

**Final Technical Report for the LEPF Small Grants Program
Elucidating Microcystin Degradation Pathway (SG514-2017)**

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Abstract

Lake Erie frequently experience cyanobacterial (blue-green algae) harmful blooms (CyanoHABs). These CyanoHABs release potent liver toxins, i.e., microcystins (MCs), which severely damage liver cells of vertebrate organisms, from fish to humans. However, available studies have been greatly biased towards the production of MCs. Much has been known on MC-producing cyanobacterial genes and taxa, as well as their influencing environmental factors. In contrast, the degradation pathway of MCs is still unclear. From our preliminary study, we found that MC-degrading bacteria in Lake Erie may use a pathway differently than what has known before (i.e., the *mlr* gene-based cleavage pathway). This project aimed to identify the hypothesized novel pathway. MC-degrading bacterial cultures that have been isolated from Lake Erie were used as models for gene knockout and enzyme assay studies. The degradation products were identified using analytical chemistry instruments (HPLC and MS). Our data showed that, as hypothesized, Lake Erie bacteria carried out an *mlr*-independent pathway for MC-LR degradation.

Introduction

Lake Erie frequently experience cyanobacterial (blue-green algae) harmful blooms (CyanoHABs), which bring a number of harmful effects, especially microcystin (MC) contamination. MCs are liver toxins to vertebrate organisms and can be accumulated in aquatic animals through the food chain and ultimately harm the health of human (Carmichael 1992; Hisbergues et al. 2003). Over 90 MC isoforms have been identified; microcystin-leucine arginine (MC-LR) is the most abundant and widely studied one. MC-LR, therefore, often serves as a model in MC related studies. MC-LR and all other MC isoforms possess a monocyclic heptapeptide structure, which makes them highly resistant to physical and chemical breakdowns under typical ranges of temperature, pH and radiation in natural environments (Chen et al. 2010). Heterotrophic bacteria are thought as major degraders of MCs in natural environments (Maruyuma et al. 2006; Kormas et al. 2013; Mou et al. 2013), however, little is known on mechanisms of MC degradation. To adjust this knowledge imbalance, this project aims to elucidate MC degradation mechanism.

Early studies on bacterially mediated MC degradation are dominated by culture-dependent work (Jones et al. 1994; Cousins et al. 1996; Saito et al. 2003). These studies have reported a number of MC-degrading bacterial isolates that are predominantly restricted within an alphaproteobacteria family, i.e., *Sphingomonaceae* (example genera are *Sphingomonas*, *Sphingopyxis* and *Sphingoscincella*) (Jones et al. 1994; Maruyuma et al. 2006; Hoefel et al. 2009). Using *Sphingomonas sp.* ACM-3962 as a model system, MC-LR degradation was first identified to follow a step-wise cleavage process that are encoded by multiple *mlr* genes (Jones et al. 1994). In this degradation pathway, microcystinase (MlrA) initiates the degradation by hydrolyzing the cyclic MC-LR into a linear intermediate, which reduces the toxicity MC-LR by 160 times (Bourne et al. 1996). Therefore, detection of microcystinase gene (*mlrA*) has been widely adopted to probe microcystin-degrading bacteria in various natural lakes (Saito et al. 2003; Somdee et al. 2013) and man-made environments such as sand filters (Hoefel et al. 2009).

However, our recent metagenomic study in Lake Erie revealed contrasting results (Mou et al., 2013). We found that MC degrading bacteria in Lake Erie are taxonomically diverse and

they employ an alternative degradation pathway other than the *mlrA*-gene based cleavage. Our results also highlight the importance of using indigenous bacteria as models to study MC degradation in Lake Erie. In the past years, our lab has successfully obtained a number of MC-degrading bacteria from Lake Erie water and sediments. Preliminary PCR assay of these isolates failed to identify *mlrA* gene using established primers (Saito et al., 2003). Negative results have also been found by other studies (Yang et al., 2014). This project aimed to use these isolates as models to identify MC degradation genes and pathways in Lake Erie. The long-term goal is to use the identified gene sequences to develop new PCR and qPCR assays for sensitive and accurate monitoring in situ MC degradation activities in Lake Erie. Together with known MC production genes, we will be able to have a timely and accurate view on MC dynamics in lake water.

Methods

Bacterial isolates

Three bacterial isolates of our lab collection were used in this study. These are *Acidovorax facilis* LEw-2, *Pseudomonas fluorescence* LEw-24, *Pseudomonas putida* LEw-1033. These bacteria were all isolated from Lake Erie. PCR assays have indicated that none of these isolates carry *mlrA* gene, the key gene involved in *mlr*-based cleavage pathway (Fig. 1).



Fig 1. PCR amplification showed negative results for *mlrA* gene presence in tested isolates.

Chemically competent cells

Bacterial isolates were grown in R2A media to the stationary phase and then treated with TSS solution to prepare chemically competent cells. These chemically competent cells were utilized for mutation using Tn5.

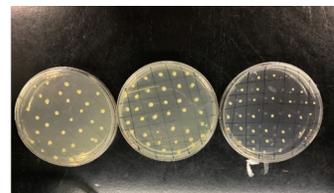


Fig. 2 Tn5 mutants on LB agar plates.

Tn5 transposon mutagenesis and Screening

Tn5 (*Kan^r*) transposon mutagenesis kit from Epibio, Madison, WI, was used for transformation. The transposon along with the transposase and the chemically competent MC-degrading cells were mutated according to the instructions on the kit. The mutants were grown to an $OD_{600} \approx 0.4$

and stored at -80 °C in 15% glycerol. A library of 15,000 mutants (Fig. 2) was prepared for screening for the presence of MC- (MC non-degrading mutant). Screening was performed using a 96 well plate analysis, with each well containing the mutant cells and MC-LR. The optical density readings for each well were taken at intervals of 0, 24 and 48 hrs. All of the mutants were screened for the ability to degrade MC-LR by growing them in nutrient rich media (R2A) to reach $OD_{600} \approx 0.1$ then transferred to BG11 media supplied with MC-LR (the sole carbon source). Inhibition of growth or death after the transfer was tracked by measure the optical density in each culturing tube. Mutants that showed no growth were labeled as MC- mutants and were used for sequencing and identification of MC-degrading genes.

Restriction endonuclease cleavage analysis

Total genomic DNA from the above MC- mutants were isolated using the Chargeswitch gDNA mini bacterial extraction kit (Thermo Fisher Scientific) and treated with the restriction enzyme HindIII and BamHI for cleaving the region of mutation. Gel electrophoresis (1% agarose gel) was performed to confirm insertion of transposons in the MC- mutants. After confirmation, the extracted DNA of MC- mutants were sequenced at the Macrogen USA (MD, USA) and Genomics Core facility of the Case Western University, Ohio.

Cell extract of MC+ strain

Cultures of the MC-degrading bacterial wild strains were inoculated in LB medium and incubated overnight at 30° C with shaking at 200 rpm. The cultures (15 ml at $OD_{600} \approx 0.3$) were harvested after 48 hrs of incubation (the time to reach the late log phase) and centrifuged at 5000 rpm to obtain a pellet. The supernatant was decanted from each tube and replaced by an equal volume of PBS (Phosphate buffer saline, pH=7). The cells were resuspended and repelleted for 3 times to wash out the residue of culturing media. The resuspended cells were then sonicated at 4 °C in a Cole Palmer 8894 sonicator. The cell debris were centrifuged at 18,000 rpm for 20 min at 4 °C, the CE (supernatant) was decanted and passed through 0.2 µm filters to remove remaining cell debris. The filtered CE was collected in a clean tube and used for enzyme assays..

Enzyme assays

CE (0.25 ml) was mixed with MC-LR (final concentration of 1 µg/ml) and PBS to reach a final volume to 1.5 ml. The mixture was briefly vortexed and then incubated at 30°C. Samples (200 µl) were taken at 0, 2, 6, 8, 12 and 24 hrs after incubation and analyzed by HPLC.

HPLC

High performance liquid chromatography (HPLC) was performed using a reverse-phase HPLC with a Spherisorb ODS-2 C18 column (5 mm; 250 by 4.6 mm). The protocol was modified based on Bourne et al. (1999). The mobile phase consisted of 32% acetonitrile (solvent A). The acetonitrile content was raised to 55% (solvent B) with a 20-min linear gradient. The column was washed with both solvents for 5 min at a flow rate of 3 ml/min before starting analysis. Samples (200 µl) were injected into the system and the compounds were detected using a UV detector at 238 nm. The degradation products were then identified based on their retention time.

Ion spray mass spectrometry (MS)

A triple-quadrupole mass spectrometer (model Burk Esquire) was used to identify compounds in the digested CE. A pneumatically assisted electrospray (ion spray) interface was used to introduce samples into the atmospheric pressure ionization source of the MS. The interface sprayer was operated at a positive potential of 5.6 kV. Degradation products were analyzed by a combination of single MS, MS-MS and liquid chromatography-MS. Data were processed by the software package MacSpec (Sciex) (Bourne et al., 1996).

Results and Discussion

The three bacterial isolates used in this study all have strong ability in degrading MC-LR (Fig. 3). By 24 hours, all added MC-LR was completely degraded from 5 µg/ml to undetectable.

Due to the budget constrain, transposon mutagenesis was only

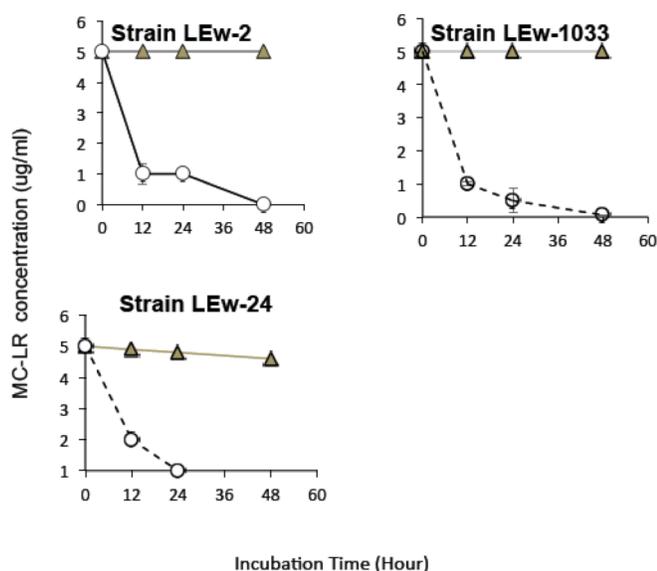


Fig. 3. The used LE bacterial isolates can degrade MC-LR within 24 hours (open triangles), while the MC-LR that added to the water stayed un-degraded (white circles).

performed for one isolates. Different from the proposal, we used *Pseudomonas fluorescence* LEw-24 instead of *Acidovorax facilis* LEw-2. This is because the Tn5 transposon mutagenesis kit suggests that it does not work well with *Acidovorax* strains. A total of 15000 mutants were generated. After screening, 6 mutants

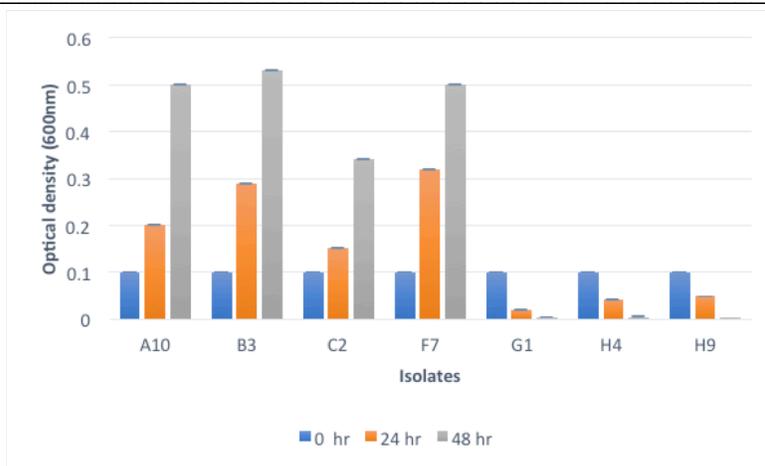


Fig. 4. Examples of four MC+ mutants (A10, B3, C2, F7) and three MC- mutants (G1, H4 and H9) identified by the growth assay.

showed the lost of MC-degradation ability and were identified as MC- mutants (Fig. 4). The MC- mutant was not able to grow in BG11 media supplied with MC-LR (the only carbon source). These MC- mutant however can still actively grow on R2A (nutrient rich medium) plates. These findings indicate that the MC-degrading genes of these mutants were interrupted. Sequencing of the mutated regions was attempted for several times with MacroGen (4 times), Genomics Core Institute (2times). MacroGen was mistakenly used 16S rRNA gene primers for sequencing and after 3 times of sequencing still didn't give out reliable data. We then switched to Genomics Core Institute. The tested run looks promising and we are expecting the sequencing results in a couple weeks. Based on the LC-MS results and previous metagenomic study (Mou et al., 2013), we suspected the knockout genes are affiliated with xenobiotic metabolism.

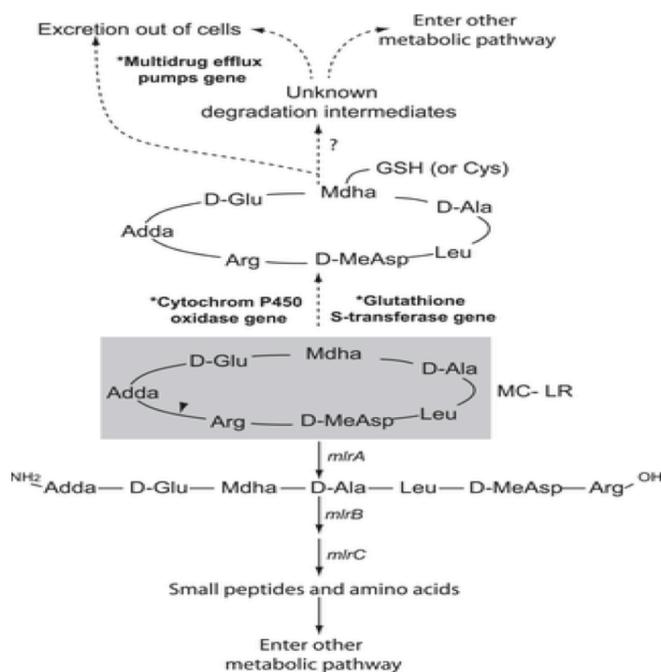


Fig. 5. Proposed MC-LR degradation pathway (dashed arrows) that is independent from *mlr* genes.

HPLC-MS analysis was performed to identify the degradation intermediates and products. Previous metagenomic analysis has suggested that in Lake Erie, bacteria might taken a novel degradation route that is independent of *mlr* genes (Fig. 5 for LEw-2).

HPLC analysis showed that MC-LR (retention time 3.3 min) gradually decreased to undetectable from T0 (beginning of the incubation) to T10 (10 h after the start of the incubation). The HPLC chromatographs (Fig. 6 and Fig.7) compared with the known *mlr*-gene dependent pathway were very different. At the T8 time point, the MC-LR peak height significantly decrease , while another three new peaks emerged, indicating that the MC-LR degraded into multiple

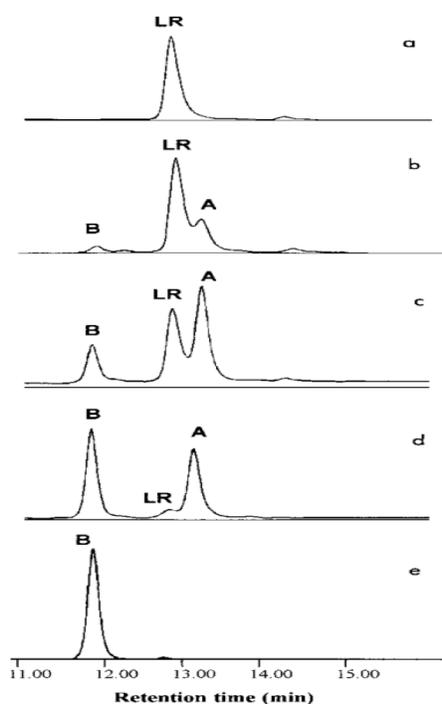


Fig. 7 HPLC analysis of the degradation intermediates and products of MC-LR by *Spingomonas* strains that carry out *mlr* genes (Bourne et al., 1996).

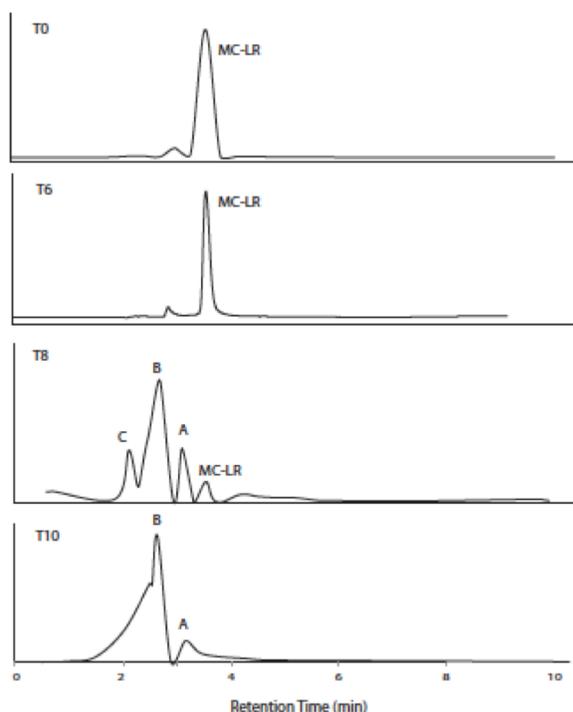


Fig. 6 HPLC analysis of the degradation intermediates and products of MC-LR by strain LEW-2

compounds. At time T0, product B still remained at high concentration, indicating it as the final products.

Further MS analysis showed confirmed the MC-LR peak (m/z 996). Further product purification and MS/MS was needed to identify compound A, B and C. This process had been slow due to the status of the instrument (was broken until late April). Initial runs has confirmed

that A (m/z 864), B (m/z 745) and C (m/z 701) were not products during *mlr*-based MC degradation, as the parent and daughter ions on the MS were different from those of the *mlr*-based MC pathway. However, further data analysis was needed to achieve final confirmation of the chemical compound structure.

Outreach activities

Results of this study are shared with the project agency advisor Heather Raymond (HAB Coordinator, Lead Hydrogeologist, Ohio EPA) and other stakeholders that have potential interests on microcystin degradation research, including superintendents of several local water treatment plants.

Acknowledgements

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References

- Carmichael W (1992) The toxins of cyanobacteria. *Sci Am* 2:78-86
- Bourne D, Jones G, Blakeley R, Jones A, Negri A, Riddles P (1996) Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin-LR. *Appl Environ Microbiol* 62:4086–4094
- Chen J, Li B, Wei Z, Shao H, Jing D, Yan F, Zhi Q (2010) Degradation of microcystin-LR and RR by a *Stenotrophomonas* sp. strain EMS isolated from Lake Taihu, China. *Int. J Mol Sci* 11: 896-911
- Cousins I, Bealing D, James H, Sutton A (1996) Biodegradation of microcystin- LR by indigenous mixed bacterial populations. *Water Res* 30:481-485

- Hisbergues M, Christiansen G, Rouhiainen L, Sivonen K, Borner T (2003) PCR-based identification of microcystin-producing genotypes of different cyanobacteria genera. *Arch Microbiol* 180:402-410
- Hoefel D, Adriansen C, Bouyssou M, Saint C, Newcombe G, Ho L (2009) Development of an *mlrA* gene directed TaqMan PCR assay for quantitative assessment of microcystin-degrading bacteria within water treatment plant sand filter biofilms. *Appl Environ Microbiol* 75:5167-5169.
- Jones G, Orr P (1994) Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *Water Res* 28:871-876
- Kormas K, Lymeropoulou D (2013) Cyanobacterial toxin degrading bacteria: who are they? *BioMed Res Int* 2013: 1-12
- Maruyama T (2006) *Sphingosinicella microcystinivorans* gen. nov. sp. nov. a microcystin-degrading bacterium. *Int J Syst and Evol Microbiol* 56: 85-89
- Mou X, Lu X, Jacob J, Heath R (2013) Metagenomic identification of bacterioplankton taxa and pathways involved in microcystin degradation in Lake Erie. *PLoS ONE* 8:1-14.e61890.
- Saito T, Okano K, Park D, Itayama T, Inamori Y, Neilan A, Burns P, Suquira N (2003) Detection and sequencing of the microcystin LR- degrading gene, *mlrA* from new bacteria isolated from Japanese lakes. *FEMS Microbiol Lett* 229:271-276
- Somdee T, Thunders M, Rucks M, Allison M, Page R (2013) Degradation of [Dha (7)] MC-LR by a microcystin degrading bacterium isolated from Lake Rotoiti, New Zealand. *ISRN Microbiol* 2013:1-8.

Yang F, Zhou Y, Yin L, Zhu G, Liang G, Pu Y (2014) Microcystin-degrading activity of an indigenous bacterial strain *Stenotrophomonas Acidaminiphila* MC-LTH2 isolated from Lake Taihu. PLoS One 9:e86216.