

Full Length Research Paper

Biodegradation of microcystin by a new *Bacillus* sp. isolated from a Saudi freshwater lake

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Accepted 23 August, 2010

A new strain of *Bacillus* sp. was isolated from a Saudi eutrophic lake containing toxic cyanobacterial blooms. Based on phylogenetic analysis of the 16S rRNA gene sequence, the new strain most likely belonged to the genus *Bacillus* with a similarity of 81%. Using polymerase chain reaction (PCR), AMRI-03 strain was shown to contain a gene homologues to mlrA that encodes the most important enzyme for microcystin degradation. The strain was capable of degrading microcystin-RR (MC-RR) (10 mg l⁻¹) in batch experiments under environmentally related conditions. The degradation of MC-RR was fully completed within 5 days after a lag period of 2 days. The MC-RR degradation by AMRI-03 strain occurred in a medium containing nitrogen and phosphorus, indicating that this could likely occur along with other organic compounds found in the environment. Therefore, the coexistence of such bacteria with MCs in the same environment can contribute to the self-purification of the ecosystem from such potent toxins.

Key words: *Bacillus* sp., 16S rRNA gene, degradation, microcystin, mlrA gene, polymerase chain reaction.

INTRODUCTION

Cyanobacterial blooms are usually formed as a result of an increased of eutrophication of freshwater supplies. Most of these blooms consist of species producing different kinds of cyanotoxins including hepatotoxins (e.g. microcystins), neurotoxins and lipopolysaccharide endotoxins (Codd et al., 2005; Holst et al., 2003). Microcystins (MCs) are a group of monocyclic heptapeptide hepatotoxins produced by species of cyanobacteria such as, *Microcystis*, *Anabaena*, *Nostoc* and *Planktothrix/Oscillatoria* (Carmichael, 1992; Keil et al., 2002; Baldia et al., 2003) and recently by *Anabaenopsis* (Mohamed and Al-Shehri, 2009).

MCs consist of seven amino acids (D-Ala-X-D-MeAsp-Z-Adda-DGlu-Mdha-) (Carmichael, 1992). Now, over

seventy varieties of MC have been known (Fastner et al., 2002). They are strongly hepatotoxic due to their potent inhibition of protein phosphatases 1 and 2A (Kuiper-Goodman et al., 1999; Sivonen and Jones, 1999).

MCs are usually retained inside cyanobacterial cells, but released into natural waters after cell lysis (Sivonen and Jones, 1999). The presence of such potent toxins in water bodies such as reservoirs, lakes and rivers can be a threat to mammals, fish, plants and invertebrates (Codd et al., 1999). Furthermore, an intake of water contaminated by MCs caused the high incidence of liver cancer in China (Ueno et al., 1996), and MCs were the cause of human death among individuals receiving hemodialysis treatment in Brazil (Jochimsen et al., 1998).

Consequently, the effective removal of MC toxins from water sources is a major goal for all water utilities and the protection of aquatic ecosystem. MCs are known to be chemically stable compounds (Lahti et al., 1997). Most conventional drinking water treatments have limited efficacy in removing dissolved MCs (Svrcek and Smith, 2004), and sometimes produce carcinogenic substances and other mutagens (Ishii et al., 2004). Therefore, bacterial biodegradation could be the promising method for the removal of MCs in natural water. It is now known

Abbreviations: PCR, Polymerase chain reaction; MCs, microcystins; NBA, nutrient broth agar; NB, nutrient broth; BLAST, basic local alignment search tool; NCBI, national centre for biotechnology information; PPIA, protein phosphatase inhibition assay; PP2A, protein phosphatase 2A; ELISA, enzyme-linked immunosorbent assay; LB, lysogeny broth.

that these hepatotoxins may undergo biological degradation by aquatic bacteria owing to enzymatic pathways, particularly by some strains of bacteria identified as pertaining to the genus *Sphingomonas* (Bourne et al., 1996; Saito et al., 2003; Ame et al., 2006).

It is found that these degradation products, such as the linearized MC-LR, the tetrapeptide and Adda, are essentially non-toxic, which strongly indicated that the microbial degradation using MC-degrading bacteria is quite effective for the detoxification of MCs (Tsuji et al., 2006). Most of the MC-degrading bacteria isolated so far belong to *Sphingomonas* sp. and *Pseudomonas* sp. or related species called "pseudomonad" (Jones et al., 1994; Takenaka and Watanabe, 1997; Park et al., 2001; Ishii et al., 2004; Tsuji et al., 2006). However, it has been shown that the presence of a gene *mlrA*, encoding the most important enzyme for MC degradation, is unique to MC degraders but not only to the genus *Sphingomonas* (Saito et al., 2003). Therefore, other MC-degrading bacteria such as *Methylobacillus* (Hu et al., 2009), *Burkholderia* (Lemes et al., 2008), *Sphingosinicella* (Maruyama et al., 2006), *Arthrobacter*, *Brevibacterium* and *Rhodococcus* (Manage et al., 2009) were successively isolated from many lakes, ponds, reservoirs, and rivers, particularly those containing toxic cyanobacterial blooms.

Tendaha Lake is about 50 acre freshwater pond in a residential area of Tendaha city located southwest of Saudi Arabia (18° 30' N and 42° 20' E). The lake is surrounded by agricultural farms of fruits and vegetables. It is usually fed with rainwater collected from the neighboring areas. The pond serves as wildlife habitat and as water source for irrigation, recreational and drinking purposes. Recently, the lake showed a toxic cyanobacterial bloom producing the hepatotoxins, microcystins, such as MC-RR (Mohamed and Al-Shehri, 2009). It is hypothesized that MC could be degraded by local bacterial community frequently exposed to cyanobacterial blooms in Tendaha Lake. Therefore, the present study aimed to isolate and identify a native bacterium capable of MC degradation, and to detect a gene *mlrA* which encodes the most important enzyme for MC degradation in this bacterium.

MATERIALS AND METHODS

Isolation of microcystin-degrading bacteria

Water samples were collected during May 2009 from Tendaha Lake which has been previously reported to have MC-producing blooms of cyanobacteria (Mohamed and Al Shehri, 2009). Diluted samples of lake water were inoculated onto nutrient broth agar (NBA) medium plates. Single colonies from these plates were transferred to liquid nutrient broth (NB) medium. Firstly, to screen the isolated bacteria for the ability of MC degradation, the grown cells of morphologically different five bacterial isolates were inoculated (5 ml) separately to 50 ml NB medium containing MC-RR (1 mg l⁻¹). All cultures of bacterial strains were maintained in an incubator at 28°C (the temperature of lake water during the sampling time) with

shaking (120 rpm) in the dark for 5 d. The remaining concentrations of MC-RR in the medium were monitored by enzyme-linked immunosorbent assay (ELISA) using the Envirologx kit for MC as described in biodegradation of MC-RR section. Among the tested isolates, only one bacterium strain showed MC degrading activity. The isolate was then identified by 16S rRNA gene sequencing according to Neilan (1995).

Identification and sequence analysis of 16S rRNA gene

Bacterial DNA of the isolate, capable of degrading MC, was extracted from 5 ml overnight cultures by using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA) with some modifications. 1 ml of the overnight cultures was placed in sterilized micro-centrifuge tubes and then centrifuged at 7550 g for 1 min. The supernatant was decanted and then the pellet was suspended in 180 µl of lysozyme solution (20 mg ml⁻¹ lysozyme in 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton), followed by incubating for 30 min at 37°C. 20 µl of Proteinase K was then added, mixed by vortexing and incubated at 56°C for 30 min. DNA amplification reactions were conducted in a BioRad MyCycler thermocycler. The oligonucleotide primers 1F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1R (5'-GGTACCTTGTTACGACTT-3') were used to amplify bacterial 16S rRNA gene. Polymerase chain reaction (PCR) program was conducted under the following condition: 3 min at 95°C, 30 cycles of 50 s at 95°C; 1 min at 50°C; and 2 min at 72°C and 10 min at 72°C. The PCR products were analyzed on 1.0% agarose gel containing 0.5 µg ml⁻¹ ethidium bromide and visualized by BioRad Gel Documentation System 2000.

The 16S rRNA gene PCR product was sequenced by Macrogen Company, Korea. Homology of the 16S rRNA gene sequence of the isolates with reference 16S rRNA gene sequences was analyzed using the Basic Local Alignment Search Tool (BLAST) algorithm in GenBank available in the National Centre for Biotechnology Information (NCBI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). Only the highest-scored BLAST result was considered for phylotype identification.

Detection of genes involved in microcystin degradation

A specific oligonucleotide primer set MF, 5'-GACCCGATGTTCAAGATACT-3' and MR, 5'-CTCCTCCACAAATCAGGAC-3' (Saito et al., 2003) were used in PCR to screen bacterial isolates containing the *mlrA* gene. Amplifications were conducted on a BioRad MyCycler thermocycler under conditions previously documented by Ho et al. (2006). PCR products were analyzed by electrophoresis on a 1% agarose gel containing 0.5 µg ml⁻¹ ethidium bromide and visualized by BioRad Gel Documentation System 2000.

Biodegradation of microcystin-RR

To study degradation of MC, the MC degrading bacterium was cultured overnight on an orbital shaker at 140 rpm in NB medium at 28°C, harvested at exponential phase and used for biodegradation experiment. The grown cells were inoculated into three sterile 100 ml Erlenmeyer flasks containing 50 ml NB liquid medium and MC-RR (95%, provided by Prof. Dr. Zakaria A. Mohamed, King Khalid University) at a concentration of 10 mg l⁻¹. Flasks with NB medium containing 10 mg l⁻¹ MC-RR without bacterial cells were used as control. Treated and control cultures were shaken (140 rpm) in the dark at 28°C. 1 ml of samples was withdrawn carefully under sterile conditions at zero time and one-day interval for 10 d. Each sample was monitored for bacterial growth by measuring the optical density

(OD) spectrophotometrically at 600 nm wavelength. The samples were then centrifuged (10,000xg) and the supernatants were kept in glass tubes for the determination of non-degraded MC by ELISA. ELISA was used with no further sample preparation and run using the Envirologix kit for MCs according to Envirologix manufacturer protocol (Carmichael and An, 1999). An ELX800 microplate reader (BioTek, Highland Park, Winooski, VT 05404, USA) was used for the absorbance measurement at 450 nm. For quantification, the competitive calibration curve (B/Bo% versus concentration of MC-RR on a semi-log scale, where B and Bo are the absorbance values of the sample and the blank, respectively) were used. The curve was drawn using the calibrator solutions contained in the kit (0.16-2.50 $\mu\text{g l}^{-1}$). All standards and samples were analyzed in triplicates. Samples with CV>15% were rejected.

The average biodegradation rate was calculated according to Lemes et al. (2008) by dividing the concentration of MC-RR as initially spiked into the samples by the number of the days until MC was no longer detected by ELISA. The half life of toxin degradation was calculated from the linear regression of the toxin decay curve. The loss of toxicity of bacterial degradation by-products was assessed by protein phosphatase inhibition assay (PPIA) using protein phosphatase 2A (PP2A) enzyme and *p*-nitrophenyl phosphate substrate purchased from Sigma. Enzyme and substrate solutions were prepared according to known procedure (Heresztyn and Nicholson, 2001). Samples (20 μl) were combined with PP2A (0.25 U ml^{-1}) solutions (20 μl) in microplate wells and incubated at 37°C for 5 min. After addition of 200 μl of substrate solution, the microplate was incubated at 37°C for 1.5 h and the absorbance of the wells were measured at 405 nm using an ELX800 microplate reader. Toxin concentrations were calculated from calibration curves plotted as percentage inhibition of PP2A, expressed as B/B₀%, versus MC-RR concentrations (0.01 – 10 $\mu\text{g l}^{-1}$). In blanks, MC standards were replaced by high-purity water such that full color development was obtained. In order to more precisely determine toxin concentrations, only the linear region of the calibration curve, that is, the region between 20 and 80% activity, was used for quantification. The IC₅₀ was the toxin concentration which resulted in 50% inhibition, that is, 50% activity.

RESULTS

Phylogenetic analysis

Among different bacterial strains isolated from Tendaha Lake during the present study, strain AMRI-03 showed the ability of degrading MC-RR. A phylogenetic tree based on bacterial 16S rRNA gene sequence showed a close relationship between the isolate AMRI-03 and the genus *Bacillus* (Figure 1). The alignment of isolate 16S rRNA gene with GenBank showed that AMRI-03 had the highest homology (81%) with *Bacillus subtilis* strain BSX5 (accession number: DQ112340). The 16S rRNA gene sequence of AMRI-03 strain was deposited in the GenBank under accession number GU294753.

Biodegradation of microcystin-RR by strain AMRI-03

Detection of MC-RR degradation began after 2 days of incubation with strain AMRI-03. Subsequently, MC was rapidly degraded with a removal of 18% of the original MC-RR concentration (10 mg l^{-1}) by the third day of

incubation. The toxin was gradually degraded to below the detectable limit by day 7 (Figure 2). The results of PPIA showed a decrease in toxicity which were correlated with the decrease in MC concentrations as determined by ELISA. No significant difference in the bacterial density was observed between toxin-treated and control cultures (Figure 3), indicating that MC-RR has no effect on bacterial growth. MC concentrations in the control (NB medium with no bacteria) did not significantly change (that is, no degradation) during all the period of incubation (Figure 2). The average biodegradation rate of MC-RR by strain AMRI-03 was about 1.45 $\mu\text{g ml}^{-1} \text{d}^{-1}$. The results of linear regression of the percentage of degraded MC-RR showed that the half-life ($D_{1/2}$) of MC-RR degradation by this strain was 4.1 d (Figure 4).

Detection of the *mlrA* gene from the microcystin degrading bacteria

Figure 5 shows the results of detection of the *mlrA* gene by PCR. Bands of the expected size (approximately 800 bp) were detected in strain AMRI-03 by PCR employing the primer set MF-MR.

DISCUSSION

Microcystins produced by cyanobacteria in aquatic environments could be released into the water by natural cell lysis or cell destruction during water treatment (Tsuji et al., 2006). These toxins come into contact with a wide range of aquatic organisms, including aquatic bacteria which could degrade such toxins. The majority of these MC-degrading organisms have been identified as *Sphingomonas* (Bourne et al., 1996, 2001; Park et al., 2001; Saito et al., 2003; Harada et al., 2004; Ishii et al., 2004; Ho et al., 2006). Recently, MC degradation has also been reported for different bacterial strains of other genera including *Pseudomonas* (Takenaka and Watanabe, 1997), *Paucibacter* (Rapala et al., 2005), *Methylobacillus* (Hu et al., 2009), *Lactobacillus* and *Bifidobacterium* (Nybom et al., 2007), *Burkholderia* (Lemes et al., 2008), *Sphingosinicella* (Maruyama et al., 2006), *Arthrobacter*, *Brevibacterium* and *Rhodococcus* (Manage et al., 2009). However, MC degradation by *Bacillus* sp. has not been reported yet.

In the present study, a *Bacillus* sp. isolated from a eutrophic lake (Tendaha Lake) containing toxic blooms of cyanobacteria, was found to degrade MC-RR. It is the first study to investigate the ability of the genus *Bacillus* to degrade MC. In addition, the 16S rRNA gene sequence data showed a homology (81%) between AMRI-03 and *B. subtilis* strains BSX5. It has been suggested that sequence similarity must be above 95% to qualify as evidence of a similar species (Amann et al., 1995; Chen et al., 2004). Consequently, the strain AMRI-

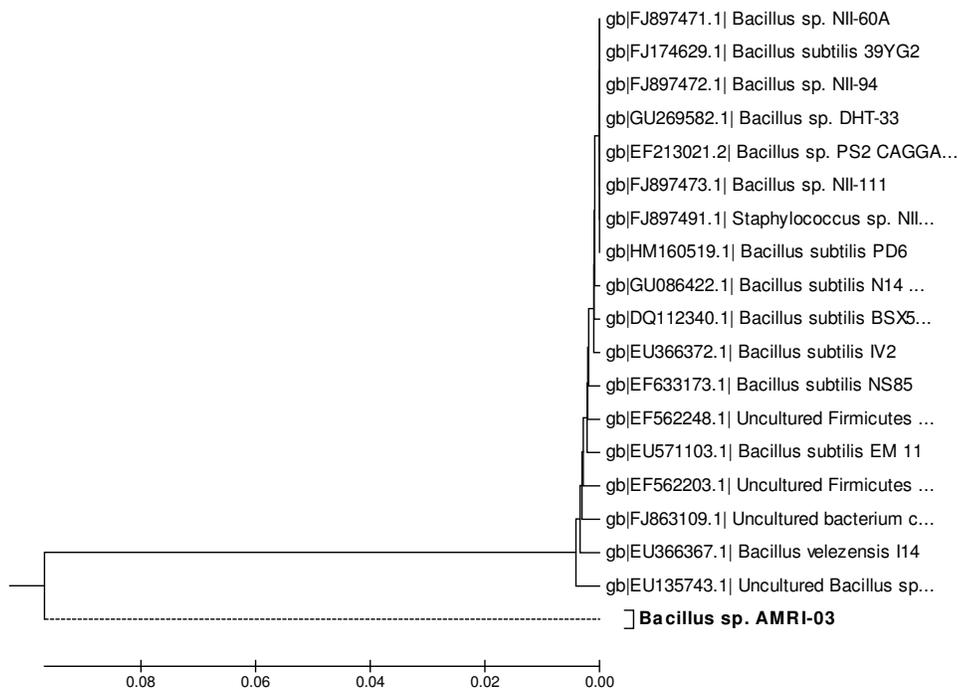


Figure 1. Phylogenetic tree showing the position of microcystin-degrading *Bacillus* sp. strain AMRI-03 within the radiation of most related species of the genus *Bacillus* from the NCBI Genbank.

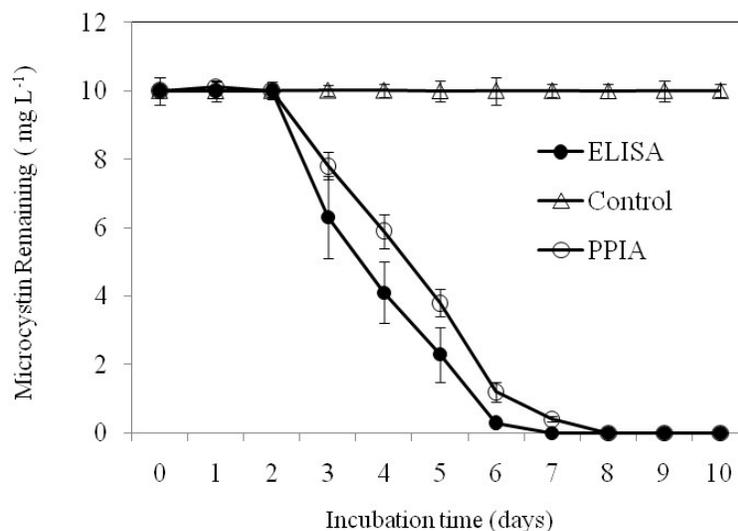


Figure 2. Degradation of microcystins-RR over a 10-day period with *Bacillus* sp. strain AMRI-03 isolated from a Saudi eutrophic lake. Changes in microcystin concentrations ($\mu\text{g ml}^{-1}$) were monitored by enzyme-linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay (PPIA).

03 could be assigned as a new species of *Bacillus*.
B. subtilis was found to be able to completely degrade aromatic compounds (Das and Mukherjee, 2007), which

may further expand the range of substrates that can be degraded. For MC, it was reported that MC degradation by bacteria is not induced by degradation substrates

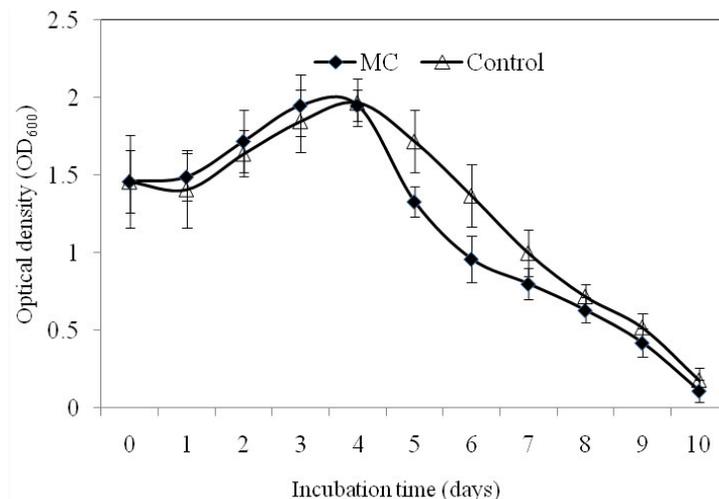


Figure 3. Growth curve of *Bacillus* sp. strain AMRI-03 in NB medium under toxin-treated and control conditions.

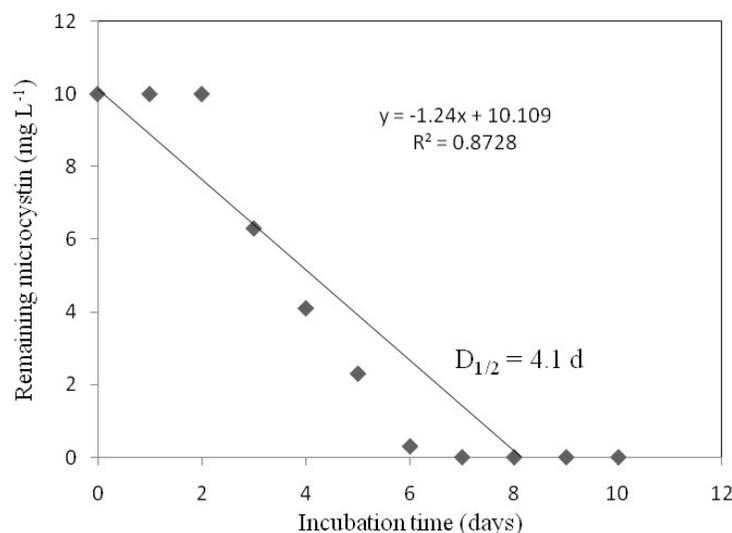


Figure 4. The half life time of microcystin-RR during the biodegradation by *Bacillus* sp. strain AMRI-03.

(MCs) but inherent; and that the substrate specificity of the hydrolytic enzymes is relatively broad and MC-degrading bacteria are effective on other cyclic peptides (Kato et al., 2007). This could be true for *Bacillus* strains degrading aromatic compounds, where it can also degrade MCs. Previously, Lemes et al. (2008) found that some *Burkholderia* strains capable of the dissimilation of aromatic compounds, are also able to degrade MCs.

Strain AMRI-03 started to degrade MC-RR after lag period of 2 d. This time is in a harmony with that reported for *Sphingopyxis* by Ho et al. (2007a). Jones et al. (1994) found lag-phases ranging from 3 to 20 d in different surfaces containing MCs. Hyenstrand et al. (2003) found also a slow initial degradation of MCs. In contrast,

Christoffersen et al. (2002) suggested that the degradation of MCs in natural waters with cyanobacterial histories occur without lag phases. In this study, although AMRI-03 strain was isolated from a lake containing toxic cyanobacteria, it showed a short lag phase for MC-RR degradation. This could be attributed to the higher initial MC concentration (10 mg l^{-1}) used in the present study. Similar observation was reported by Ho et al. (2007b) who found a short lag period of 2 d at an initial MC concentration of $10 \text{ } \mu\text{g l}^{-1}$, while no apparent lag period was observed using the lowest MC concentration of $3 \text{ } \mu\text{g l}^{-1}$. The time period in which total biodegradation was observed (7 d) during the present study lies within the range obtained from previous studies (4-15 d) (Matthiensen

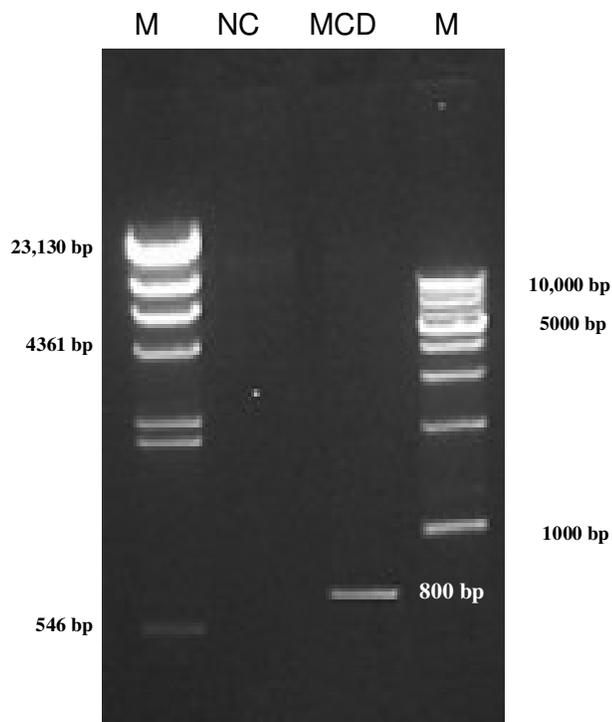


Figure 5. Detection of *mlrA* gene fragments by PCR in microcystin-degrading strains (MCD) and negative control (NC). Amplification of PCR product using the primer set MF-MR.

et al., 2000; Park et al., 2001; Ho et al., 2007b; Lemes et al., 2008). It seems that the difference in the total biodegradation time could be attributed to the difference in bacterial strains, MC variants and initial toxin concentration.

The degradation rate of MC-RR by strain AMRI-03 was high ($1.45 \text{ mg l}^{-1} \text{ d}^{-1}$ at initial concentration 10 mg l^{-1}) when compared to the degradation rate ($0.12 \text{ mg l}^{-1} \text{ d}^{-1}$ at initial concentration $200 \text{ } \mu\text{g l}^{-1} \text{ d}^{-1}$) of MC-RR by *Sphingomonas* CBA4 (Ame et al., 2006). However, the degradation rate of MC-RR by AMRI-03 strain was lower than that of Japanese strain (Y2) of *Sphingomonas* ($8.3 \text{ mg l}^{-1} \text{ d}^{-1}$ at initial concentration 10 mg l^{-1}) (Park et al., 2001). The difference in degradation rate of MC-RR may be due to the difference in bacterial strains and/or initial MC concentration used. The biodegradation rate of MC-RR by AMRI-03 strain afforded a calculated half-life of 4.1 d in this experiment. This half-life time is about 5 times higher than that obtained (18 h) for MC-RR degradation by *Sphingomonas* CBA4 using $200 \text{ } \mu\text{g l}^{-1}$ initial concentration of MCs (Ame et al., 2006). Once again, the difference in half-life times of toxin degradation between the two studies may be due to the difference in bacterial species used in these studies or the difference in initial concentrations.

The results of present study also showed that no losses of MC-RR were evident in control flasks containing no bacteria, confirming that the observed degradation

occurred through the bacterium present in the culture; and there was no abiotic degradation due to physical and/or chemical factors. In addition, the degradation of MC-RR by AMRI-03 occurred in the complete medium (NB medium) indicates that this strain was able to degrade MC-RR irrespective of the presence of carbon and nitrogen sources. These results agree with those of Ishii et al. (2004) reporting that *Sphingomonas* 7CY isolated from of Lake Suwa, can degrade MCs in both minimal medium (M90) and lysogeny broth (LB) medium containing nitrogen and carbon sources. This finding indicates that the bacterial enzyme involved in MC-RR degradation is constitutive. This hypothesis is not a surprise as bacteria can degrade MC along with other organic compounds frequently found in the environment (Christoffersen et al., 2002).

The results of the present study also revealed that AMRI-03 strain possessed a *mlrA* gene and encodes a hydrolytic enzyme to open the cyclic peptide of MCs. The detection of the homologous *mlrA* gene in this strain suggests that MC-RR degradation by the genus *Bacillus* most likely followed a degradation pathway similar to one previously reported by Bourne et al. (1996, 2001) where the enzyme encoded by the *mlrA* gene cleaves the cyclic structure of MC. The presence of this gene in *Bacillus* sp. confirms the hypothesis that *mlrA* gene is unique to MC degraders but not to the genus *Sphingomonas* (Saito et al., 2003). The resultant linear structure of MC is then sequentially degraded by two additional enzymes, encoded by the *mlrB* and *mlrC* genes, respectively. It is also found that the degradation products are essentially non-toxic, which strongly indicated that the microbial degradation using MC-degrading bacteria is quite effective for the detoxification of MCs (Tsuji et al., 2006; Hu et al., 2009). Similarly, this study showed that the toxicity of by-products as determined by PPIA decreased gradually with incubation time and ultimately no detectable amounts of MC was found in the culture medium. Previously, Ho et al. (2007b) reported a good conformity between the results of PPIA and high performance liquid chromatography (HPLC) as methods for the determination of the toxicity of by-products generated from the biodegradation of MC-LR and MC-LA by *Sphingopyxis* sp. LH21.

Conclusion

A new strain of the genus *Bacillus* was isolated from Saudi freshwater lakes capable of MC degradation. In addition, this study is the first to report the degradation of the cyanobacterial hepatotoxins, MCs, by *Bacillus*. The AMRI-03 strain was able to degrade MC-RR at a concentration of 10 mg l^{-1} as it possessed a gene *mlrA*, which encodes the most important enzyme for MC degradation. AMRI-03 was able to degrade MC in a medium with nitrogen and carbon sources, and with nontoxic by-products. This could likely occur along with

other organic compounds found in the aquatic environment. Therefore, the coexistence of such bacteria with MCs in the same environment can contribute to the self-purification of the ecosystem from such potent toxins.

ACKNOWLEDGMENT

The author would like to acknowledge and thank King Khalid University for supporting this study.

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