had an open reading frame (ORF) of 1008 nucleotides and encoded putative 336 amino acid residues with the calculated molecular weight of 36.6 kDa. Sequence analysis showed 92.5% similarity to *mlr*A (GeneBank accession No. AF411068). Its sequence of deduced 336 amino acid residues has 83% positives to *mlr*A (Fig. 1). Among the putative 336 amino acid sequences, one alanine and one leucine for the cleavage of microcystin LR were also found at the 26th and 27th positions compared with that of *mlr*A.

The expressed plasmid pGEX-4T-1/*USTB-05-A* was constructed by inserting the PCR product into pGEX-4T-1 and then transformed into *E. coli* DH5α. The positive strain was identified first by digesting its expression plasmid with endonucleases *BamH* I and *Xho* I. The sequencing result showed that *USTB-05-A* gene was correctly inserted into the pGEX-4T-1 and the expressed protein with the molecular weight between 66.2 and 43 kDa containing a GST (25 kDa) was highly expressed in recombinant *E. coli* DH5α induced by IPTG.

2.2 Biodegradation of MC-LR

To confirm the enzymatic activity of the expressed protease, CE of recombinant *E. coli* DH5α was prepared and used to biodegrade MC-LR. As shown in Fig. 2, the retention time of the MC-LR peak was at 8.34 min and its area decreased with time course. In contrast, a new peak (peak A) appeared and increased at the retention time of 4.34 min. The reaction was almost finished within 60 min, and during the reaction, only the height (or area) of the MC-LR peak and the height (or area) of peak A

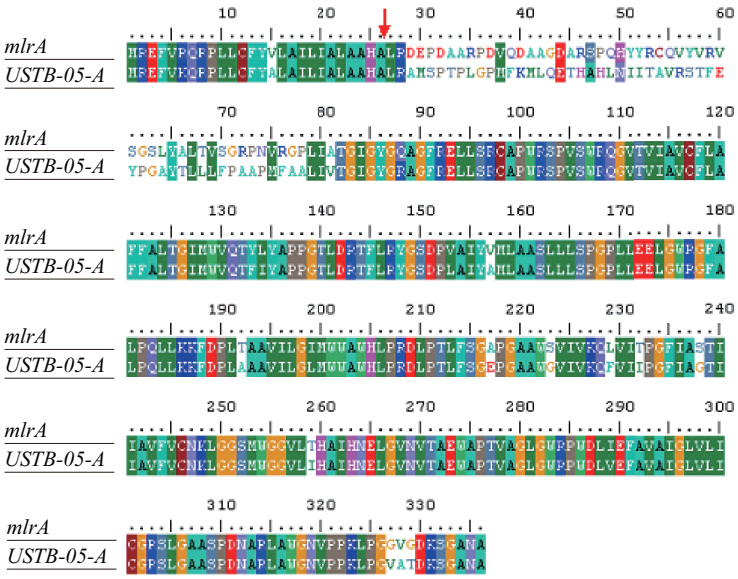

Fig. 1 Alignment of deduced amino acid sequences between gene *mlr*A and gene *USTB-05-A*. The site of amino acids in positions 26th and 27th for the cleavage of microcystin LR (MC-LR) is shown by a red arrow.

were changed. The absorbance profile of peak A was very similar to that of MC-LR and the maximum absorbance for both was at 239 nm or so. This indicates that the protein encoded by *USTB-05-A* has enzymatic activity, and the peak A was the first product (product A) of MC-LR catalyzed by a recombinant enzyme. The expressed protease encoded by *USTB-05-A*, which has been initially constructed and expressed in *E. coli* DH5α, is thus capable of biodegrading MC-LR.

2.3 LC-MS analysis of MC-LR and its biodegradation product

The liquid chromatogram-mass spectrum was used to measure the mass/charge ratios of both MC-LR and its product (Fig. 3). Based on the retention time and UV chromatogram in the HPLC profiles, the peaks of the total ion chromatogram (TIC) (Fig. 3a) at retention time 3.67 and 8.05 min were indicated as product A and MC-LR, respectively. The mass spectral analysis of MC-LR revealed a major ion at m/z 995.5, corresponding to the $[M+H]^+$ protonated molecular ion (Fig. 3b).

Product A showed a protonated molecular ion at m/z 1013.5 and a main peak at m/z 862.4 (Fig. 3c, Table 1). The ion at m/z 1013.5 indicated that product A was linearized MC-LR ($[M+H_2O+H]^+$, M+18). The peak at m/z 862.4 (M+18-151) corresponded to the loss of the terminal phenylethymethoxy group (MW: 135) and the amino NH_2 group (MW: 16) from Adda. The presence of this peak was evidence that the linearized MC-LR product contained N-terminal Adda. The presence of carboxy-terminal arginine was demonstrated by protonated ion m/z 571.2 ($[Mdha-Ala-Leu-Masp-Arg-OH+H]^+$) and m/z 488.1 ($[Ala-Leu-Masp-Arg-OH+H]^+$) (Table 1).

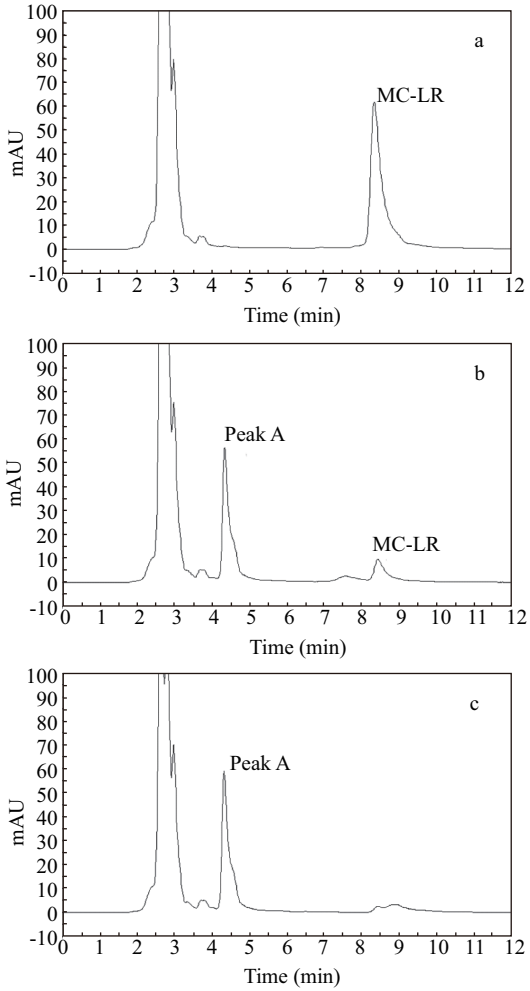

Fig. 2 High performance liquid chromatography (HPLC) profiles for the biodegradation of MC-LR by cell-free extracts (CE) containing expressed protein encoded by gene *USTB-05-A* with time course: (a) 0 min; (b) 10 min; (c) 60 min. HPLC profiles at 2, 8, and 24 hr are not shown here because of their unchanged HPLC profiles. Experimental conditions: initial MC-LR concentration of 40 mg/L; acetonitrile 35% (V/V) containing 0.05% (V/V) of trifluoroacetic acid, and flow-rate of 1.0 mL/min.

3 Discussion

Biodegradation is one of the essential processes for the reduction of MCs in natural eutrophic lakes and reservoirs.

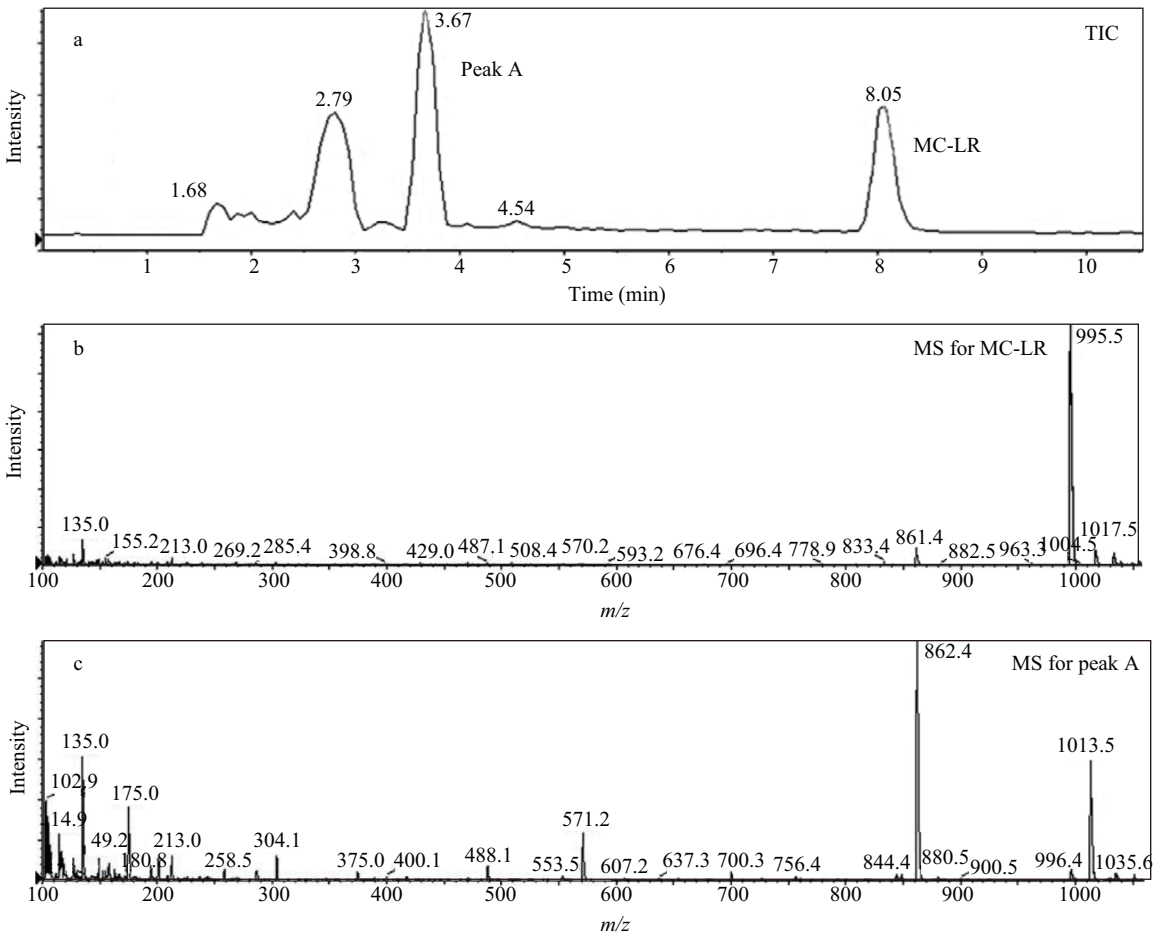

Fig. 3 Liquid chromatogram-mass spectrum (LC-MS) profile of MC-LR and its product. (a) total ions chromatogram (TIC) of MC-LR and its product; (b) mass spectrum (MS) for MC-LR; (c) MS spectrum for peak A. Experimental conditions: curtain gas = 15, ionspray voltage =5000, temperature = 450, ion source gas 1 (N_2) = 70, ion source gas 2 (N_2) = 50, declustering potential =100, entrance potential = 10.)

Table 1 Liquid chromatogram-mass spectrum (LC-MS) protonated molecular ion for product A

m/z	Identity
1013.5	[Mass (M)+H_2O*+H]$^+$
995.5	[M+H]$^+$
862.4	[M+H_2O-PhCH_2CHOCH_3-NH_2+H]$^+$
571.2	[Mdha-Ala-Leu-Masp-Arg-OH+H]$^+$
488.1	[Ala-Leu-Masp-Arg-OH+H]$^+$
304.1	[Masp-Arg-OH+H]$^+$
213.0	[Glu-Mdha+H]$^+$
175.0	[Arg-OH+H]$^+$
135.0	[PhCH_2CHOCH_3+H]$^+$

* M+H_2O corresponds to the linearized (hydrolyzed) microcystin LR.

The bacterial strains of *Sphingomonas* sp. have been widely regarded as the predominant strains able to biodegrade MC-LR. Previously, we reported that *Sphingopyxis* sp. USTB-05, isolated from sediment of Dianchi Lake in China, was a promising microcystin-biodegrading bacterium (Wang et al., 2010). Further studies have revealed that the strain *Sphingopyxis* sp. USTB-05 possessed at least three proteins encoded by genes involved in the biodegradation pathway of MCs (Wang et al., 2010). To confirm the mechanism of the first step in the biodegradation of MC-LR by USTB-05, the gene *USTB-05-A* was successfully cloned and expressed in *E. coli* DH5α (Fig. 1), which would provide evidence for the first step of MC-LR biodegradation.

During the biodegradation of MC-LR catalyzed by the recombinant enzyme, only one product was produced in the first step (Fig. 2). The initial MC-LR concentration of 40 mg/L was almost completely converted to product A within 1 hr, which was a much higher rate than those shown by bacteria. Although many studies have focused on the bacterial biodegradation of MCs (Takenaka and Watanabe, 1997; Lemes et al., 2008; Manage et al., 2009; Wu et al., 2011), the biodegradation efficiency of MC-LR can be much improved by using enzymes.

Product A at m/z 1013.5 ([M+H_2O+H]$^+$, M+18) was produced as the first product in the biodegradation of MC-LR catalyzed by an enzyme encoded by gene *USTB-05-A*, and was defined as linearized MC-LR (Fig. 3c). The MS spectra also revealed a major peak at M+18-151 (862.4) rather than M+18-135, corresponding to a loss of PhCH_2CHOCH_3 from Adda plus the Adda NH_2 group of hydrolyzed MC-LR because of electron delocalization from the 4, 6-conjugated diene (Bourne et al., 1996), which indicates that the product has an N-terminal Adda, which is consistent with the previous report by Choi et al. (1993). We confirm that Arg is carboxy-terminal from the MS spectra ions at m/z 571.2 (Mdha-Ala-Leu-Masp-Arg-OH), 488.1 (Ala-Leu-Masp-Arg-OH), 304.1 (Masp-Arg-OH) and 175.0 (Arg-OH). Therefore, it has been identified that

the linearized MC-LR product is NH_2-Adda-Glu (iso)-Mdha-Ala-Leu-Masp-Arg-OH. Therefore we inferred that the first step in the biodegradation of MC-LR began with the breakage of the Adda-Arg bond in the ring of MC-LR, which was catalyzed by the first protein of *Sphingopyxis* sp. USTB-05. One hydrogen was added on the NH_2 group of Adda and one hydroxyl was linked on the carboxyl group of Arginine in MC-LR, and the cyclic MC-LR was converted to linear MC-LR as its first product (Fig. 4), which is similar to the result of ACM-3962 (Bourne et al., 1996). The sequence alignment of *mlr*A with the enzyme of ACM-3962 involved in the initial pathway by the BLASTP program exhibited a high sequence identity of 81%, which suggests that the enzymes responsible for hydrolysis of parent MCs might have a common functional domain.

Harmful cyanobacterial blooms are a growing environmental problem worldwide in natural waters and biodegradation is found to be the most efficient method for removing MCs produced by harmful cyanobacteria. Although many studies have focused on the isolation of bacterial strains for biodegrading MCs and on the biodegradation pathway (Bourne et al., 1996, 2001; Takenaka and Watanabe, 1997; Lemes et al., 2008; Manage et al., 2009; Wu et al., 2011), less information has been provided on the cloning and expression of biodegradation genes. Here a gene for the first and most important step in the biodegradation of MC-LR was successfully cloned and expressed, which encoded a protease that is responsible for the conversion of cyclic MC-LR to linear MC-LR. Bourne et al. (1996) reported that linear MC-LR is less toxic than the cyclic form. It is known that the conjugated diene on Adda, with 4E, 6E stereochemistry, and the intact glutamate residue, are essential features for maintenance of protein phosphatase interaction and toxicity (An and Carmichael, 1994). Alterations to other portions of the molecule have little effect on toxicity, except in the case of the doubly arginine-substituted molecule MC-RR, which is about 5 to 10 times less toxic than most other MCs (An and Carmichael, 1994). Because linear MC-LR is still toxic to humans, the gene for the second step in the biodegradation of MC-LR by *Sphingopyxis* sp. USTB-05 is being further cloned and expressed, which is also very important for the elucidation of the biodegradation mechanism.

4 Conclusions

Biodegradation is one of the essential methods 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 *USTB-05-A* containing 336 amino acid residues, was successfully cloned and expressed in *E. coli* DH5α. The encoded and expressed enzyme *USTB-05-A* is responsible for cleaving the target peptide bond between Adda and arginine in the cyclic structure of MC-LR. Linear MC-LR is produced as the first product that is much less toxic than cyclic MC-LR. These findings

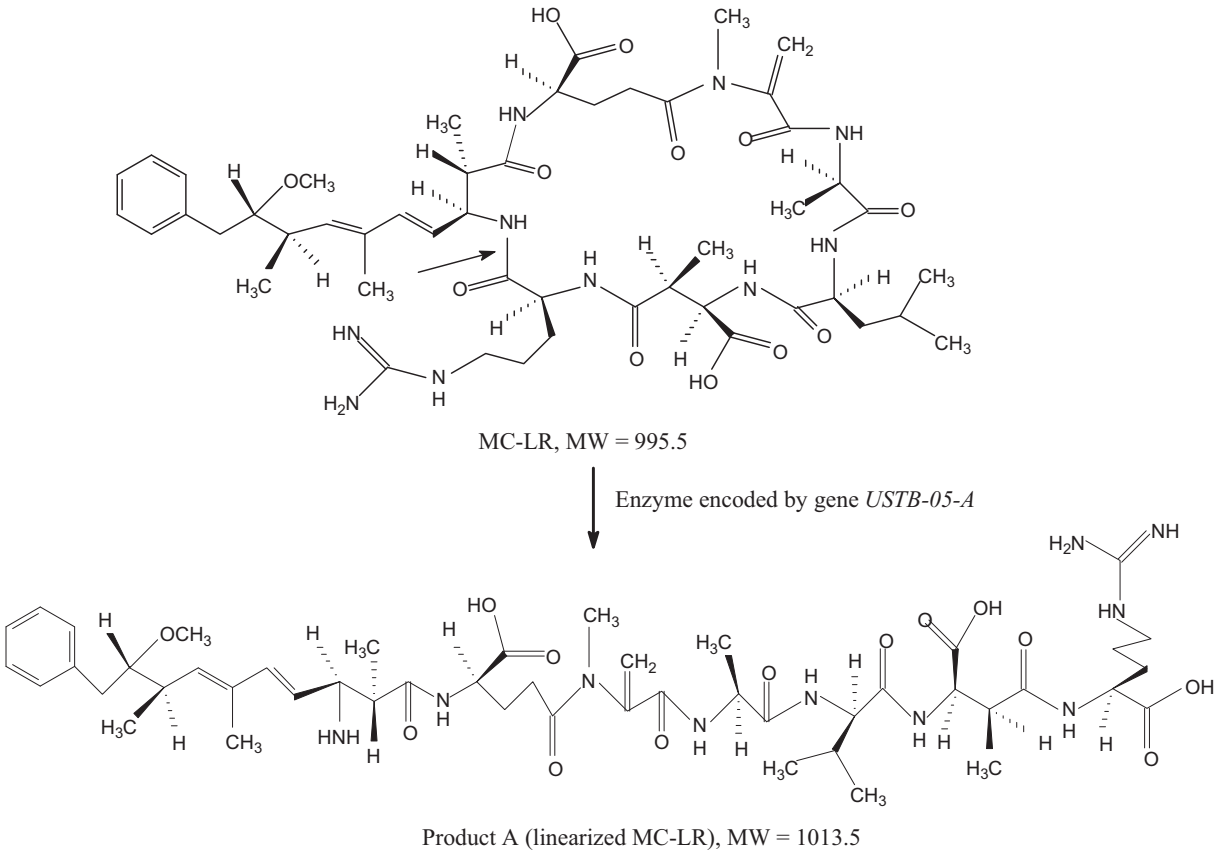

Fig. 4 First step involved in the enzymatic pathway of MC-LR biodegradation by the enzyme of *Sphingopyxis* sp. USTB-05. The one hydrogen (H) and one hydroxyl (OH) added during Adda-Arg bond breaking are boldfaced and italicized respectively (Adda-Arg bond broken position indicated by thick arrow).

are very important in understanding the pathway and mechanism of MC-LR biodegradation by USTB-05 and lay a foundation for constructing a genetically engineered bacterium to remove MC-LR efficiently using the gene of *Sphingopyxis* sp. USTB-05 involved in the biodegradation of MC-LR.

Nucleotide sequences accession number

(http://www.ncbi.nlm.nih.gov)

The nucleotide sequence of the gene *USTB-05-A* described here has been submitted to NCBI and its GenBank databases and assigned accession No. HM245411.

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