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ORIGINAL ARTICLE

Characterization of the second and third steps in the enzymatic pathway for microcystin-RR biodegradation by *Sphingopyxis* sp. USTB-05

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Abstract Microcystin-RR (MC-RR) is a dominant variant of microcystin (MC) worldwide. We previously isolated *Sphingopyxis* sp. USTB-05, which can efficiently biodegrade MC-RR. At least three enzymes encoded by MC-degrading genes are involved. Based on the successful expression of the first gene *USTB-05-A*, the second (*USTB-05-B*, KC513423) and third (*USTB-05-C*, KC573527) genes involved in the biodegradation of MC-RR were further cloned from *Sphingopyxis* sp. USTB-05 and expressed in *Escherichia coli* BL21 (DE3). After purification, the MC-degrading enzymes encoded by these genes were used for the catalytic degradation of MC-RR. The results demonstrated that the second enzyme encoded by *USTB-05-B* could convert linear MC-RR to a tetrapeptide by breaking the Ala–Arg bond. The third enzyme encoded by *USTB-05-C* could cleave Adda-Glu peptide bonds of both linear MC-RR and the tetrapeptide of Adda-Glu-Mdha-Ala, producing Adda as their common product. These findings will help better understand the biodegradation mechanism of MCs by *Sphingopyxis* sp. USTB-05.

Keywords Microcystin-RR · *Sphingopyxis* sp. USTB-05 · Cloning and expression of MC-degrading genes · Biodegradation pathway

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Introduction

Microcystins (MCs), which are produced mainly by *Microcystis*, *Anabaena* and *Nostoc* are potent hepatotoxins and tumor promoters (Falconer et al. 1994; Chen et al. 2002; Hu et al. 2002). The presence of MCs in water and their accumulation in aquatic organisms creates a potential risk to humans exposed to toxins either directly or through the food chain (Mazur and Plinski 2001). MCs are chemically stable and recalcitrant to traditional water treatment technology (Falconer 1999; Ho et al. 2006; Yan et al. 2006); however, MCs can be biodegraded by a range of aquatic bacteria. In 1994, the first MC-biodegrading bacterium, identified as *Sphingomonas* sp., was isolated from Australian surface water bodies (Jones et al. 1994). Afterwards, several bacterial strains of *Sphingomonas* sp. (Bourne et al. 1996; Ho et al. 2007; Wang et al. 2010; Xiao et al. 2011), *Ralstonia solanacearum* (Yan et al. 2004), *Pseudomonas* sp. (Takenaka and Watanabe 1997), *Stenotrophomonas* sp. (Chen et al. 2010), *Burkholderia* sp. (Lemes et al. 2008), *Arthrobacter* sp. (Manage et al. 2009) and *Kurthia gibsonii* (Wu et al. 2011) have also been shown to biodegrade MCs. Previously, we isolated a *Sphingopyxis* sp. named USTB-05 from the sediment of eutrophic Dianchi Lake in China (Wang et al. 2010). *Sphingopyxis* sp. USTB-05 had a strong ability in the biodegradation of microcystin-RR (MC-RR) and microcystin-LR (MC-LR) (Wang et al. 2010; Yan et al. 2012b).

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; Shimizu et al. 2012). The first enzyme, MlrA, 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, MlrB, is responsible for the conversion of linear MC-LR to the tetrapeptide NH_2-Adda-Glu (iso)-Mdha-Ala-OH; the third enzyme, MlrC, is

responsible for cleaving this tetrapeptide into individual amino acids (Bourne et al. 1996). However, Shimizu et al. (2012) found that MlrC also degraded linear MC-LR to Adda. Yan et al. (2012a, 2012b) confirmed that there were three genes (*USTB-05-A*, *USTB-05-B* and *USTB-05-C*) involved in the biodegradation of MC-LR in the strain USTB-05 and studied the first step involved in enzymatic pathway for MC-RR as well as MC-LR. In a word, most research on this topic to date has focused on MC-LR. There are only a few reports on the degradation pathway and its molecular mechanism of MC-RR, whose structure differs from that of MC-LR in its variable L-amino acids, although there were some suggestions that MC-RR might be degraded similarly to MC-LR (Bourne et al. 2001; Shimizu et al. 2012).

The aim of the present study was to determine the characteristics of *USTB-05-B* and *USTB-05-C* and to elucidate the MC-RR degradation pathway by enzymes encoded by *USTB-05-B* and *USTB-05-C* based on the successful cloning and expression of *USTB-05-A* (Yan et al. 2012a).

Materials and methods

Bacterial strains and vectors

The strain *Sphingopyxis* sp. USTB-05, previously isolated by the authors (Wang et al. 2010) was used. Competent *E. coli* BL21 (DE3) and competent *E. coli* DH5α were purchased from Tiangen Biotech (Beijing, China). Standard MC-RR ($C_{49}H_{75}N_{13}O_{12}$, MW: 1,038.2) with the purity of 95 % was obtained from Taiwan Algal Science, Yangmei City, Taiwan, China). The prokaryotic expression vector pET30a(+) Easy was from Promega (Madison, WI). Plasmids were isolated from *E. coli* DH5α using the small plasmid extraction kit from Bio Basic, China). DNA of *Sphingopyxis* sp. USTB-05 was extracted using a genomic DNA extraction kit (Tiangen Biotech).

Cloning of target genes

Primers to obtain the sequence *USTB-05-B* and *USTB-05-C* were designed according to information from the GenBank database. Primer (*USTB-05-B*): forward 5′-CGGGATCC-ATGACTGCAACAAAGCTTTTCCTGG-3′ (*Bam*HI underlined) and reverse 5′-CCGCTCGAGCTACGGAAGC CGTCTGAACTCTAT-3′ (*Xho*I underlined). Primer (*USTB-05-C*): forward 5′-AATCGAGCTCATGGAGATGCAGCG GCTTGCTG-3′ (*Sac*I underlined) and reverse ATAAGAAT GCGGCCGCCTAGGCTGAAAAGTCGAC-3′ (*Not*I underlined) were synthesized by Sunbiotech (Beijing, China). High fidelity polymerase chain reactions (PCR) were performed in a 50-μL volume containing 1×PCR buffer (TransStart FastPfu), 250 μM of each dNTP, ~ 1 μM of each primer and 2.5 U DNA polymerase. Cycling parameters were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C 1 min, 50 °C 1 min, 72 °C 3 min, and a final extension for 10 min at 72 °C.

PCR products were analyzed by electrophoresis on 1 % agarose gel stained with Goldveiw (HGV-II, SBS Genetech, China). The target bands were extracted from the gel using an agarose gel DNA extraction kit (Sangon Biotech, China) and inserted into the cloning vector pET30a(+) by T4 DNA ligase. The recombinant plasmids were transformed into competent *E. coli* strain DH5α, which was cultured with agitation overnight in Luria-Bertani (LB) liquid medium [1.0 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl] at 37 °C, 200 rpm. Positive clones were screened out by restriction enzyme analysis: *Bam*HI, *Xho*I and *Sac*I, *Not*I (Takara Biotech). The recombinant plasmids were sent to Sunbiotech (China) for sequencing. The gene sequences and nucleotide sequences of the inserted *USTB-05-B* and *USTB-05-C* were analyzed by the bio-information analysis software package Vector NTI 10. The recombinant plasmids pET30a(+)/*USTB-05-B* and pET-30a(+)/*USTB-05-C* were extracted and transformed into competent *E. coli* BL21 (DE3).

Expression of recombinants and detection of enzymatic activity

LB liquid (1 L) medium supplemented with ampicillin (50 μg mL^{-1}) was inoculated with 25 mL overnight *E. coli* BL21 (DE3) containing recombinant plasmid culture. The inoculated culture were grown with agitation at 37 °C, 200 rpm to an OD_{600nm} of approximately 0.6, induced with isopropyl b-D-thiogalactoside at final concentration of 0.1 mM and grown constantly at 30 °C, 200 rpm for 3 h. Cell free extracts (CE) of recombinant pGEX-4 T-1/*USTB-05-A* were used to produce the first biodegradation product of MC-RR (linear MC-RR) (Yan et al. 2012a).

Linear MC-RR was added to CE (0.35 mg mL^{-1}) containing enzyme B (encoded by *USTB-05-B*) and CE (0.35 mg mL^{-1}) without enzyme (control) sequentially, keeping the final concentration of linear MC-RR at approximate 40 μg mL^{-1}. Meanwhile, CE (0.35 mg mL^{-1}) containing

Table 1 Solvent gradient programme of ultra performance liquid chromatography (UPLC) analysis

Time (min)	Flow rate (mL/min^{-1})	A (%)	B (%)
0.0	0.4	95.0	5.0
1.0	0.4	95.0	5.0
10.0	0.4	0.0	100.0
11.0	0.4	0.0	100.0
11.0	0.4	95.0	5.0
14.0	0.4	95.0	5.0

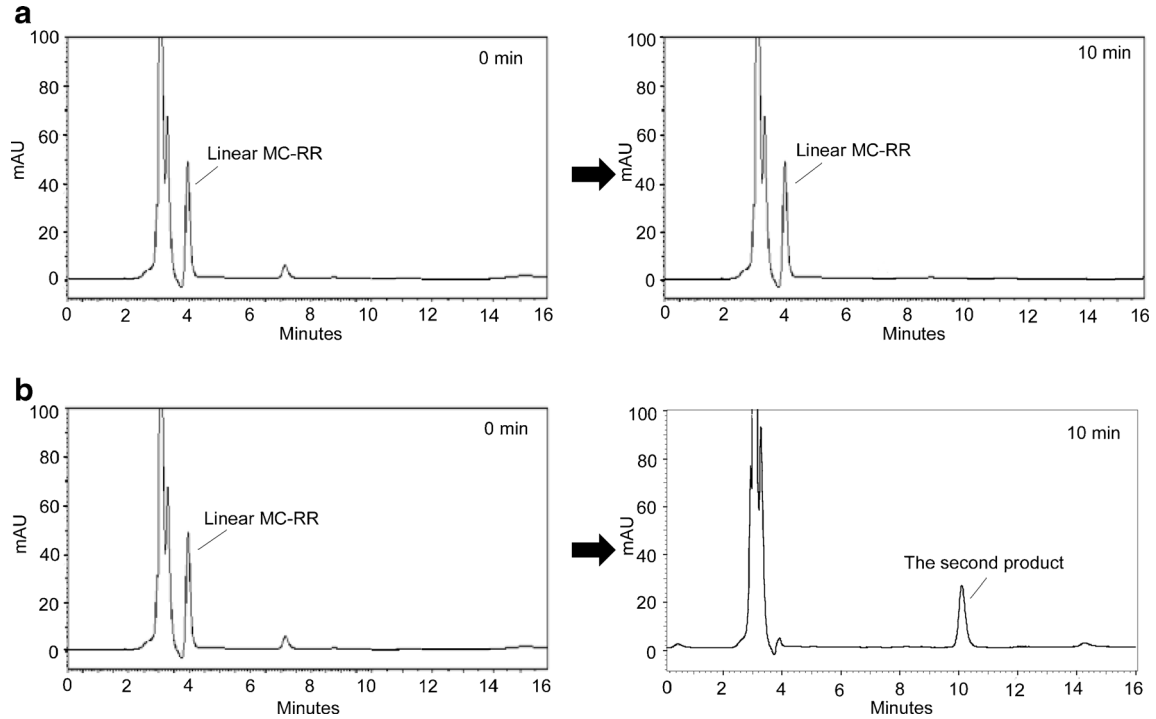

Fig. 1a,b HPLC chromatogram of linear microcystin-RR (MC-RR) degradation by the enzyme encoded by gene *USTB-05-B*. **a** Cell free extracts (CE) without target enzymes, **b** CE containing enzyme encoded by gene *USTB-05-B*. The *arrows* indicate the biodegradation time

enzyme C (encoded by *USTB-05-C*) and CE (0.35 mg mL^{-1}) without enzyme (control) were added to phosphate buffer solution (PBS) in the presence of linear MC-RR and the second product (catalyzed by enzyme B), respectively. After incubating at 30 °C with shaking at 200 rpm, samples of 200 μL were taken at 0 min, 10 min and 60 min, into which 2 μL concentrated hydrochloric acid was added to stop the reaction at various reaction time points, respectively. All

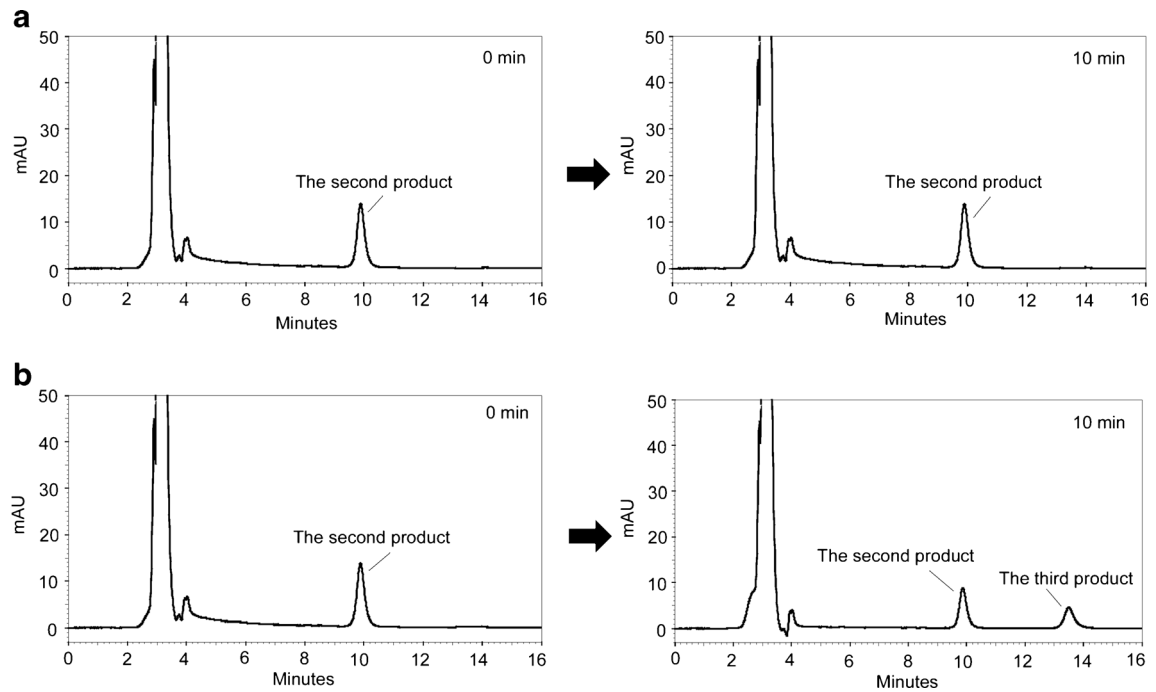

Fig. 2a,b HPLC chromatogram of second product degradation by the enzyme encoded by gene *USTB-05-C*. **a** CE without target enzymes, **b** CE containing enzyme encoded by gene *USTB-05-C*. The *arrows* indicate the biodegradation time

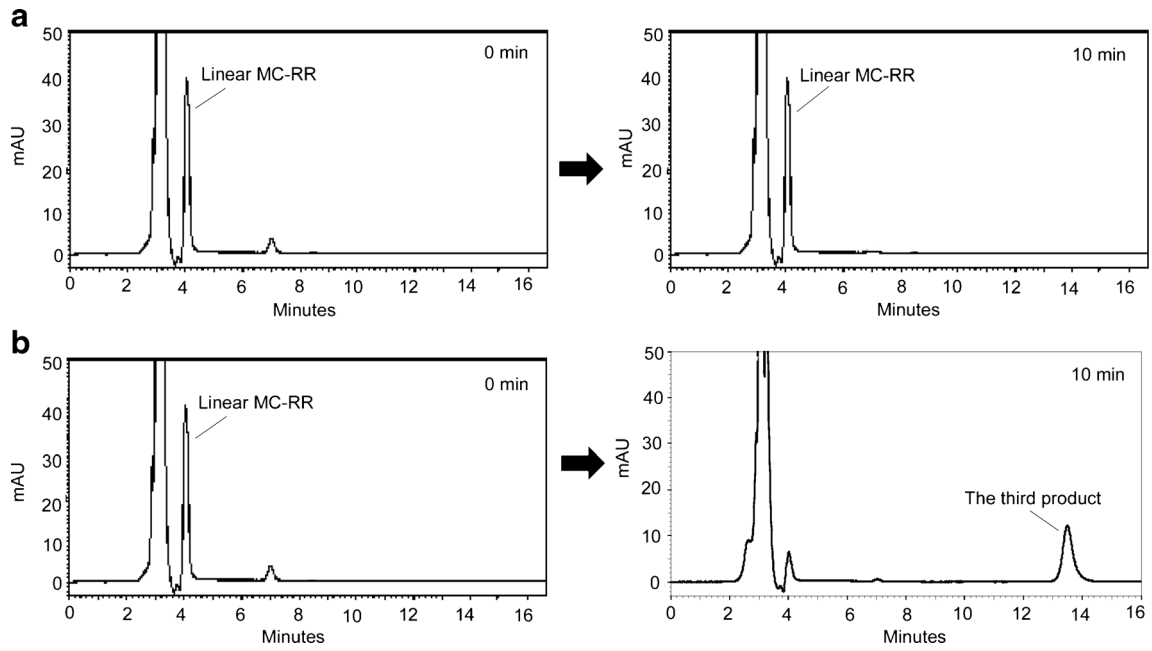

Fig. 3a,b HPLC chromatogram of linear MC-RR degradation by the enzyme encoded by gene *USTB-05-C*. **a** CE without target enzymes, **b** CE containing enzyme encoded by gene *USTB-05-C*. The *arrows* indicate the biodegradation time

samples were centrifuged at 12,000 rpm for 10 min. The samples at 0 min, 10 min were used for high performance liquid chromatography (HPLC), and the samples at 60 min were purified and concentrated using a C18 solid-phase extraction cartridge (OASIS^{TM} HLB, Waters Corporation, Milford, MA) and used for liquid chromatography tandem mass spectrometry (LC-MS).

The HPLC system (Shimadzu LC-l0ATVP, Shimadzu, Tokyo, Japan) was equipped with an ultraviolet Diode Array Detector at 239 nm using Agilent TC-C18 column (4.6 mm× 250 mm) (1200 series, Agilent Technologies, Santa Clara, CA), in which the mobile phase was 38 % (v/v) acetonitrile (chromatographic grade)-water solution containing 0.05 % (v/v) trifluoroacetic acid, with a flow rate of 1.0 mL min^{-1} and an injection amount of 20 μL (Yan et al. 2012b).

Analysis of linear MC-RR biodegradation products

LC-MS data were produced by employing the Waters ACQUITY UPLC® and XEVO-G2QTOF systems (Waters Corporation) and controlled by the software of Masslynx V4.1. A. The system was equipped with an electrospray ion source and hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer with the MS^{E} model. We injected a 5-μL aliquot of sample solution into an ACQUITY UPLC BEH C18 Column (2.1 mm×50 mm, 1.7 μm) held at 45 °C, in which a linear gradient system of A (0.1 HCOOH: 100 H_2O) and B (acetonitrile) shown in Table 1 were contained in the mobile phase. The full-scan data were acquired in positive ion mode from 50 Da to 1200 Da within a 0.2 s scan time under a capillary voltage of 3000 V, desolvation temperature of

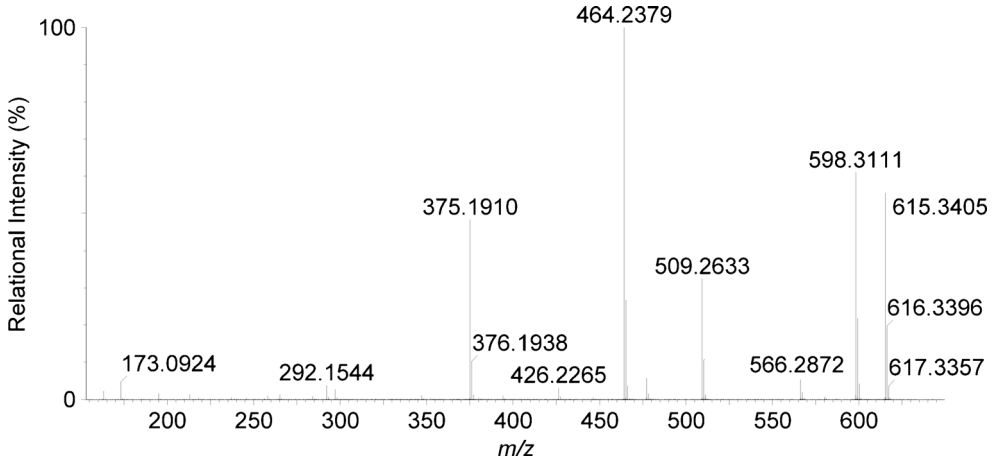

Fig. 4 Mass spectrometry (MS)/MS analysis of degradation of the second product

Table 2 Liquid chromatography tandem mass spectrometry (LC-MS)/MS protonated molecular ion for the second product

m/z	Identity
615.3	M+H (Adda-Glu-Mdha-Ala-OH+H)
598.3	M ($-NH_3$)+H
566.3	Adda ($-NH_2$–MeOH)-Glu-Mdha-Ala-OH+H
509.3	Adda ($-NH_2$)-Glu-Mdha+H
464.2	M ($-PhCH_2CHOMe–NH_2$)+H
426.2	Adda ($-NH_2$)-Glu+H
375.2	$C_{11}H_{14}O$-Glu-Mdha+H

350 °C, sample cone voltage of 40 V, extraction cone voltage of 4 V, source temperature of 100 °C, cone gas flow of 50 L h^{-1}, and desolvation gas flow of 600 L h^{-1}. We calibrated the mass spectrometer within the mass range of 50−1200 Da using a solution of sodium formate. Data were centroided and mass corrected during acquisition using an external reference (Lock-Spray™) consisting of a 0.2 ng mL^{-1} solution of leucine enkephalin infused at a flow rate of 20 μL min^{-1} via a lockspray interface, generating a reference ion at 556.2771 Da $[M+H]^+$. The lockspray scan time was set to be 0.5 s with an interval of 15 s, and data were averaged over three scans.

MS and MS/MS data were acquired using two interleaved scan functions in the MS^E mode. The first scan function was set at 6 V to collect information on the intact precursor ions in the sample, and the second scan function was ramped from 10 V to 30 V to obtain the fragment ion data from the ions in the preceding scan.

Result

Phylogeny analysis for gene *USTB-05-B* and *USTB-05-C*

DNA fragments *USTB-05-B* (1626 kb) and *USTB-05-C* (1587 kb) were obtained from PCR amplification of total DNA of *Sphingopyxis* sp. USTB-05. Then, sequence similarity was analyzed using the software of vector NTI 10. The result showed *USTB-05-B* was 97.4 % similar to *mlrB* of *Sphingopyxis* sp. C-1 (AB468059) (Shimizu et al. 2012) and 65.7 % similar to *mlrB* of *Sphingomonas* sp. ACM-3962 (AF411069) (Bourne et al. 2001). *USTB-05-B* encoded a putative protein of 542 amino acid residues. The deduced amino acid sequence of USTB-05-B was 65.9 % similarity to MlrB of ACM-3962 and 100 % similarity to MlrB of the strain C-1, respectively. *USTB-05-C* was 99.2 % similar to that of *mlrC* of strain C-1 and 87.6 % similar to that of ACM-3962. The deduced amino acid sequence USTB-05-C was 99.1 % similar to MlrC of strain C-1 and 90.0 % similar to that of ACM-3962.

Enzymatic activity of recombinant *USTB-05-B* and *USTB-05-C*

As shown in Fig. 1b, the retention time of the linear MC-RR peak was 3.98 min and its peak decreased with time. In contrast, a new peak with a retention time of 10.78 min appeared and increased. However, no new peak appeared in the control group (Fig. 1a). These results demonstrated that the protein encoded by *USTB-05-B* had an enzymatic activity to biodegrade linear MC-RR, which is related to the second product.

When the second product was added to enzyme B, the peak of the second product decreased with time while a new peak of the third product appeared (Fig. 2b). Similarly, enzyme C resulted in a decrease in linear MC-RR and the second product, accompanied by a rise in appearance of the third product (Figs. 2b, 3b), whose retention times were almost the same. These findings indicated that the enzyme B encoded by *USTB-05-B* had enzymatic activity to biodegrade linear MC-RR, and the enzyme C encoded by *USTB-05-C* could biodegrade both linear MC-RR and the second product of MC-RR, which is similar to the results of Shimizu et al. (2012).

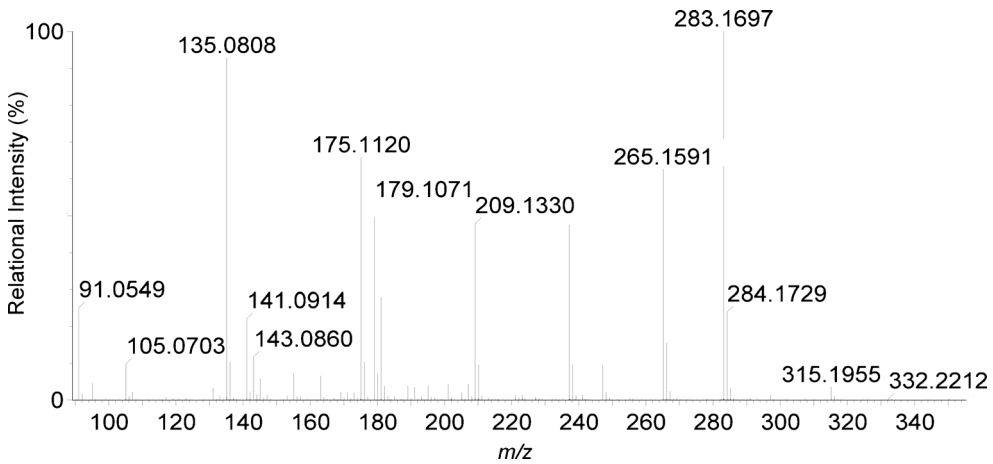

Fig. 5 MS/MS analysis of degradation of the third product

Table 3 LC-MS/MS protonated molecular ion for the third product

m/z	Identity
332.2	Adda+H
315.2	Adda+H – NH_3
135.1	$PhCH_2CHOMe$

The 4,6-conjugated diene of the Adda amino acid side chain on MCs contributes to the maximum absorbance at around 239 nm (Bourne et al. 1996). Linear MC-RR, the second product and the third product all have similar scanning profiles in the wavelength range 200–300 nm, which indicated that the group of Adda was kept intact in both the second and third products.

Product analysis

LC-MS/MS was used to measure the mass-to-charge ratios of the second and third product. According to the results presented in Fig. 4 and Table 2, the protonated molecular ion of the second product was detected at *m/z* 615.3 $[M+H]^+$ resulting from the loss of Tyr-MeAsp-Arg from linear MC-YR. The peak at *m/z* 464.2 $[M+H-151]^+$ corresponded to the loss of the terminal phenylethylmethoxy group (MW: 135) and the amino NH_2 group (MW: 16) from Adda (Namikoshi et al. 1992), and the ions at *m/z* 509.3 were caused by the loss of the Adda amino group from the proposed parent compound. So, it was confirmed that the structure of the second product of MC-YR was tetrapeptide Adda-Glu-Mdha-Ala.

Figure 5 shows that mass-to-charge ratios of the third product are at *m/z* 332.2, *m/z* 315.2 and *m/z* 135.1, which relate to M+H, the loss of the amino NH_2 group (MW: 16) from Adda and the $PhCH_2CHOMe$ part of Adda, respectively (Table 3). Therefore, it was inferred that the third product was Adda. This result is in agreement with the report by Dziga et al. (2012).

Discussion

Sphingopyxis sp. USTB-05 has a strong ability in the biodegradation of MC-RR (Wang et al. 2010). Bourne et al. (2001) performed cloning and gene library screening of a *Sphingomonas* sp. strain and detected the MCs-biodegrading gene cluster, *mlrA*, *B*, *C* and *D*. Previously, *USTB-05-A*, the first gene of *Sphingopyxis* sp. USTB-05, involved in the biodegradation of MC-RR was cloned and expressed, and linear MC-RR was found to be the first biodegradation product of MC-RR (Yan et al. 2012a).

The amino acid sequences of *USTB-05-B* (1626 bp) and *USTB-05-C* (1587 bp) were highly homologous to those of strain ACM-3962 (Bourne et al. 1996) and strain C-1(Shimizu et al. 2012). HPLC chromatograms indicated that the enzymes encoded by *USTB-05-B* and *USTB-05-C* all had the original function of biodegrading the first product of linear MC-RR (Figs. 1, 3). And the enzyme encoded by *USTB-05-C* had the original function of biodegrading the tetrapeptide (Fig. 2). The Adda group was kept intact in all three products of MC-RR. Although the products of MC-LR and the first product of MC-RR have been characterized (Bourne et al. 1996; Yan et al. 2012a; Yan et al. 2012b), the second and the third biodegradation products of MC-RR have not been identified. In this study, we successfully cloned and expressed the second and third genes involved in the biodegradation of MC-RR by

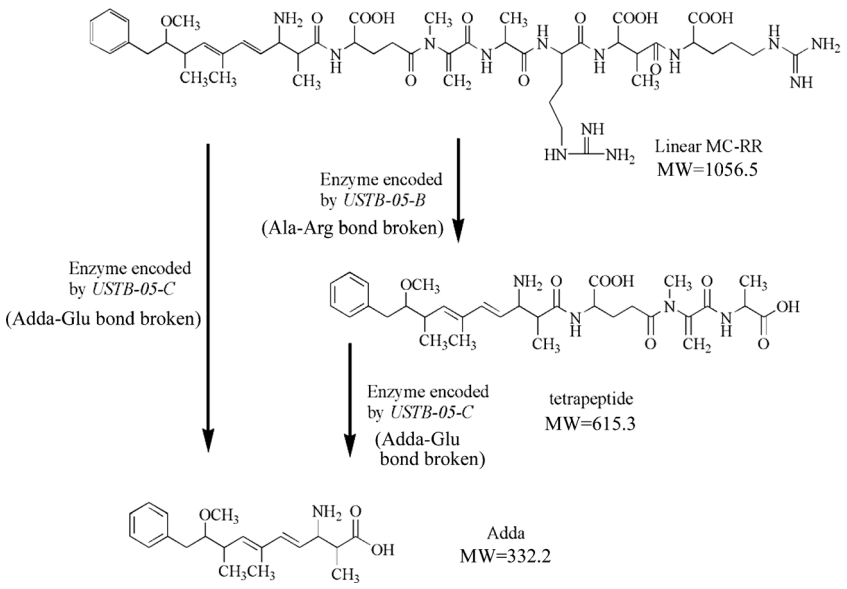

Fig. 6 The second and third steps involved in the enzymatic pathway for the biodegradation of MC-RR by *Sphingopyxis* sp. USTB-05.The *arrows* indicate the biodegradation route

Sphingopyxis sp. USTB-05. The two genes encoded highly active enzymes that could biodegrade linear MC-RR and the tetrapeptide. In addition, we showed that the third enzyme encoded by *USTB-05-C* could cleave the Adda–Glu peptide bond of both linear MC-RR and the tetrapeptide Adda-Glu-Mdha-Ala, and Adda was produced as their common product (Fig. 6), which is similar to the progress of MC-LR degradation by *Sphingopyxis* sp. C-1 (Shimizu et al. 2012). It suggested that strains USTB-05 and C-1 have the same function to degrade MC-LR and MC-RR. Bourne et al. (2001) and Imanishi et al. (2005) reported that the tetrapeptide has a linear structure, but its chain length differs from that of linear MC-LR. We therefore confirmed that the enzyme encoded by *USTB-05-B* recognized the Ala–Arg bond to degrade linear MC-RR to a tetrapeptide, and that the enzyme encoded by *USTB-05-C* recognized the Adda–Glu bond to degrade both linear MC-RR and the tetrapeptide to Adda.

Investigating the biodegradation mechanism can help us construct genetically engineered bacteria to degrade MCs efficiently. Up to now, the three genes of *Sphingopyxis* sp. USTB-05 involved in the biodegradation of MC-RR have all been cloned and expressed, and the biodegradation pathway of MC-RR by *Sphingopyxis* sp. USTB-05 has also been identified. This study is very important in both the basic and applied research pertaining to the elimination of MCs in lakes, reservoirs, and water treatment plants.

Conclusions

We cloned and expressed the second (*USTB-05-B*, 1626 bp) and third (*USTB-05-C*, 1587 bp) genes involved in the biodegradation of MC-RR by *Sphingopyxis* sp. USTB-05. We found that the enzyme encoded by *USTB-05-B* could convert the linear MC-RR to a tetrapeptide. The enzyme encoded by *USTB-05-C* could cleave Adda–Glu peptide bonds of both linear MC-RR and the tetrapeptide Adda–Glu–Mdha–Ala. Adda was produced as their common product. This study is not only a significant for understanding the biodegradation pathway of MCs, but also provides insight into an important mechanism to remove harmful MCs from drinking water sources.

The nucleotide sequence of the *USTB-05-B* (GenBank database accession No.: KC513423) and *USTB-05-C* (GenBank database accession No.: KC573527) described here have been submitted to NCBI.

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