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Degradation of Microcystin-RR by *Sphingomonas* sp. CBA4 isolated from San Roque reservoir (Córdoba – Argentina)

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Abstract

We report the aerobic biodegradation of Microcystin-RR (MC-RR) by a bacterial strain isolated from San Roque reservoir (Córdoba – Argentina). This bacterium was identified as *Sphingomonas* sp. (CBA4) on the basis of 16S rDNA sequencing. The isolated strain was capable of degrading completely MC-RR (200 $\mu g\ l^{-1}$) within 36 h. We have found evidence that MC-RR biodegradation pathway by this *Sphingomonas* sp. strain would start by demethylating MC-RR, affording an intermediate product, which is finally biodegraded by this strain within 72 h. Our results confirm that certain environmental bacteria, living in the same habitat as toxic cyanobacteria, have the capability to perform complete biodegradation of MC, leading to natural bioremediation of waterbodies. The bacterium reported here presents genetic homologies with other strains that degrade MC-LR. However, initial demethylation of MC-RR has been not described previously, raising questions on the probable presence of different biodegradation pathways for different MC variants.

Introduction

Toxic blooms of cyanobacteria occur worldwide in eutrophic lakes, ponds and reservoirs. Microcystins (MC) are the most commonly detected cyanobacterial toxins. These toxins have the general structure cyclo-(D-Ala-*X*-D-MeAsp-*Z*-Adda-D-Glu-Mdha-), in which *X* and *Z* represent variable L-amino acids, and Adda refers to the β-amino acid residue of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Over 70 structural analogues of MC have been identified. These structural variants differ primarily in *X*-*Z* amino acids and in methylation or demethylation on MeAsp and MDha (Codd et al. 1999; Fastner et al. 2002). When L-aa at position 2 and 4 are both Arginine, the MC is indicated as MC-RR (Figure 1).

MC are toxic to mammals, fish, plants and invertebrates (Codd et al. 1999; Pflugmacher et al. 1999; Pflugmacher & Wiegand 2001; Wiegand & Pflugmacher 2005). At the molecular level, MC bind irreversibly to and inhibit the serine/threonine protein phosphatases 1 and 2A (PP1, 2A). Microcystin-LR has been shown to be a liver-tumor promoter (Humpage & Falconer 1999; Ito et al. 2002).

MC are known to be chemically stable compounds (Lahti et al. 1997). It is, therefore not surprising that conventional drinking water treatments have only limited efficacy in removing dissolved MC (Svrcek & Smith 2004). Many studies have been conducted to solve this important

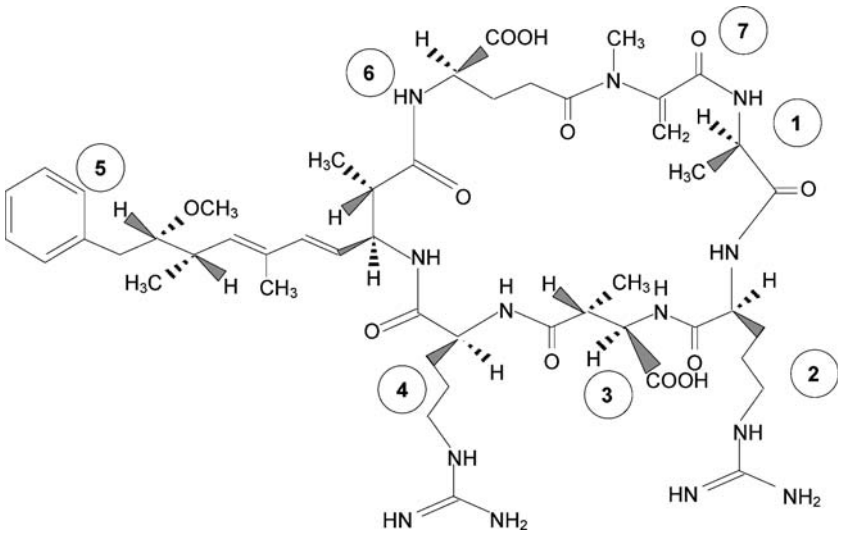

Figure 1. Chemical structure of Microcystin-RR showing the two variable amino acids (arginine) at positions 2 and 4.

trouble during the last years (Chen et al. 2005; Jurczak et al. 2005; Qiao et al. 2005; Shi et al. 2005; Svrcek & Smith 2004).

Recent concerns about chronic exposure of humans to MC in drinking water have led to a recommendation by the World Health Organization, advising that MC concentration in drinking water should not exceed 1 μg l^{-1} (WHO 1998).

The potential persistence of MC is crucial for the evaluation of their effect on aquatic organisms, livestock and humans that could be exposed in the natural environment. Furthermore, the understanding of their persistence and degradation is a prerequisite for risk assessment.

As possible carbon and nitrogen supply, MC may have a high nutritional value as source of either amino acids or energy to microorganisms. However, the cyclic structure of MC makes them resistant to many common bacterial proteases (Harada 1996), indicating that the capability to degrade microcystins by microorganisms requires rather specific enzymes.

Degradation of dissolved MC from temperate waterbodies has been studied in several cases. Lam et al. (1995) reported the degradation of MC-LR using a sewage effluent as inoculum. Cousins et al. (1996) found total degradation of MC-LR by reservoir water, while Jones et al. (1994) reported degradation of MC-LR in different surface waters, starting with different concentrations of MC. Holst et al. (2003) and Hyenstrand et al. (2003) confirmed MC biodegradation with ^{14}C-microcystin-LR. Holst et al. (2003) described that aerobic as well as anaerobic microorganism in sediments of a water recharge facility can efficiently remove MC, while, Hyenstrand et al (2003) found carbon dioxide as a major end product of MC degradation.

Bourne et al. (1996) described an enzymatic pathway for the degradation of MC-LR by a strain of *Sphingomonas* sp. Afterwards, they performed cloning and gene library of the *Sphingomonas* sp. strain detecting the MC-degrading gene cluster, *mlrA*, *B*, *C* and *D*. The enzyme encoded by the *mlrA* gene can cleave the Adda-arginine peptide bond in MC-LR and open the cycled structure. After opening 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 MC into the cell (Bourne et al. 2001).

San Roque reservoir is the main drinking water supply for Córdoba city (Argentina), showing cyanobacterial blooms for about 30 years. Recently, we have demonstrated that most of these blooms present evidence of toxicity. Thus, we confirmed the presence of MC-LR and MC-RR in 97% of studied cases, with concentrations ranging from 5.8 to 2400 μg MC g^{-1} of freeze-dried material. Though the occurrence was very similar for both toxins, the highest concentrations correspond to MC-RR (Amé et al. 2003).

Since MC-RR is the most abundant MC in this waterbody, the main goal of the present study was to examine the capability of its native microbial community to degrade this cyanotoxin. We hypothesized that MC-RR could be degraded by local bacterial community frequently exposed to cyanobacterial blooms.

We report the isolation from San Roque reservoir of a strain identified as *Sphingomonas* sp., which was able to perform complete aerobic biodegradation of MC-RR. Furthermore, we will examine on the genetic homology of this strain with other strains reported as MC degraders as well as differences with previous reported metabolic pathways. Finally, we discuss on the consequences of biodegradation on MC breakdown from the aquatic environment.

Materials and methods

Chemicals

Microcystin-RR was purchased from Sigma-Aldrich (purity ≥95% by HPLC). Solvents and other chemicals used were all reagent-grade.

Acclimation

A water sample from San Roque reservoir was used as microorganism source. This sample was taken during a bloom of *Microcystis* (Amé et al. 2003). The initial acclimation of this wild microorganisms population was carried out in 250 ml sterile flasks containing the water sample (inoculum), mixed with sterile mineral salts medium (MSM) containing per litre: 112 mg $MgSO_4 \cdot H_2O$, 5 mg $ZnSO_4 \cdot H_2O$, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, 340 mg KH_2PO_4, 670 mg $Na_2HPO_4 \cdot 7H_2O$, 14 mg $CaCl_2$ and 0.13 mg $FeCl_3$. The pH of MSM was adjusted to 7.0 and sterilized before use. Controls were prepared in the same way and stem sterilized (Pesce & Wunderlin, 2004; Spain & Nishino 1987).

MC-RR was added as the sole carbon and nitrogen source to reach a starting concentration of 200 $\mu g\ l^{-1}$. Inoculated samples contained an initial bacterial concentration of 1×10^6 CFU ml^{-1}. Both inoculated media and sterile controls were shaken continuously at 23 ± 2 °C. Aerobic mesophilic microbial counts (APHA 1992) and MC-RR quantification were performed daily to assess microbial survival and MC consumption. Fresh MC-RR was added to restore the initial concentration after exhaustion. Acclimated microorganisms were obtained from serial subcultures by transferring 20% v/v of the starting culture to an Erlenmeyer flask containing MSM and MC-RR (200 $\mu g\ l^{-1}$). Four serial subcultures were done, using the last one for bacterial isolation, purification, and biodegradation experiments (Pesce & Wunderlin 2004).

Isolation of Microcystin-RR degrading bacteria

Microorganisms obtained from the last subculture during acclimation experiments were isolated on one-tenth strength nutrient tryptone soy agar (Biokar Diagnostics, France), supplemented with MC-RR (200 $\mu g\ l^{-1}$). Different types of colonies were isolated from the Petri dish on the basis of size, color and morphology. Subsequent characterization was done considering 16S ribosomal RNA sequence analysis (Lane et al. 1985).

DNA samples were obtained from each pure strain culture by washing three times with distilled water, suspended in 10 mM Tris pH 8.0, and boiled 10 min. Bacterial gene encoding 16S rRNA was amplified by PCR using universal primers as previously described (Watt et al. 2001). Nucleotide sequence data were obtained by DNA sequencing (Macrogen Inc., Korea) employing the same primers used for PCR amplification. A multiple alignment was obtained, and a phylogenetic tree was constructed according to the neighbor-joining method by CLUSTAL W software (Tompson et al. 1994). This analysis was built according to Ishii et al. (2004) in order to compare our results with previous reports on bacteria capable of performing MC biodegradation.

Biodegradation of Microcystin-RR

The capability of the isolated bacterial strains to degrade MC-RR was examined by inoculating them into MSM containing MC-RR (200 $\mu g\ l^{-1}$). MC-RR biodegradation was analyzed by following changes in its concentration. Bacterial growth was also followed by aerobic mesophilic microbial counts on one-tenth strength nutrient tryptone soy agar (Biokar Diagnostics, France) (APHA 1992).

Assays were carried out using the same methods described for the acclimation procedure.

Analysis of Microcystin-RR

To evaluate MC-RR consumption, 5–15 ml culture was filtered through a 0.22 μm sterile membrane filter. Filtrate was adjusted to a final concentration of 1.5% acetic acid and applied to a C-18 SPE cartridge (LiChrolut RP-18,500 mg, Merck, Germany). SPE extract was suspended in 200 μl of methanol and analyzed by HPLC, in according to our previous experience (Amé et al. 2003).

HPLC was run on a Hewlett-Packard system equipped with a KONIC UV-VIS spectrophotometer, using a 4.6×250 mm Microsorb-MV 100 C18 (VARIAN) column, with acetonitrile: 0.05% trifluoracetic acid (50:50) as mobile phase, flow rate: 0.8 ml min^{-1}, column temperature: 20 °C and UV detection at 238 nm.

Extraction and HPLC performances were verified using culture medium supplemented with 50, 100, 200 $\mu g\ l^{-1}$ MC-RR. The average recovery percentage for MC-RR was (85 ± 3%).

Metabolites arising from biodegradation experiments were analyzed by HPLC and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). MALDI-TOF-MS instrument was equipped with a nitrogen laser operating at 337 nm, and a two-state ion source operating in the delayed extraction mode (PerSeptive Biosystems, Framingham, MA, USA).

Samples verified by MALDI-TOF-MS were analyzed using the method described in Pflugmacher et al. (1998). Briefly, 2 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (in 3:2 v/v acetonitrile-0.1% trifluoroacetic acid) were premixed with 2 μl of the microcystin-extract. Afterward, 2 μl of this mixture were applied to the sample plate, and air-dried at 24 °C. Measurements were performed at an acceleration voltage of 20 kV using reflector mode, allowing the determination of monoisotopic mass values. Each spectrum refers to the sum of 100–200 individual laser shots. All analyses were carried out in duplicate.

Results

Acclimation of microorganisms

Experiments conducted to verify the capability of native microorganisms to grow using MC-RR (200 $\mu g\ l^{-1}$) as the sole carbon and nitrogen source, demonstrated that at the end of the 14-day aerobic incubation, the MC-RR concentration had decrease to an undetectable level. Successive addition of MC-RR, to restore its initial concentration, shows that the toxin was exhausted earlier by the inoculum during acclimation period (Figure 2). We assumed complete acclimation after 22 d, by which time microorganisms were able to degrade MC-RR in 3 d. There was no evidence of chemical degradation of MC in sterile controls (Figure 2).

Isolation and identification of MC-RR degrading bacteria

Based on the hypothesis that MC-RR could be degraded by diverse microorganisms present in the acclimated inoculum, bacterial strains were isolated from cultures supplemented with MC-RR. Three bacterial strains, named CBA2, CBA3 and CBA4, were isolated considering their external morphological characteristics. These strains were inoculated into 100 ml MSM containing MC-RR (200 $\mu g\ l^{-1}$) to test the degradation capability by each isolated strain. The aptitude of pure strains to degrade MC-RR was tested using the same protocol described for inoculum acclimation.

Among the three isolated strains, only CBA4 was capable of degrading MC-RR. CBA4 was identified by 16S rRNA as described in materials and methods. DNA fragments of 850 bp were obtained from PCR reaction. The sample was purified and sequenced in both directions by Macrogen Inc., Korea. The nucleotide sequence

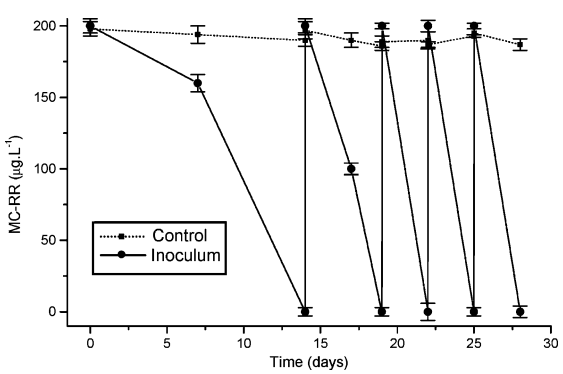

Figure 2. Die away curve for MC-RR during acclimation assay.

was compared with NCIB-BLAST Database (http://www.ncbi.nlm.nih.gov).

CBA4 shows an identity of 97% with genes encoding 16S rRNAs for *Sphingomonas* sp.

The closest relative bacteria to CBA4 was proved to be *Sphingomonas* sp. ACM-3962 (97% homology), which has been previously reported as capable of degrading MC-LR (Bourne et al. 1996). CBA4 also presents 93% homology with 7CY and Y2, *Sphingomonas* sp. strains reported to degrade MC (Ishii et al. 2004; Park et al. 2001).

To get more information on the taxonomic and phylogenetic position of CBA4, we constructed the phylogenetic tree. Its position in the tree was distinctively distant from the cluster corresponding to 7CY and Y2 strains, but close to *Sphingomonas* sp. ACM-3962, as expected. This result confirms the classification of CBA4 as a member of the genus *Sphingomonas* as deducted from the 16S rRNAs homology. The Genbank accession number for the 16S rDNA sequence of strain CBA4 is AY920497.

Biodegradation of MC-RR by Sphingomonas sp. CBA4

Figure 3 shows the die away curve for MC-RR by *Sphingomonas* sp. CBA4. These experiments, carried out using MC-RR (200 μg l^{-1}), showed that *Sphingomonas* sp. CBA4 was capable of degrading almost completely this toxin within 36 h incubation, confirming its capability to use MC-RR as sole carbon and nitrogen source under aerobic conditions. This plot also shows an approximately linear correlation between remaining MC-RR and time ($r^2 = 0.990$). Using a starting concentration of 200 μg l^{-1} MC-RR we calculated an approximate half life of 18 h, although it should be consider that zero-order reactions are dependent on the initial substrate concentration. Thus, the MC-RR half-life reported here is only valid for the condition reported here.

The decrease in MC-RR concentration was accompanied by a slight increase in the bacterial density (from 1.2×10^6 to 5.75×10^6 CFU ml^{-1}), proving absence of inhibition on the bacterial growth, and that MC-RR is used as the sole carbon and nitrogen source.

Figure 4 shows HPLC chromatograms of MC-RR and its biotransformation products. It can be observed that the peak corresponding to MC-RR decreased gradually, while two peaks, called A and B, were observed as probable biotransformation products. The peak of MC-RR had completely disappeared within 48 h incubation (Figure 4), while peaks A and B disappeared within 72 h (data not shown).

Extracts corresponding to 24 and 48 h incubation were analyzed by MALDI-TOF-MS looking

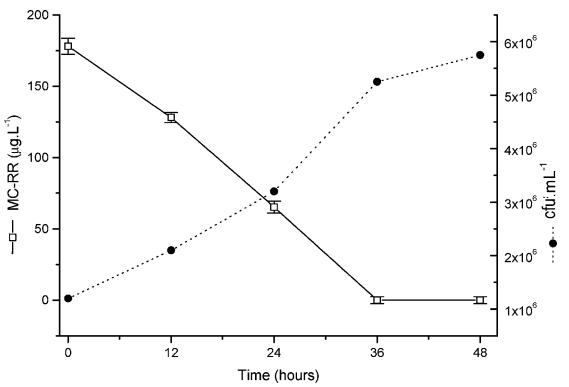

Figure 3. Changes in the MC-RR concentration and bacterial growth (CFU ml^{-1}) during the biodegradation of MC-RR by *Sphingomonas* sp. (CBA4).

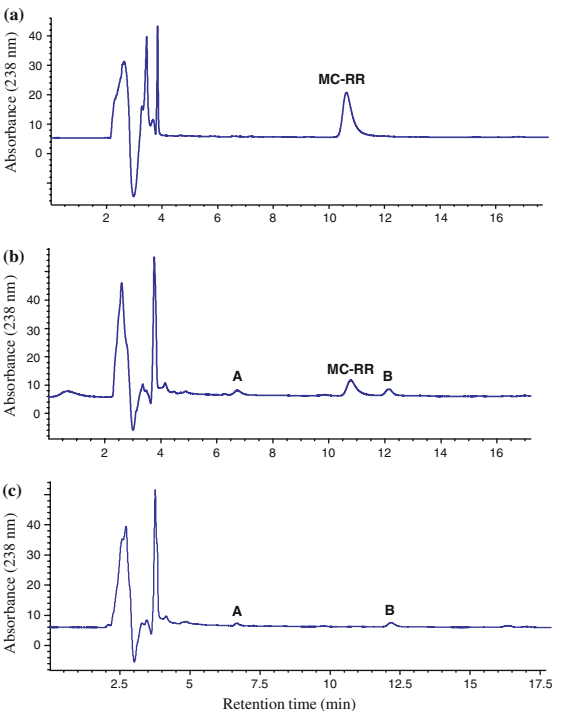

Figure 4. HPLC chromatograms obtained during biodegradation of MC-RR by *Sphingomonas* sp. (CBA4) at time zero (a), 24 h (b), and 48 h (c). Peaks A and B show biotransformation products of MC-RR.

to identify biotransformation products. Analysis of MALDI-TOF-MS corresponding to the 24 h extract affords three major peaks (Figure 5). One of them is compatible with MC-RR (m/z 1038, remaining toxin).

The MALDI-TOF MS spectrum for 48 h sample did not show any major peak, precluding us to get further insight in the possible biodegradation pathway.

Discussion

Cyanotoxins can naturally occur either cell-bounded (intracellular) or water-dissolved (extracellular). Toxin occurrence into water may be due to its natural release, caused by cell lysis, and also by cell destruction during water treatment (induced release).

In any case, the dissolved toxin comes into contact with a wide range of aquatic organisms, including aquatic bacteria. As MC are oligopeptides, it is likely that they could use this cyanotoxins as carbon and nitrogen source.

Most of the work on the degradation of cyanobacterial hepatotoxins has been done on MC-LR (Bourne et al. 1996, 2001; Cousins et al. 1996; Harada et al. 2004; Holst et al. 2003; Hyenstrand et al. 2003; Jones & Orr 1994; Lam et al. 1995; Saito et al. 2003; Takenaka & Watanabe 1997). Only few studies are available on other MC (Christoffersen et al. 2002; Imanishi et al. 2005; Ishii et al. 2004; Park et al. 2001).

We were particularly interested in studying the biodegradation of MC-RR considering its high occurrence in San Roque reservoir (Amé et al. 2003) and the probable consequences of its occurrence.

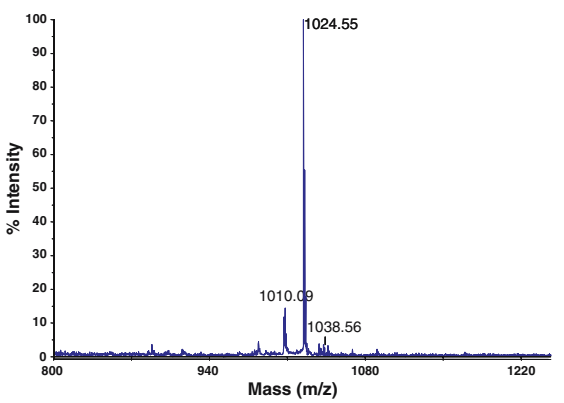

Figure 5. MALDI-TOF mass spectra of MC-RR and its biotransformation products after 24 h biodegradation in the presence of *Sphingomonas* sp. CBA4.

Several studies showed a prolonged lag phase until starting the degradation of MC, similarly to the biodegradation of other toxins by native bacteria in natural environments. It could be expected that the bacterial community, arising from a waterbody where cyanobacteria are common, can degrade microcystin along with other organic compounds frequently found in the environment (Christoffersen et al. 2002).

When the biodegradability of MC was experimentally tested using natural microbial communities, the persistence of toxins ranged from a few days to several weeks. In our case, adaptation of the native community to perform biodegradation of MC-RR was evident after 14 days of aerobic incubation. This time is about coincident with that reported by Lam et al. (1995), who found a lag phase of 10 days for degradation of MC-LR with sewage effluent as inoculum. Jones et al. (1994) found lag-phases ranging from 3 to 20 days in different surface waters and at different initial concentrations of MC-LR, while Cousins et al. (1996) found total degradation within less than 1 week in reservoir water. Hyenstrand et al. (2003) found also a slow initial degradation of MC while Christoffersen et al. (2002) confirm the degradation of MC-LR and -RR in natural waters with previous cyanobacterial histories without lag phases. These differences in the duration of the lag phase could be due to different bacterial community composition, its degree of adaptation, etc. However, these results indicate that most of native microbial community can be adapted to perform biodegradation of these toxins quite rapidly.

Lahti et al. (1998) tested the ability of a large number of bacterial strains, isolated from lake water and sediment, to degrade MC in laboratory experiments and found that only 17% of the strains had this ability.

Our results show that only one out of three bacterial strains isolated from San Roque reservoir was able to biodegrade MC-RR. Since several strains have been isolated and characterized from different water bodies, it appears that MC degrading bacteria are distributed all over the world (Welker et al. 2001). However, it is remarkable that *Sphingomonas* was frequently found among the feasible genus occurring in

freshwater. For instance, *Sphingomonas* sp. ACM 3962 was isolated from Lake Centenary-Australia (Bourne et al. 1996); *Sphingomonas* sp. Y2 was isolated from Lake Suwa-Japan (Park et al. 2001) as *Sphingomonas* sp. 7CY (Ishii et al. 2004). Therefore, our results reinforce the concept that the genus *Sphingomonas* is particularly widespread all over the world, having the enzymatic system necessary to degrade these natural toxins.

In accordance to our results, Saito et al. (2003) detected in *Sphingomonas* sp. a MC-LR degrading gene (*mlrA*), however, they report that even when this gene is exclusive to MC degraders it is not limited to the genus *Sphingomonas*.

Figure 3 shows the die away curve for MC-RR by *Sphingomonas* sp. CBA4. The calculated degradation rate was 4.98 $\mu g\ l^{-1}\ h^{-1}$ (0.12 $mg\ l^{-1}\ d^{-1}$). This rate could be considered equivalent with the one obtained by Park et al. (2001). This biodegradation rate affords a calculated half-life of 18 h in presence of acclimated bacteria under the conditions used in our experiments.

The biodegradation pathway for MC-RR has not been completely elucidated yet. Recently Imanishi et al. (2005) evaluated if MC-RR, as well as other MC and nodularin, suffers the same biodegradion pathway than MC-LR by a previously isolated *Sphingomonas* sp. strain B-9. The degradation pathway proposed for MC-LR consists of sequential hydrolysis starting at the Arg-Adda bond, which affords a linearized microcystin-LR, followed by the Ala-Leu bond (Ala-Arg for MC-RR) producing a tetrapeptide, with further hydrolysis of the Adda-Glu bond with production of Adda (Bourne et al. 1996; Harada et al. 2004; Imanishi et al. 2005).

The mass spectrum of the linearized (acyclic) MC-LR degradation product presents an m/z 1013.5 (MC-LR m/z $995.5 + H_2O + H^+$). If the degradation of MC-RR would follow the same way, an analogous degradation product of MC-RR would be expected at m/z 1057.5. However, we did not find evidence of this reaction.

Our results show that MALDI-TOF-MS of the 24 h extract affords three major peaks. The main one was observed at m/z 1024, which can be assigned as a product arising from the demethylation of MC-RR. This assignation is based on the molecular weight for 3 and 7 demethyl MC-RR reported in the literature (Barco et al. 2004; Sivonen & Jones 1999). The third peak of interest has an m/z of 1010, and was assigned to bi-desmethyl MC-RR, which has been also formerly reported (Sivonen & Jones 1999).

In a recent report, Imanishi et al. (2005) did not find presence of the linearized MC-RR during the study of MC-RR biodegradation by *Sphingomonas* sp. strain B-9, in good agreement with our results. On the contrary, we did not find neither the tetrapeptide (m/z 615.3) nor the Adda residues (m/z 332.2) as described in the same report.

Furthermore, to the extent of our knowledge, this is the first report of such demethylation products during biodegradation of MC. This result raise questions on the possibility to obtain different biodegradation pathways from different bacterial strains, even when they show high genetic homology. It is likely to think in small genetic differences among degrading strain, leading to diverse enzymatic systems, which afford distinctive pathways. On the other hand, it could be possible that different MC variants induce small changes on biodegradation enzymatic system of a given bacterium during the acclimation phase. Additional research is needed to elucidate these questions.

Unfortunately, demethylation products seem to retain the structure, which is responsible for MC toxicity. Sivonen & Jones (1999) summarized some data for MC showing that MC-RR has a LD_{50} of 600 $\mu g\ kg^{-1}$ in mouse, while LD_{50} for [D-Asp^3] MC-RR and [Dha^7] MC-RR are 250 and 180 $\mu g\ kg^{-1}$ respectively, and positive for [D-Asp^3,Dha^7] MC-RR in a non-quantitative mouse bioassay. In our case, intermediate demethylation products are finally biodegraded by *Sphingomonas* sp. CBA4 within 72 h incubation.

Conclusion

Natural remediation process is triggered when the environment faces with either natural toxins or xenobiotics. During this process bacteria community changes in order to enable the dominance of species capable of performing an efficient detoxification. Thus, the native bacterial community of San Roque reservoir is adapted to perform biodegradation of MC-RR. From the entire community, we isolated one strain, identified as *Sphingomonas* sp. CBA4, which was able to biodegrade MC-RR at a concentration of 200 $\mu g\ l^{-1}$.

Sphingomonas sp. CBA4 presents important genetic homologies with other strains proposed to degrade MC-LR.

Distinctively, this strain is capable of demethylate MC-RR, probably as the first step within the catabolic pathway leading to complete toxin degradation. This initial biodegradation pathway for MC-RR has not been previously reported, and seems to be markedly different from the previously described for MC-LR.

As we did not observe natural or chemical degradation, it should be mentioned that biological degradation appears as an important route in the break down of MC in the environment. This fact reinforces the need of a complete understanding of MC degradation in aquatic ecosystems, which requires further studies in population composition of MC-degrading bacteria as well as elucidation of its biodegradation pathways, looking to answer questions rose during this study.

Acknowledgements

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