Final Technical Report for the Lake Erie Protection Fund Small Grants Program
Isolation of Microcystin-degrading Bacteria (SG450-13)

Xiaozhen Mou
Department of Biological Sciences, Kent State University, Kent OH 44242

Abstract

Harmful cyanobacterial (blue-green algal) blooms (CyanoHABs) invade Lake Erie ecosystems and have caused a number of ecological and health consequences. This includes the “drinking water crisis” in Toledo Ohio last summer, which has affected nearly half million residents. Bacterially mediated processes are believed to be the major means to remove microcystins in natural waters. However, taxa and mechanisms involved in microcystin degradations have not been well studied in Lake Erie. This project aimed to isolate bacteria pure culture from Lake Erie and use them as models to study microcystin degradation pathway and its regulating factors. We obtained about 300 bacterial pure isolates. About half were gram positive and the other half were gram negative. Growth assays, BIOLIO analysis and ELISA measurements identified 27 bacterial cultures that carried out microcystin degradation functions. 16S rRNA gene sequencing revealed that microcystin degrading bacteria consisted of Bacillus, Lysinibacillus of Firmicutes; Pseudomonas and Stenotrophomonas of Gammaproteobacteria. Phenotypic and physiological assays were done to examine difference of these microcystin-degrading bacteria in response to nutrient, pH and temperature variations. The obtained microcystin-degrading bacteria strains can serve as good candidates for future bio-engineering procedures to build biofilters for microcystin removal from water systems.

Introduction

The heath of Lake Erie ecosystem is under great pressures created by human activities. The high nutrient loading from Lake Erie tributaries has resulted eutrophic conditions in Lake Erie, especially its Western Basin. As a consequence of eutrophication, harmful cyanobacterial (blue-green algal) blooms (CyanoHABs) occur in Lake Erie every summer with increasing affected area, frequency and intensity (Stumpf et al., 2012). One important harmful effect of these HABs is production and release of high loads of toxic secondary metabolites, such as microcystins, in Lake Erie. Microcystins (MCs) have strong hepatotoxicity and can severely damage mammalian liver cells. The concentration of microcystins in Lake Erie frequently exceeds the World Heath Organization’s guideline values (1 μg/L for drinking water, 20 μg/L for moderate risk for recreation bathing water use) (http://www.doh.wa.gov/Portals/1/Documents/4400/334-177-recguide.pdf). In August 2014, microcystins were detected in drinking water systems of Toledo, Ohio and led to a two-day “tap water ban” for about half million residents (Arenschield, 2014).

The toxicity of microcystins is determined by their concentrations in water, therefore processes that produce and remove microcystins are equally important in controlling the standing concentrations of microcystins, which in turn determines the overall harmfulness of an ongoing CyanoHAB. In Lake Erie, microcystins are mainly produced by cyanobacteria in the genus of *Microcystis*. Extensive studies have been made to understand the factors that control the growth of *Microcystis* and their production of microcystins (Rinta-Kanto and Wilhelm, 2006). Significant restoration efforts have been undertaken by local, state and federal agencies to avoid, control and/or mitigate *Microcystis* blooms in Lake Erie. However, after a short period of success (~20 years), *Microcystis* dominated CyanoHABs returned to Lake Erie in the 1990s (Stumpf et al., 2012). Based on current nutrient and climate model, CyanoHABs are predicted to continue in Lake Erie at least in the near future. Therefore, understanding mechanisms that remove microcystins from water is in great need, as this knowledge is required in developing practical and environmental friendly approaches for cyanotoxin bioremediation.

Microcystins have over 80 variants and all of them share a cyclic structure. This makes them chemically stable under the environmental range of pH, light radiation and temperature (Manage et al., 2010). The removal of microcystins in natural environments is believed to be mainly through biological processes, especially microbially mediated degradation (Edwards and Lawton, 2009). Studies on microcystin-degrading bacteria are relatively limited and most of them have been conducted in artificial environments, such as water treatment plants. These studies have similarly suggested that MC degradation is carried out by a narrow group of bacteria, which are mainly affiliated with the order *Sphingomonadales* of alphaproteobacteria (Ho et al., 2010). Studies on pure cultures of MC-degrading *Sphingomonadales* have demonstrated that MC degradation in these bacteria involves *mlr*-gene based cleavage (Bourne et al. 1996).

However, our recent metagenomic study suggested that MC-degrading bacteria in Lake Erie consist of a diverse group of bacteria (Mou et al., 2013). Our study also indicated that, in Lake Erie, *Methylophilales* and *Burkholderiales* (both are Betaproteobacteria) are important microcystin degraders, while *Sphingomonadales* may only play a minor role in microcystin degradation. Examination of genes induced by microcystins suggested that Lake Erie bacteria may employ an alternative degradation pathway other than the *mlr*-gene based cleavage that are ubiquitously used by *Sphingomonadales*. Therefore, understanding the mechanisms that regulate microcystin degradation in Lake can only be accurately studied using local bacteria as the study systems. The specific aim of this project was to isolate indigenous microcystin-degrading bacteria from Lake Erie and identify their taxonomic identity and factors that affects their growth and degradation activities.

Methods

Sample collection and processing. Water samples were taken from Sandusky Bay, Sandusky Sub-basin and Western Basin of Lake Erie at sites with visible HABs in summer 2013 and 2014 (Figure 1). The water samples were kept on ice and transported back to lab. Immediately after arrival, water samples were filtered through 3.0 μm filters to remove most cyanobacteria, bacteriovores and big particles. The filtrates, which contained the bacterioplankton fraction, were used as inocula to isolate microcystin degrading bacteria.

Figure 1. Sampling stations (yellow dots) in the Lake Erie Western Basin, and Sandusky Bay and Sandusky Subbasin. Map generated by Google Earth.

Isolation of microcystin bacteria. Two approaches were used to obtain microcystin degrading bacteria.

1) *Microcystin enrichment*. In this approach, bacterioplankton in the pre-filtered water were transferred into microcosms (1 L flasks), incubated in the dark at room temperature (~ 22°C) for 7 days with inorganic nutrients (nitrate, ammonium and phosphate). This pre-incubation was to remove the labile organic substrates from the water and created a carbon-limited condition. The pre-incubated cells were then fed with 50 μg/L MC-LR or MC-RR (the most common and

abundant MCs in Lake Erie) and incubated in the dark at 35°C for another 7 days. The total cell abundance in the microcosms was tracked during the incubation using fluorescence microscopy. The consumption of added microcystin compounds were measured using ELISA kits (Abraxis). At the end of the incubation, bacterial pure cultures were isolated from these enrichment samples using both the plate streaking and dilution to extinction methods. The obtained pure cultures were then subjected to further functional screening to confirm their ability in microcystin degradation.

2) *General cultivation followed by functional screening*. Bacterioplankton cells were cultured on a number of solid nutrient media, including ½ and ¼ strength LB media, R2A media, low-nutrient media DR2A and PE03 (Tamaki et al., 2005). Each liter of DR1A contained 0.05 g each of yeast extract, peptone; acid hydrolysate of casein, dextrose, and soluble starch; 0.03 g each of dipotassium phosphate and sodium pyruvate; and 0.0024 g of magnesium sulfate and 15 g of agar. Each liter of PE03 contained 0.05 g each of sodium glutamate, sodium succinate, sodium acetate, yeast extract, casamino acids, sodium thiosulfate, and ammonium sulfate; 5 ml of basal salts solution; and 0.2 ml of vitamin solution (Hanada et al., 1995) and 15 g of agar. Since actionobacteria may account for over half of the freshwater bacterioplankton community, we have also used selective media to specifically cultivate Actinobacteria (Zhang et al., 2010). The cells were incubated at 35°C in the dark. Cells with different colony morphology were selected for further functional screening.

Functional screening. The pureness (or axenicity) of the cultures were be examined microscopically. The pure cultures were then screened for MC-degrading capability by re-inoculate them in BG11 media supplemented with microcystins (microcystin LR or microcystin RR) as the sole carbon sources. Cells with positive growth were individually transferred into wells of BIOLOG MT2 plates. Each well of the BIOLOG MT2 plate contained minimal medium and microcystin-LR or microcystin-RR (0, 10 and 100 μg/L, final concentration). Consumption of microcystins was indicated by color change (into purple) of the wells. Consumption of microcystins was also confirmed by tracking microcystin concentrations using Abraxis microcystin ELISA kits.

DNA extraction and PCR-RFLP analysis. Isolated MC-degrading bacteria were regrown to exponential phase in R2A liquid media. Part of the cells (2 ml) were mixed with freezing media and store at -80 °C. The rest of the cells (~10 ml) were processed for DNA extraction. 16S rRNA genes will be amplified and the PCR amplicons were digested with restriction enzyme cfoI for gel electrophoresis analysis to examine the restriction fragment length polymorphism (RFLP) patterns. Bacterial isolates that have unique RFLP patterns, i.e., cells of different species, were selected for 16S rRNA gene sequencing at the Ohio State Plant-Microbe Genomics Facility. The taxonomic identities of sequenced isolates were determined using the RDP Taxonomy Identifier

Phenotypic characterization of MC-degrading isolates. The phenotypic characteristics of sequenced microcystin-degrading bacteria were examined based on the pigmentation, Gram staining, morphology and motility.

Results and Discussion

A total of 30 enrichment microcosms were set up. And despite the sampling locations, after incubation all of the Lake Erie microcosms showed consumption of added microcystin. After the pre-incubation and following the microcystin addition, most of the samples showed 90% of microcystin consumption with 48 hours. This indicated that indigenous Lake Erie bacterioplankton community contained members that can effectively degrade microcystins.

We have obtained ~300 bacterial isolates. Gram stain assays revealed that 171 were Gram positive and 145 isolates were Gram negative bacteria. Most of the isolates had white colonies and a few appeared red, yellow or pink. Twenty-seven isolates were identified as microcystin degraders (Table 1) by growth assay in minimum media (Figure 1), BIOLOG (Figure 2) and ELISA measurement (Figure3).

Table 1. Obtained microcystin-degrading bacterial isolates.

Strain number	Isolation medium	Sample sites	Gram stains	Colony Color	Microcystin degradation	
					BIOLOG	ELISA
1006	M5	S19	G-	red	+	+
1011	M8	S8	G-	yellow	+	+
1012	M8	S8	G-	white	+	+
1029	M8	S8	G+	white	+	+
1033	M6	S8	G+	white	+	+
1040	M2	S19	G-	white	+	+
1046	M8	S13	G-	yellow	+	+
1047	M2	S4	G-	red	+	+
1049	M6	S4	G-	pink	+	+
1069	M6	S10	G+	white	+	+
1132	M12	S21	G+	white	+	+
1152	M12	S10	G-	white	+	+
2010	M3	S17	G+	white	+	+
2029	M3	S14	G+	white	+	+
2051	M3	S10	G+	white	+	+
2053	M8	S8	G+	white	+	+
2055	M2	S15	G+	white	+	+
2056	M3	S10	G+	white	+	+
2070	M2	S16	G+	white	+	+
2074	M4	S8	G+	white	+	+
2079	M12	S23	G+	white	+	+
2082	M1	S15	G+	white	+	+
2087	M9	S16	G+	white	+	+
2089	M1	S15	G+	white	+	+

2113	M9	S10	G+	white	+	+
2119	M4	S20	G+	white	+	+
2166	M13	S5	G+	white	+	+
2174	M11	S10	G+	white	+	+
2179	M11	S22	G+	white	+	+

Figure 2. Bacterial isolates were grown in BG11 media with microcystin LR or microcystin RR as the sole carbon sources. Cells were incubated in the dark at 35°C, the optical density were checked to monitor the growth of cells. Cells with significant OD value increase and with the corresponding negative controls (no addition of microcystins) stayed clear, were confirmed to be microcystin degradation positive bacteria.

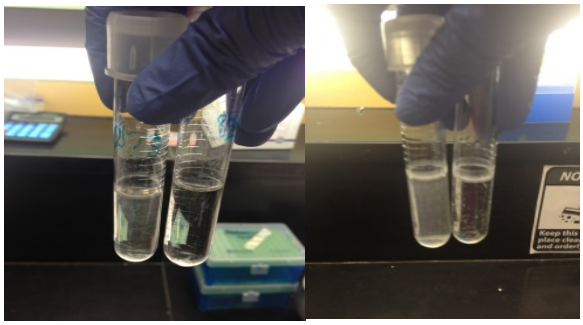

Figure 3. Bacterial cells were grown in the BIOLOG MT2 plates with different concentrations of microcystins. For each bacterial cells, 9 wells were used, three as negative controls (no microcystin addition), three as low microcystin treatments (10 μg/L), and the rest three as high microcystin treatments (100 μg/L). Wells that turned purple indicating the consumption of microcystins.

According to RFLP data, we have selected 16 cells for 16S rRNA sequencing. Sequences were annotated using the RDP Classifier. Two isolates were identified as unclassified bacteria and will be re-sequenced to further examine their taxonomic identity. Six isolates were proteobacteria and all were from the class of Gammaproteobacteria. Five of the Gammaproteobacteria were affiliated with the genus of *Pseudomonas*, the other one was affiliated with *Stenotrophomonas*. There were 8 sequences affiliated with the phylum of Firmicutes all in the class of Bacillales. Five out of these sequences were assigned to the genus level; one was affiliated with *Lysinibacillus*, four with *Bacillus* (Table 2).

Our previous metagenomic study has suggested that betaproteobacteria may play a significant role in microcystin degradation in Lake Erie (Mou et al., 2013). However our isolation effort did not obtain microcystin degraders that are affiliated with Betaproteobacteria. In addition, although Alphaproteobacteria, especially *Sphingomonas* have been repeatedly suggested as important microcystin degraders, we didn’t obtain such taxon in our collection of microcystin degrading bacterial isolates.

Table 2. Taxonomic identities of microcystin-degrading cultures obtained from this and previous studies.

Organisms	**Taxa**	**Reference**
Arthrobacter spp.	Actinobacteria	Manage et al. 2009
Brevibacterium sp.	Actinobacteria	Manage et al. 2009
Microbacterium sp.	Actinobacteria	Ramani et al., 2011
Rodococcus sp.	Actinobacteria	Manage et al. 2009
Bacillus	Firmicutes	Alamri 2012 **This study**
Lysinibacillus	Firmicutes	**This study**
Novosphingobium sp.	Alphaproteobacteria	Jiang et al., 2011
Rhizobium gallicum	Alphaproteobacteria	Ramani et al., 2012
Sphingomonas sp.	Alphaproteobacteria	Bourne et al., 2001 ; Saito, 2003; Somdee et al., 2013; Ho et al., 2007; Chen et al.,2010
Sphingopyxis sp.	Alphaproteobacteria	Ho et al., 2007; Saito et al., 2003; Okano et al., 2009
Bordetella sp.	Betaproteobacteria	Yang et al., 2014
Burkholderia sp.	Betaproteobacteria	Lemes et al., 2008
Methylobacillus sp.	Betaproteobacteria	Hu et al., 2009
Ralstonia solanacearum	Betaproteobacteria	Yan et al., 2004
Paucibacter toxinivorans	Betaproteobacteria	Rapala et al., 2005
Pseudomonas	Gammaproteobacteria	Takenaka et al., 1997; **This study**
Stenotrophomonas sp.	Gammaproteobacteria	Chen, 2010; **This study**

Conclusion

Indigenous Lake Erie bacterioplankton community contains members of microcystin degraders. *Gammaproteobacteria* and *Firmicutes* may dominate the cultural microcystin-degraders. Microcystin-degrading abilities varied among isolates and are affected by temperature and nutrient supplies. The obtained bacterial isolates and knowledge on factors that regulate their toxin degradation activities can potentially be used to develop biofiltration units for effective removal of microcystins from water systems.

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