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Isolation and Characterization of Microcystin-Degrading Bacteria from Lake Erie

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Abstract

Heterotrophic bacteria are suggested as the major agents that degrade microcystins (MCs), a major cyanotoxins, in natural environments. However, little is known of the taxonomic and functional diversity of MC-degrading bacteria in Lake Erie of the Laurentian Great Lakes, the largest freshwater system on earth. This study obtained six bacterial pure isolates from Lake Erie with an ability to use MCs as the sole carbon and energy sources. MC degradation rates of the isolates were impacted by temperature and pH. The key gene for MC degradation (*mlrA*) were failed to be PCR amplified from for all 6 MC degraders, indicating they may possess a novel MC degradation pathway. In addition for potentials used in MC bioremediation, two isolates maybe can offer extra benefits as biofertilizers.

Keywords Microcystins · HAB · CyanoHAB · Bacterial degradation · Lake Erie

Cyanobacterial harmful blooms (CyanoHABs) frequently develop in eutrophic waters and lead to multiple negative effects (Paerl and Otten 2013). One of the harmfulness is the production of cyanotoxins, which causes significant ecological and health concerns, especially in waters that serve as drinking water supplies and recreational grounds (Bláha et al. 2009; Paerl and Otten 2013). Cyanotoxins produced during freshwater CyanoHABs are dominated by a group of liver toxins known as microcystins (MCs; Hisbergues et al. 2003). Over 90 MC isoforms have been identified; microcystin–leucine arginine (MC–LR) is the most abundant and widely studied one (Paerl and Otten 2013). MC–LR and all other MC isoforms possess a monocyclic heptapeptide structure, which makes them highly resistant to physical and chemical breakdowns in natural environments (Chen et al. 2010). MC degradation in nature is primarily carried out by heterotrophic bacteria (Kormas and Lymperopoulou 2013).

Early studies on MC biodegradation are dominated by culture-dependent work, which have reported a number of MC-degrading bacterial isolates that are predominantly restricted within the *Sphingomonadaceae* family (such as *Sphingomonas, Sphingopyxis* and *Sphingoscinicella*) (Jones and Orr 1994; Maruyama 2006; Hoefel et al. 2009). Using *Sphingomonas* sp. ACM-3962 as a model system, MC–LR degradation was first identified to follow a stepwise cleavage process that is initiated by microcystinase (MlrA) (Bourne et al. 1996); therefore, its gene (*mlrA*) has been widely adopted to probe MC-degrading bacteria in various natural lakes (Somdee et al. 2013) and man-made environments (Hoefel et al. 2009). However, the ubiquity of *Sphingomonadaceae* and *mlr* genes to MC degradation is in question. Recent studies have identified many non-*Sphingomonadaceae* MC degraders, and many of them do not carry *mlrA* genes (Manage et al. 2009; Somdee et al. 2013; Yang et al. 2014). Moreover, a recent metagenomic study indicates that a diverse group of bacterial taxa, especially those affiliated with betaproteobacteria, and xenobiotic metabolism-related genes may govern MC degradation in Lake Erie (Mou et al. 2013b). However, no direct evidence is available.

Lake Erie is the shallowest and most southern lake among the five Laurentian Great Lakes, which represent the largest surface freshwater system on earth. However, like most surface freshwaters, Lake Erie has become eutrophic and host periodic blooms of MC-producing CyanoHABs (Michalak et al. 2013). A bloom event in Lake Erie in August 2014

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resulted in a shutdown of public water supply from 500,000 residents (Henry 2014). Due to the obvious ecological, economic and health significance, MC transformation has been a hot research and remediation topic in Lake Erie and CyanoHAB-impacted freshwaters worldwide. However, majority studies have been focused on mechanisms that regulate the synthesis of MCs, leaving MC degradation relatively understudied.

This study aimed to obtain one of the first collections of MC-degrading bacterial pure cultures from Lake Erie and examine their potential diversity in taxonomy, phenotype, physiology and MC-degrading efficiency and pathway (i.e., whether or not involve *mlr* genes).

Materials and Methods

Samplings were performed once every month in Lake Erie from July to September 2013 in areas with frequent and long-lasting CyanoHABs (HAB Bulletin, NOAA, 2017). Surface water samples were collected and filtered through 5 µm pore-size membrane filters (Whatman membrane filters, Pittsburgh, PA, USA). The resulting filtrates for each sample were amended with NH_4NO_3 and KH_2PO_4 (both at 0.05 mM, final concentrations) and incubated in the dark at 30°C with a rotation rate of 200 rpm for 7 days. Afterwards, the incubated water was amended with 1 µg/mL MC–LR (Cayman Chemicals, Ann Arbor, Michigan) and incubated at the same condition for another 7 days. Aliquots of 50 µL of incubated water were then spread onto agar plates prepared with R2A, IPS-I or LB media and incubated at 30°C in the dark for 48 h. Single colonies were re-streaked for at least five times to obtain pure isolates.

Bacterial pure cultures were re-inoculated in liquid R2A media and incubated at the same condition for 48 h. Cells were harvested by centrifugation and washed three times with PBS before being resuspended in BG11 media and incubated without carbon source for 24 h. The starved cells were transferred into BIO-LOG MT2 plates (Hayward, CA, USA) containing MC–LR (0, 0.1, 1 and 10 µg/mL; final concentrations). Samples without bacterial cells were also included. The MT2 plates were incubated in the dark at 30°C for a total of 48 h. Color changes were tracked by measuring the absorbance at 630 nm using a Synergy Multimode Reader (Biotech, VT, USA).

MC–LR degradation was also confirmed by growing carbon-starved bacterial pure cultures in 1 µg/mL of MC–LR in BG11 media. Bacterial growth was tracked by measuring optical density (OD_{600}) during the incubation. MC–LR consumption in the samples was also measured using a Microcystins Nodularins ADDA ELISA kit (ABRAXIS Warminster, PA, USA) following the manufacturer's protocol.

Carbon-starved cells were prepared following procedure described above and mixed with BG11 media that contain 5 µg/mL (final concentration) of MC–LR. Cells were then incubated at various temperatures or pH and tracked for MC degradation as above. The intial measurements for the pH were made before the incubation (pH 123 m, Hannah Instruments, MI, USA) and tracked during the experiment. No significant change was identified in any treatment during the experiment.

Phenotypic characteristics of putative MC-degradation bacterial isolates, including colony pigmentation, cell morphology and gram nature, were examined using standard procedures (Clark 1976). Motility of strains was examined by wet mount and Leifson's staining technique for observation of the presence of flagella and cell movement using light microscopy (Clark 1976).

Genomic DNAs of pure isolates were extracted using an UltraClean GelSpin DNA Extraction Kit (MoBio, Carlsbad, CA, USA). 16S rRNA genes were amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) following a PCR program that included 95°C for 3 min, followed by 30 cycles at 95°C for 1 min, at 59°C for 1 min, at 72°C for 1 min, and a final step at 72°C for 10 min. PCR amplicons were examined using agarose gel (1%) electrophoresis and then purified using an Ultra Clean Gel Purification Kit (MoBio, Carlsbad, CA, USA) before sequenced for near-full length at the Macrogen Corporation, USA. The obtained 16S rRNA gene sequences were deposited into the NCBI GenBank under the accession numbers of KX185385–KX185387, KX185392, KX185397 and KX753361.

PCR amplification of *mlrA* gene were used the primers MF (5′-CCTCGATGACCTCGTAGC-3′) and MR (5′-CGG CCATCTTCAGCAAT-3′) following a PCR program that included 94°C for 1 min followed by 30 cycles at 94°C

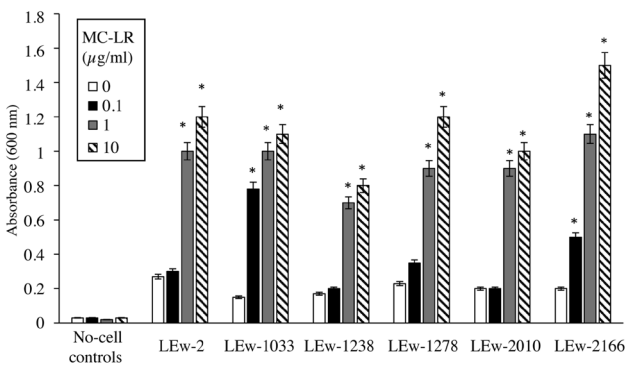

Fig. 1 MC–LR degradation at different concentrations by isolated bacterial strains revealed by BIOLOG-MT2 screening. Asterisks are to label samples with significant higher absorbance value than their corresponding no-MC-addition controls ($p < 0.05$)

for 20 s, at 60°C for 10 s, and at 72°C for 30 s (Saito et al. 2003). PCR amplicons were examined using agarose gel (1%) electrophoresis. A synthetic positive control of *mlrA*-containing plasmid was obtained from the USGS Michigan-Ohio Water Science Center (Dr. D. France).

All statistical analyses were performed using R (R core team, 2013). *t* test was used to compare the absorbance values among the different MC–LR treatments in the MT2 BIOLOG assay. Two-way Analysis of Variance (ANOVA) was used to compare bacterial growth and MC–LR consumption during the growth assays under different temperatures and pH values. Pearson's product moment correlation analysis was performed to examine potential correlations between the MC–LR degradation rate and temperature or pH.

Results and Discussion

The culturing effort obtained a total of 500 bacterial strains from Lake Erie water samples. Six of these isolates showed increased absorbance values in BIOLOG MT2 assays when supplied with MC–LR (10 µg/mL) as the single carbon source ($p < 0.05$; Fig. 1). Meanwhile, corresponding negative controls of these six isolates maintained low absorbance values (Fig. 1). These six bacteria were putatively identified as MC-degrading bacteria. When MC–LR supply was reduced to 1 µg/mL, the degradation rate of all six isolates significantly reduced and at 0.1 µg/mL, only half of the isolates still showed positive consumption of MCs within 48 h ($p < 0.05$). This indicate that substrate availability affects

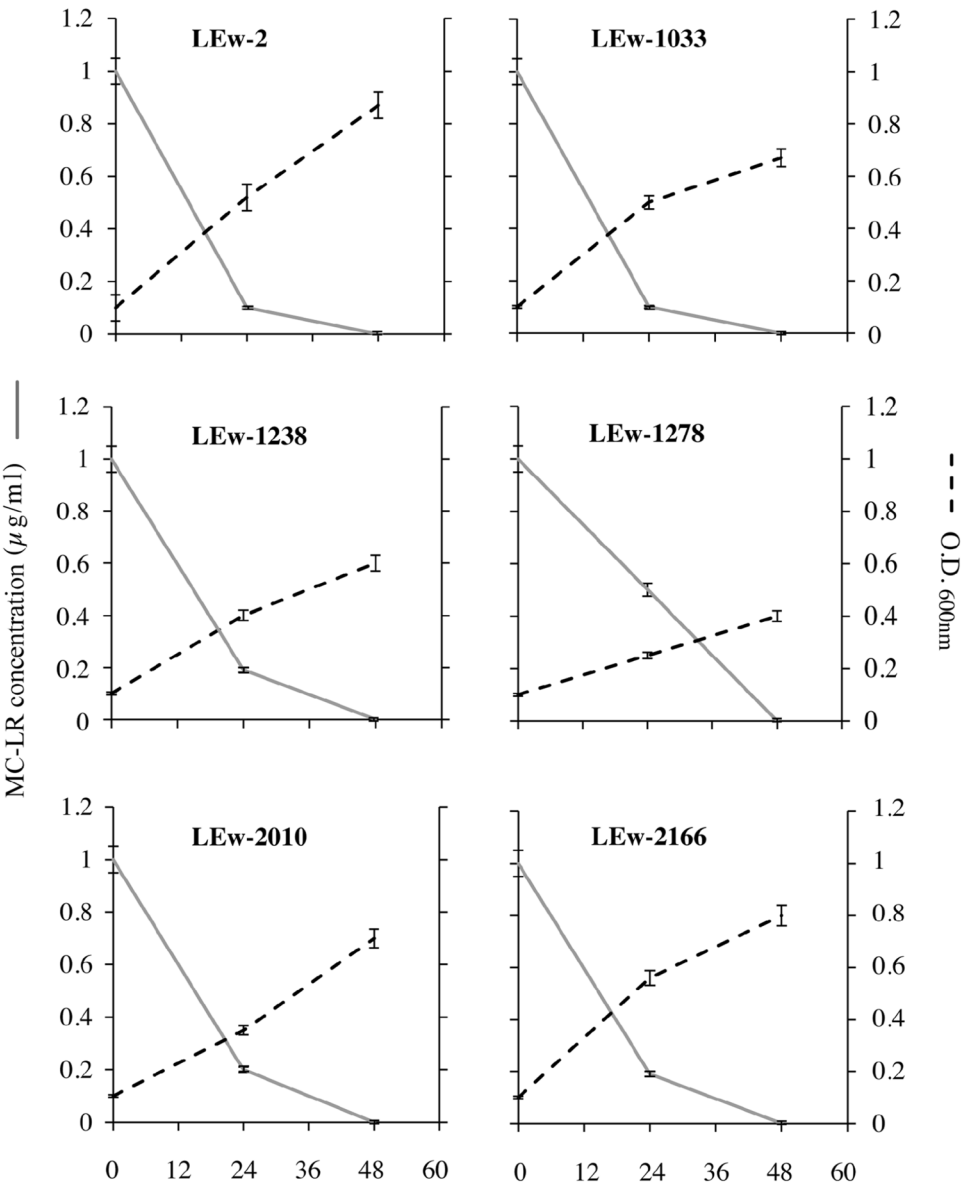

Fig. 2 Changes of MC–LR concentration (left axis, solid gray line) and optical density OD_{600} (right axis, dash black line) in BG11 growth media supplied with 1 µg/mL of MC–LR and bacterial isolates

MC-degradation rate of bacteria and that bacteria may have varied affinity to MC–LR.

The MC-degradation ability of the six putative MC-degrading strains were confirmed by growth assays. With the same starting OD_{600} value (0.5), volume (5 mL) and incubation conditions, all six bacterial isolates fully consumed MC–LR (Fig. 2). Coincident with consumptions of MC–LR, bacterial cell number significantly increased for all of the tested isolates (*t* test; $p < 0.05$). LEw-1278 showed a close to linear degradation of MC–LR over time, while the other five isolates had a faster consumption of MC–LR in the first 24 h than the second 24 h. Based on measurements at 24 h, the average MC–LR consumption rate for the six isolates was 0.03 µg/mL/h.

Obtained MC-degrading bacterial isolates showed a range of phenotypic traits in cell colony pigmentation, cell morphology, and gram nature (Table 1).

In accordance with the diversity of phenotypic tratis, near full-length 16S rRNA genes sequences revealed that the six MC-degrading bacterial isolates were broadly affiliated with different bacterial taxa (Fig. 3). Four isolates were affiliated with proteobacteria, either in the beta (LEW-2) or the gamma-classes (LEw-1033, LEw-1278 and LEw-2166). Isolate LEw-2 was affiliated with *Acidovorax facilis* of Burkholderiales (betaproteobacteria). Members of Burkholderiales accounted for ~20% of bacterioplankton community in the western basin of Lake Erie (Mou et al. 2013a) and have been suggested as important MC-degraders in Lake Erie (Mou et al. 2013b). This is the first report for a *Acidovorax* member to degrade MCs, although several *Acidovorax* species have been reported to degrade complex cyclic organic compounds, such as monocyclic and polycyclic aromatic hydrocarbons (PAHs) (Singleton et al. 2001). The species *A. facilis* is a common soil taxon and some strains are used as plant growth promoting (PGP) inoculants in bio-fertilizers (such as Accomplish LM by Loveland Products) to increase the yield of corn and soybean crops (Adesemoye et al. 2017). This is partly because *A. facilis* carry nitrilases that can degrade organic nitrogen to carboxylic acid and ammonia, which in turn can be readily used by plants (Wu et al. 2007). Application of *A. facilis*-containing bio-fertilizers has been studied in cornfields of Northwestern Ohio (Lentz 2015);

Table 1 Phenotypic characteristics of microcystin-degrading bacterial isolates obtained from water samples of Lake Erie

Isolate ID	Colony color	Shape	Gram nature	Flagella	*mlrA* (+/−)
LEw-2	Pink	Cocci	−	−	−
LEw-1033	Yellow	Coccobacilli	-	−	−
LEw-1238	White	Cocci	+	+	−
LEw-1278	Orange	Bacilli	−	−	−
LEw-2010	White	Cocci	+	−	−
LEw-2166	White	Coccobacilli	−	−	−

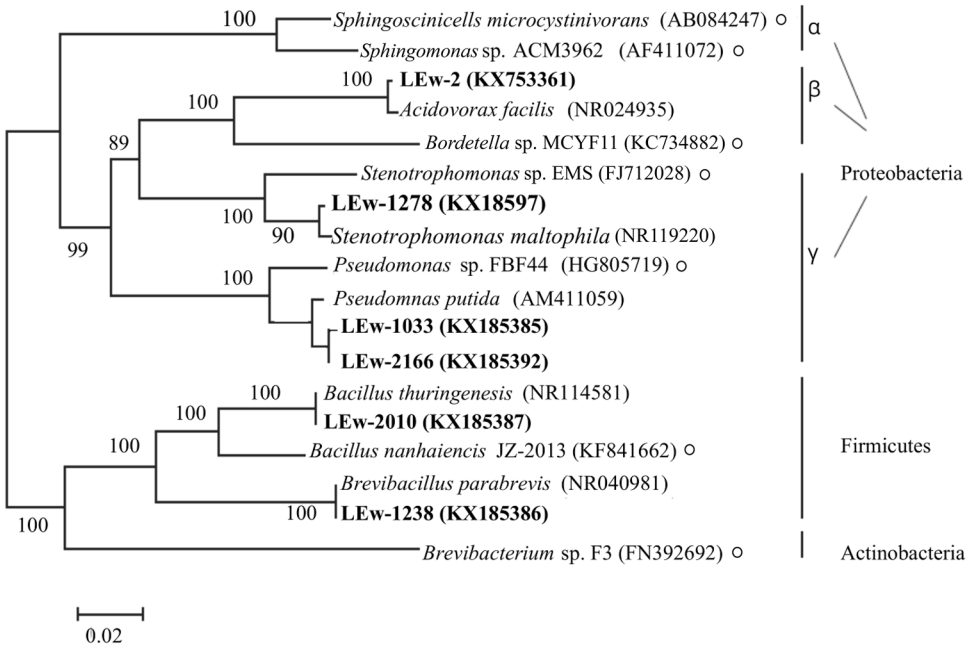

Fig. 3 A neighbor-joining phylogenetic tree based on partial sequence of 16S rRNA gene showing the relationship sequences of isolated MC-degrading bacteria and their closely related relatives. Gray circles are to label previously identified MC-degrading bacteria. The GenBank accession numbers of the sequences are shown in parentheses. Bootstrap values that are higher than 50% are show at the branch nodes (1000 resampling). The scale bar represents 0.02 nucleotide substitutions per position

runoff from these and other agriculture fields is a potential source of this taxon in Lake Erie.

For MC-degrading isolates that were affiliated with gammaproteobacteria, LEw-1033 and LEw-2166 were both closely affiliated with *Pseudomonas putida. Pseudomonas* species, have shown abilities to degrade recalcitrant aromatic compounds (Guerin and Boyd 1995; Morono et al. 2004), including MC–LR (Yang et al. 2014). Consistent with our findings, *mlrA* has not been identified in any of the previously isolated MC-degrading gamma-proteobacteria species. Isolate LEw-1278 was affiliated with *Stenotrophomonas maltophila*. A MC-degrading *S. maltophila* has also been isolated from Lake Taihu, China (Yang et al. 2014) with a slightly slower MC degradation rate.

We also obtained two Gram-positive MC-degrading isolates in the phylum of Firmicutes, i.e., LEw-1238 (*Brevibacillus brevis*) and LEw-2010 (*Bacillus thuringiensis*). These species can degrade multiple refractory compounds, such as polyethylene and pyrene (Alhassani and Ashraf 2007; Nehra et al. 2016), but this is the first time to show their MC degradation abilities. Like *A. facilis* (LEw-2), *B. brevis* strains have been recommended to serve as PGP inoculants (Nehra et al. 2016). Therefore, applying *A. facilis* and *B. brevis* strains to farmland could potentially offer extra benefits in bio-remediating microcystin contaminations, which is a raising concern in areas that use Cyano-HAB impacted water for irrigation.

It is noted that none of the obtained isolates was affiliated with or close relative to the *Sphingomonadaceae* of Alphaproteobacteria (Fig. 3), which family contains most of the reported cultured MC-degrading isolates and perform MC degradation via *mlr* genes-based cleavage pathway (Ho et al.

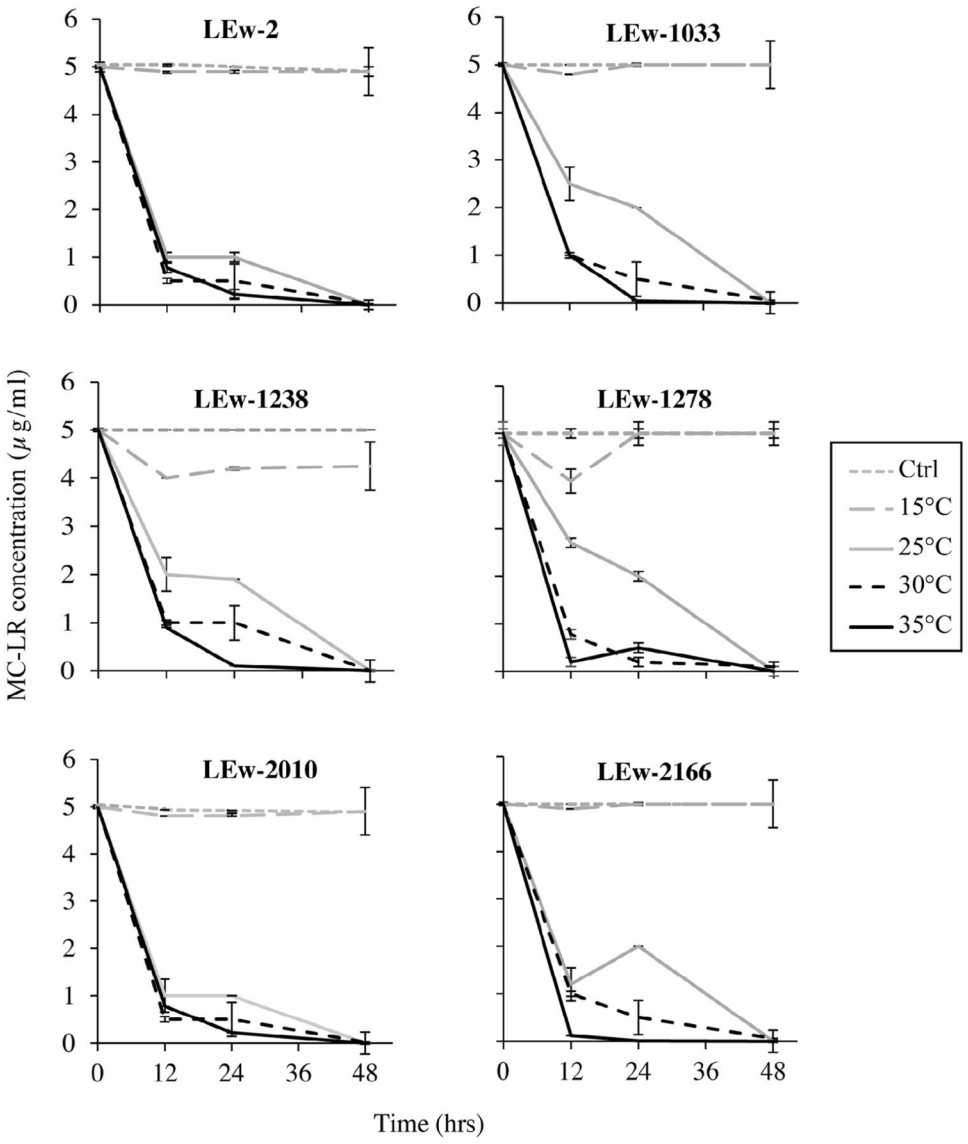

Fig. 4 Effect of temperature on MC–LR degradation by MC-degrading bacteria

2007). In our subsequent PCR analysis, *mlrA* genes were not detected in any of our six isolates. In contrast, PCR was consistently successful when amplifying *mlrA* from the positive control (data not shown). Therefore, both of the taxonomic and functional gene make up of our MC-degrading isolates suggest alternative pathway(s) exist and sometime even be more important than *mlr*-based cleavage in MC degradation in freshwater systems. This is consistent with the increasing evidence from both culture-dependent and independent studies (Mou et al. 2013b; Kansole and Lin 2016). Our isolates can serve as models to elucidate the proposed novel pathways and genes involved in the breaking down of MC–LR.

For each isolate, both of the bacterial growth activity and MC–LR degradation rate were positively correlated with temperature ($r = 0.75$; $p < 0.05$; Fig. 4). At 15°C, only LEw-1238 showed significant MC–LR degradation. As incubation temperature increased (25°C, 30°C and 35°C), isolates gradually increased MC–LR degradation rate and consumed 100% of added MC–LR (5 µg/mL) in 48 h. MC–LR degradation activities were observed at the highest rate when pH values were at 7 and 8 (Fig. 5). The degradation activity was significantly inhibited at either higher or lower pH conditions (ANOVA; $p < 0.05$), except for LEw-2. For LEw-2, MC–LR degradation rate was maintained at all tested basic pH conditions; MC degradation was only inhibited at acidic pH conditions (Fig. 5).

The suggested effects of T and pH on MC degradation were consistent with findings of previous culture studies and field observations (Phujomjai et al. 2015; Zhang et al. 2015). A faster degradation rate at higher T could potentially

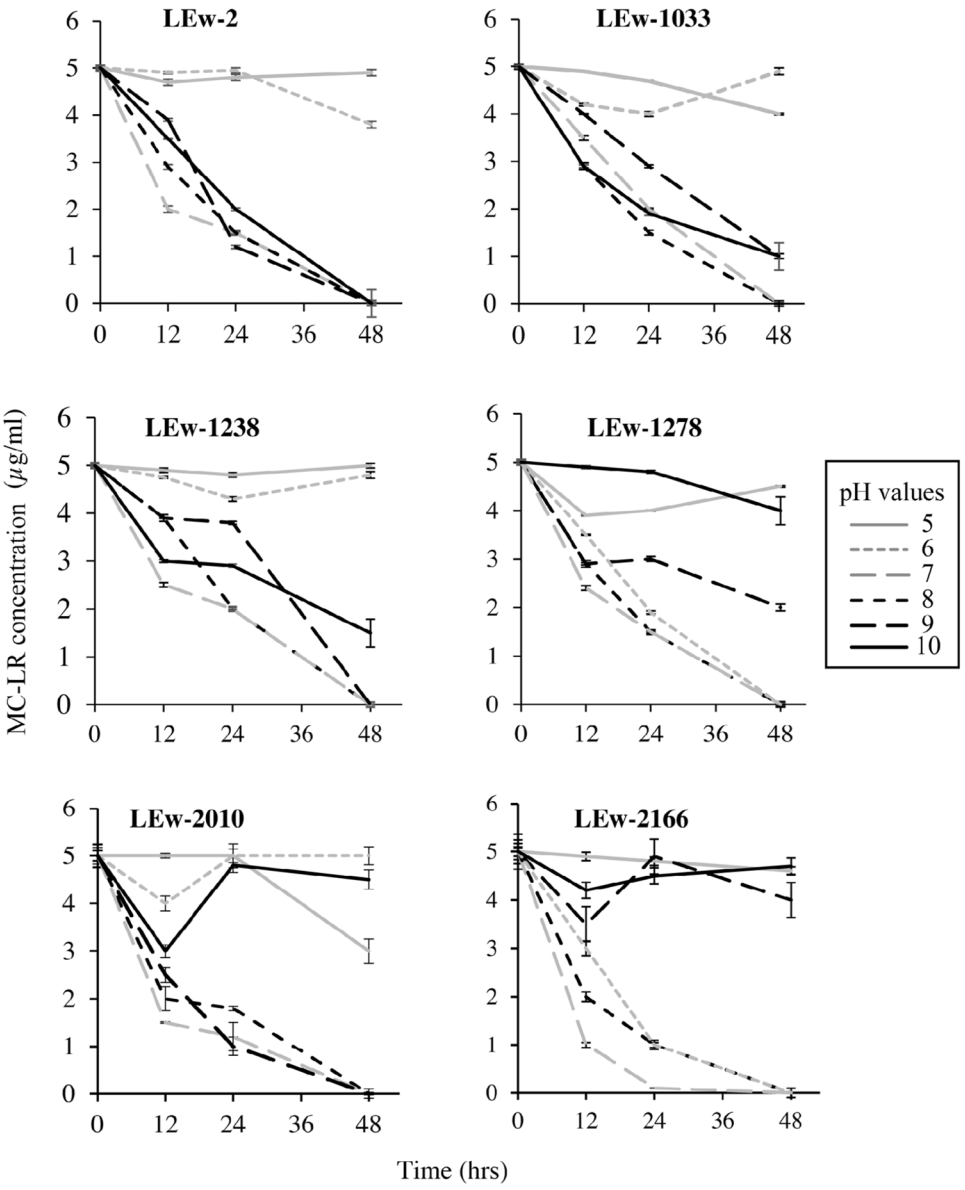

Fig. 5 Effects of pH on degradation of MC–LR by MC-degrading bacteria

offset an increased production of MC by cyanobacteria during warm weather conditions. The optimal pH for our Lake Erie isolates in MC–LR degradation was at pH 7–8, which matches the typical pH range during CyanoHAB blooms in Lake Erie (Mou et al. 2013a). In addition, during drinking water treatment process, water under treatment is typically maintained at a low basic pH range (Stevik et al. 1999). Our isolates bacteria have the potential to serve as inoculatants to augment MC degradation during the filtration step in drinking water treatment processes.

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