

# Isolating microcystin-LR degrading enzymes for algal bloom toxin remediation\*

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*Harmful algae blooms negatively affect aquatic ecosystems and communities that rely on those bodies of water for drinking water. Many algal blooms contain cyanobacteria which produce harmful toxins. Many algal blooms, like those in Lake Erie, contain the cyanobacteria *Microcystis aeruginosa* which produces the toxin microcystin-LR. This toxin disrupts cellular pathways and inhibits the removal of phosphates. As a result, the toxin causes liver and kidney damage in humans which can lead to death. To combat the harmful effects of microcystin-LR produced from algal blooms, our group intends to target the toxin, microcystin-LR, in the aquatic environment. Our previous design of an enzyme spray containing *mlrA*, *mlrB*, *mlrC*, *mlrE*, and *mlrF* will be sprayed in affected ecosystems to degrade the toxin. These enzymes will be produced by inserting the enzyme genes isolated from *Sphingomonas* sp. and *Sphingopyxis* sp. into *Escherichia coli* which will mass produce the enzymes. In this study, our group grew *Sphingomonas* sp. PS-60 in an attempt to isolate the *mlrA* gene.*

Keywords: microcystin-LR, *mlrA*, *Sphingomonas* sp. PS-60, algal blooms



**H**armful algal blooms (HABs), which have been documented for centuries, affect many ecosystems (hab.who.edu, 2022). Lake Erie has warm, shallow waters and is surrounded by agricultural lands which create large amounts of fertilizer run-off into Lake Erie. These characteristics provide the perfect environment for algal growth (Ohio Sea Grant College, 2022). Algal blooms establish dangerous habitats by consuming oxygen, blocking sunlight, and producing detrimental toxins. Thus, algal blooms impact human health activity in Lake Erie's surrounding communities by impacting the biological functions of domestic animals through drinking water containing cyanobacteria and increasing the chance of liver tumors in

humans (Butler et al., 2009). In Toledo, Ohio, algal blooms in Lake Erie and the Maumee River poison the community that sources its water directly from the lake and the river (Rogener, 2020). In 2014, a combination of heavy winds and an HAB prompted citywide orders to pause the use of all forms of tap water for three days. The harsh winds caused waves of water to directly flow into water treatment facilities, raising the level of the algal blooms in Lake Erie to a quantity the World Health Organization deems toxic for humans. This halt in access to water was a significant burden on the eleven million people who depend on Lake Erie water (Ohio Sea Grant College, 2022).

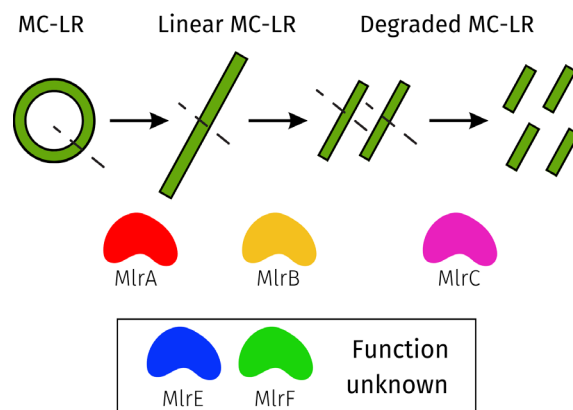
Algal bloom toxicity originates from Microcystin-LR (MC-LR). Released by *M.*

\* The authors were mentored by Beth Pethel from Western Reserve Academy, Hudson OH. Please direct correspondence to: pethelb@wra.net. This is an Open Access article, which was copyrighted by the authors and published by BioTreks in 2023. It is distributed under the terms of the Creative Commons Attribution License, which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited.

*aeruginosa* when its cell walls burst during death, MC-LR is a cyclic heptapeptide made up of seven amino acids: D-alanine (D-Ala), L-amino acid (L-Leu), D-Methylaspartic acid (D-Me Asp), L-amino acid (L-Arg),  $\beta$  amino acid (Adda), D-Glutamic acid (D-Iso Glu), and N-Methyl Dehydroalanine (Moha) (Cyanosite, n.d.). The stable structure of MC-LR makes it resistant to common chemical breakdown mechanisms such as hydrolysis or oxidation (Butler et al., 2009; Cyanosite, n.d.). MC-LR denatures enzymes, known as protein phosphatases, inhibiting them from performing common steps in many biological pathways. For example, it disrupts the process of removing phosphates from amino acids like serine and threonine, thus causing a build-up of phosphorylated protein, which induces cell apoptosis and, accordingly, liver cell death (Yoshida et al., 1997).

*Sphingomonas* bacteria, which reside primarily in soil and water, produce a set of enzymes that degrade MC-LR—MlrA, MlrB, MlrC, MlrD, MlrE, and MlrF—to obtain a carbon source (Savola, 2016). MlrA linearizes the cyclic nature of MC-LR through hydrolysis, breaking the peptide bond between  $\beta$  amino acid (ADDA) and L-arginine (L-Arg) to form a linearized product (Savola, 2016). Following this breakdown, MlrB, a serine protease, breaks apart the bond between the Leucine (L-Leu) and D-alanine amino acids to form a tetrapeptide (Massey & Yang, 2020). MlrC, a metalloprotease, further degrades the tetrapeptide into amino acids (Savola, 2016). MlrD aids in the transport of microcystin-LR into the cell (Massey & Yang, 2020). The specific functions of MlrE and MlrF are unknown, but they are critical to microcystin-LR degradation (Metzloff, 2019a; 2019b). We inserted genes for enzymes MlrA, B, C, E, and F into five different DH5 $\alpha$  *E. coli* cultures optimized for plasmid production (MCLAB, n.d.; **Figure 1**). We did not include MlrD because this enzyme imports the toxin into a cell. Instead, the transformation will include the enzymes produced within DH5 $\alpha$  *E. coli* to break down the microcystin toxin.

The project aims to mass-produce and purify the enzymes, allowing us to test them for optimal enzymatic activity against the toxin. We envision using these enzymes to



**Figure 1.** The breakdown of microcystin-LR through MlrA, MlrB, and MlrC enzymes. The function of MlrE and MlrF enzymes is unknown.

directly remediate toxin-producing algal blooms and bolster filtration mechanisms in water treatment facilities. As past experiments observed successful heterologous expression of MlrA in *E. coli* (Lee et al., 2006), we designed our first experiment to transform MlrA from *Sphingomonas* sp. PS-60 into *E. coli*. The experiment will determine the presence of the MlrA gene in *Sphingomonas* sp. PS-60. Unfortunately, we were unable to isolate the MlrA gene from *Sphingomonas* sp. PS-60. Instead, we designed our experiment to transform MlrB from *Sphingosinicella microcystinivorans* Y2 into *E. coli*.

## Materials and methods

### Overview

The final goal of our project was to extract the genes encoding for the Microcystin-degrading enzymes—mlrA, mlrB, mlrC, mlrE, and mlrF—and move them into DH5 $\alpha$  *E. coli*, which we tested for its ability to decompose toxins released by algal blooms. Our experiments aim to test the effects of one of the five MC-degrading enzymes on disintegrating microcystin-LR.

To begin this process, we attempted to confirm the chosen bacterial strain contained the MlrA MC-degrading enzyme. The ideal choices of bacteria include *Sphingopyxis* sp.

USTB-05, *Novosphingobium* sp. THN1, and *Sphingomonas* sp. ACM-3962, which have been tested for containing MC-biodegrading gene cluster mlrABCD by Wei et al. (2021). However, we were neither able to find a site to purchase any of these bacteria nor obtain them from a laboratory that has worked on them in the past. Instead of locating a specific strain, we focused on finding a strain within the desired genus.

Since the majority of MC-degrading bacteria isolated from Lake Taihu, China, belong to *Sphingomonas* and *Sphingopyxis* (Wang et al., 2021), we decided to use *Sphingomonas* sp. PS-60 for our initial testing of MC-degrading enzymes. Since this bacterium is still mostly unexplored by scientists, we want to begin with checking that the mlrA gene exists within the bacteria, since there is a higher chance that other MC-degrading enzymes are contained in this bacterium if we are able determine that the mlrA gene is contained in this bacterium.

### First Experiment

#### Growing *Sphingomonas* sp. PS-60

Once we received bacteria numbered ATCC 31461, we followed the directions provided by ATCC to rehydrate the bacteria. We allowed the bacteria to grow in a tube with 6 mL of nutrient broth and a 194 mL flask of nutrient broth overnight in a 30°C shaking incubator. We used Carolina Biological nutrient broth which is composed of beef extract and pancreatic digest of gelatin. Upon visible turbidity, we streaked 2 nutrient agar plates using the flask and 2 nutrient agar plates using the tube. Using isolated colonies from those plates, we streaked two additional plates on nutrient agar.

#### DNA Extraction and Purification

We followed the DNA Purification procedure for gram-negative bacteria, Thermo Scientific GeneJET Genomic DNA Purification Kit #K072 (Ravintheran et al., 2019). Purification included centrifugation, digestion, proteinase K digest, vortexing, RNase A, vortexing, incubation, Lysis solution, vortexing, 50% ethanol, vortexing,

purification column, wash buffer I, centrifugation, wash buffer II, centrifugation, elution buffer, incubation, storing the purified DNA at -20 °C (Ravintheran et al., 2019).

### PCR

We attempted to obtain the gene mlrA from the *Sphingomonas* sp. PS-60 DNA that we extracted. We used the following mlrA primers: Forward: 5'-GACCCGATGTTCAAGATACT-3' and Reverse: 5'-CTCCTCCCACAAATCAGGAC-3' (Zhang et al., 2017). All primers were purchased from ThermoFisher. We created our PCR mix (Table 1) with the end goal of a 50µl sample, labeled one PCR tube as *Sphingomonas* mlrA and the other as a negative control, and proceeded to add 25µl of taq PCR Master Mix to each tube.

Table 1. Table showcasing the components, volume, and end concentration of each component used in PCR.

Component	Volume	Concentration (µmol/µl)
Taq PCR Master Mix	25 µl	2.5 units Taq DNA Polymerase 1x QIAGEN PCR Buffer* 200 µM of each dNTP
Forward 10x primer mix	1 µl	0.4 µM
Reverse 10x primer mix	1 µl	0.4 µM
Template DNA	5 µl	0.1µg/ul
RNase free water	18µl	-
<b>Total</b>	<b>50 µl</b>	-

To achieve the ratio of 0.2µM of each primer in the sample, we added 1µl of the forward 10x primer mix and 1µl of the reverse primer mix. We added 5µl of template DNA and 18µl of RNase free water to the PCR tube labeled *Sphingomonas* mlrA. We added 23µL of RNase free water negative control. We briefly centrifuged the

two tubes and placed them in the PCR machine (BIO RAD T100 Thermal Cycler) and ran the following PCR program listed in **Table 2**.

*Table 2. This table shows the times, temperatures, and number of run cycles used in our PCR protocol.*

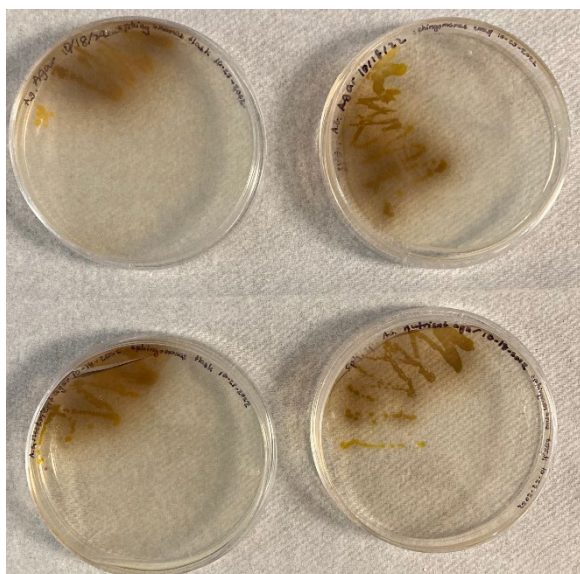
Step	Time	Temperature
<b>Initial</b>	3 minutes	94°C
<b>Denaturing</b>		
<b>3-Step Cycling:</b>		
Denaturing	45 seconds	94°C
Annealing	45 seconds	50°C
Extension	1 minute	72°C
<b>Number of Cycles</b>	34	
<b>Final</b>	10 minutes	72°C
<b>Extension</b>		

### DNA Gel Electrophoresis

We analyzed our PCR products using gel electrophoresis. We used 12.5uL per sample and a control with 4uL of minipcr 1kb DNA ladder. We ran the gel for 30 minutes at 90 volts.

### BLASTn

We performed a BLASTn alignment of the



*Figure 2. An image of streaked plates shows the dark brown substance produced by Sphingomonas sp. PS-60 after four days of incubation.*

full mlrB sequence and that of *S. microcystinivorans* Y2 with the default settings of BLASTn.

## Results

### Growing Sphingomonas sp. PS-60

Following supplier guidelines, our liquid cultures reached turbidity after two days. Similarly, our streaked plates appeared dark brown with a haze around the colonies after three days (**Figure 2**). After two days, the plates appeared more orange yellow with the color appearing darker closer to the initial streak (**Figure 3**).

### PCR and Gel Electrophoresis

As seen in **Figure 4**, no bands were present. After repeating DNA extraction, PCR, and gel electrophoresis no bands appeared in the negative control or sample lanes. Since there were no visible bands in the bacterial or negative control lane in any of our three trials, this suggests a high possibility that *Sphingomonas* sp. PS-60 does not contain the mlrA gene.



*Figure 3. After two days, the colonies on this streaked plate appeared a dark yellow. The original streak appeared a darker orange yellow while the most isolated colonies appeared yellow.*

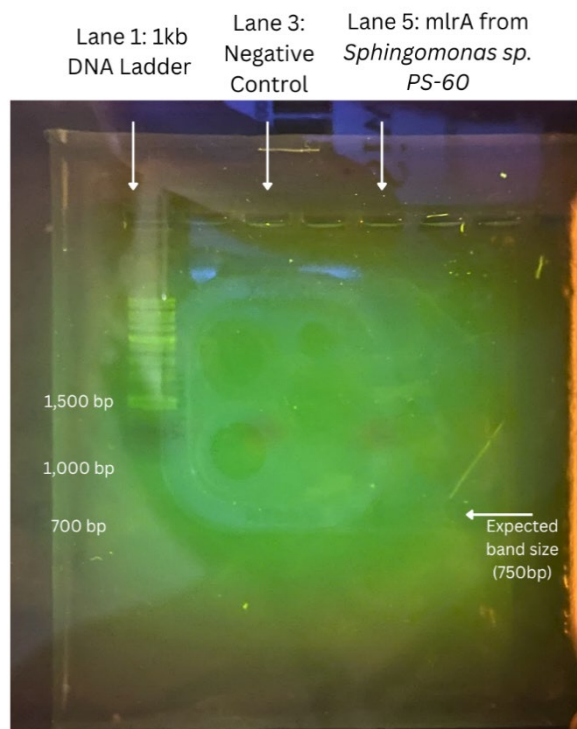


Figure 4. An image of the electrophoresis gel shows that only the ladder appeared. There are no bands in the negative control lane or the lane with bacterial DNA

### BLASTn Results

According to BLASTn results (Figure 5), there is an 88% identity between the genome sequence of *Sphingosinice microcystinivorans* Y2 and the DNA sequences for the mlrB gene, which gives some promise in the presence of the mlrB gene in *S. microcystinivorans* Y2 (Altschul et al., 1990).

## Discussion

The negative result of our gel electrophoresis has several implications. First, the DNA extraction estimated that we harvest up to  $2 \times 10^9$  bacterial cells. Due to limited resource availability, we weren't able to measure the concentration of our bacterial culture using either fluorescence techniques (e.g. nanodrop or Qubit) or a particle counter. Therefore, we roughly estimated the amount of liquid culture, around 1  $\mu$ L, for purification. In turn, we likely used

insufficient DNA samples for PCR, resulting in very low concentrations for starting DNA in our PCR (Life Technologies, n.d.). However, since we conducted the experiment three times with different amounts of DNA samples, insufficient DNA samples might not be the most probable cause of a negative result for our PCR.

Primer design could be another source of error. We took the primer sequence for *Sphingomonas* PS-60 from Zhang et al. (2017), which analyzed the roles of mlrA to mlrD from *Sphingopyxis* sp. in MC-LR degradation, because *Sphingopyxis* sp. is a gram-negative bacterium from the same genus as our chosen bacteria. However, the primers for *Sphingopyxis* sp. could be unsuitable for *Sphingomonas* PS-60, which can cause either the synthesis of an unrelated gene fragment or no reaction at all as no parts of the primer could bind with the bacterial sequence. This source of error remains under investigation, but it could be a potential factor that we need to examine further in future experiments. Moreover, the purity of extracted DNA may also negatively influence the test result of electrophoresis (Purity - Does, n.d.). Inhibitors and proteins left in purified nucleic acids will lead to the inhibition of enzymatic reactions, including PCR and ligation.

## Next steps

Upon discovering that *Sphingomonas* sp. PS-60 may not contain the genes necessary for our design, we found that the bacteria *S. microcystinivorans* Y2 may instead contain the genes we seek to isolate. In future experiments, we intend to grow *S. microcystinivorans* Y2, which we have obtained from ATCC. Once we receive the bacteria, we will grow it, then extract and purify the DNA, and eventually, run a PCR to amplify and isolate the mlrB gene.

## Author contributions

This project is based off Western Reserve Academy's 2022 Biobuilder Team's project "Mass-producing microcystin-LR-degrading enzymes for algal bloom toxic remediation".

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RID: ZHWYRBV911R
Job Title:RBWX01000008.1 Sphingosinicella microcystinivorans
Program: BLASTN
Query: RBWX01000008.1 Sphingosinicella microcystinivorans strain DSM 19791 Ga0244538_102, whole genome shotgun sequence ID:
lcl|Query_353135(dna) Length: 678046
Subject:None ID: lcl|Query_353137(dna) Length: 1626

Sequences producing significant alignments:

Query E Per. Acc. Scientific Common Max Total
Description Name Name Taxid Score Score
cover Value Ident Len Accession
None provided 0 2090 2090 0%
0.0 88.50 1626 Query_353137

Alignments:
>
Sequence ID: Query_353137 Length: 1626
Range 1: 1 to 1626

Score:2090 bits(2317), Expect:0.0,
Identities:1439/1626(88%), Gaps:0/1626(0%), Strand: Plus/Minus

Query 83962 TTATGGAAGCCACCGGAACATAAGTCTGTTTCAGATTGACGGTGTGAAATGTAACACTCTG 84021
||||| ||||| || ||||| | | ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1626 TTATCGAAGCCGCTGAACACTATCCCGTTCAGATTTACGGTGTGAAATGTAAGATTTG 1567

Query 84022 AGGACCAGTTGCATGCAATGAAAATGCGGCGTTCGGGGCCACCAGGTCATCCGCCAGCGC 84081
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1566 AGGACCAACAGCATGTAATGAAAATGCGGCGTTCGGGGCCACAAGGTCATCTCCAGCGC 1507

Query 84082 AGCAACGAGCGGAACCCGACCCCGTGGAGTATTCAGCGTCCATCCATTGCAATCCCTTGA 84141
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1506 AGCAGCTAACGGCACCCGCCCTGCCGGAGTATCCAGCGACCAACCCTTCGTATCCCTTGA 1447

Query 84142 CAGGGTTACAGTGACACCGAGTCATCGCTCCGATATCGACCGGGCCTGAACTGCTTGGC 84201
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct 1446 CAGGGTTACGGTAATACCAAGCTCATCGCTGTGATATTGTCCGGGCCAAAACAACCTTGGC 1387

```

Figure 5. The BLASTn result and sequence alignment for *mlrB* and *S. microcystinivorans* Y2.

All authors contributed to the research, writing, and proofreading of this paper. R.R. was the principal designer and creator of the video.

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