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Genomic signatures of Lake Erie bacteria suggest interaction in the *Microcystis* phycosphere

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Abstract

Microbial interactions in harmful algal bloom (HAB) communities have been examined in marine systems, but are poorly studied in fresh waters. To investigate HAB-microbe interactions, we isolated bacteria with close associations to bloom-forming cyanobacteria, *Microcystis* spp., during a 2017 bloom in the western basin of Lake Erie. The genomes of five isolates (*Exiguobacterium* sp. JMULE1, *Enterobacter* sp. JMULE2, *Deinococcus* sp. JMULE3, *Paenibacillus* sp. JMULE4, and *Acidovorax* sp. JMULE5.) were sequenced on a PacBio Sequel system. These genomes ranged in size from 3.1 Mbp (*Exiguobacterium* sp. JMULE1) to 5.7 Mbp (*Enterobacter* sp. JMULE2). The genomes were analyzed for genes relating to critical metabolic functions, including nitrogen reduction and carbon utilization. All five of the sequenced genomes contained genes that could be used in potential signaling and nutrient exchange between the bacteria and cyanobacteria such as *Microcystis*. Gene expression signatures of algal-derived carbon utilization for two isolates were identified in *Microcystis* blooms in Lake Erie and Lake Tai (*Taihu*) at low levels, suggesting these organisms are active and may have a functional role during *Microcystis* blooms in aggregates, but were largely missing from whole water samples. These findings build on the growing evidence that the bacterial microbiome associated with bloom-forming algae have the functional potential to contribute to nutrient exchange within bloom communities and interact with important bloom formers like *Microcystis*.

Introduction

Cyanobacterial harmful algal blooms (cHABs) occur annually in both freshwater and marine systems. These blooms have the potential to be disruptive to aquatic ecosystems due to both the scale of accumulated biomass and the release of secondary metabolites that have metabolic consequences for other organisms [1, 2]. *Microcystis* is a pervasive genus of cyanobacteria that

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forms blooms on every continent except Antarctica [2]. Some species of *Microcystis* produce microcystins, potent hepatotoxins that can limit access to potable water [3, 4]. The threats to ecosystem services and public health posed by cHABs have resulted in numerous and diverse mitigation and management strategies ranging from simple aeration of small freshwater systems [5] to application of chemicals [6, 7] or barley straw [8]. In the last decade, considerable advancements have been made in the application of biotic solutions that inhibit cHABs, including those based on microorganisms, although these technologies have yet to be successfully validated beyond laboratory-scale, albeit environmentally relevant, studies [9]. Several bacteria have been identified that are capable of degrading microcystins produced by cyanobacteria or are algicidal [10–12]. Previous work suggests such antagonistic interactions may occur in the *Microcystis* phycosphere [13, 14], a microenvironment that surrounds phytoplankton cells analogous to the rhizosphere in plant roots [15]. However, bacterial-phytoplankton interactions are not exclusively antagonistic, as evidence from marine phytoplankton studies suggests mutualistic relationships also exist [16]. The phycosphere provides a nutrient-rich environment for heterotrophic bacteria due to the release of organic molecules by the phytoplankton, including dissolved organic carbon [17]. In the early phases of cell growth, phytoplankton release lower molecular weight molecules, such as amino acids and carbohydrates, while higher molecular weight molecules, such as polysaccharides, nucleic acids, and proteins, can be released into the phycosphere during lysis [17]. The release of molecules by phytoplankton can attract heterotrophic bacteria to the phycosphere, ultimately leading to a potential exchange of nutrients between the bacteria and phytoplankton [16, 17].

The physiology of *Microcystis* spp. makes them well-suited for nutrient exchange with heterotrophic bacterial partners. *Microcystis* spp. are colonial cyanobacteria surrounded by an exopolysaccharide layer with which bacteria are tightly coupled [18–20]. Previously, multiple strains of bacteria have been found to impact the formation of *Microcystis* colonies and exopolysaccharide production [21]. Furthermore, the species of bacteria associated with the *Microcystis* phycosphere differ based on whether they are particle associated ($>$ 10 μm) or free-living [22]. While we know that heterotrophic bacteria are closely associated with *Microcystis* colonies [23], the potential mechanisms of exchange between the partners in the freshwater cHAB phycosphere have yet to be characterized. In the current study we provide genomic data that support the previously proposed interaction by which heterotrophic bacteria (heterobionts) utilize the carbon released by *Microcystis*, while *Microcystis* may benefit from nutrient or vitamin products released by the heterotrophic bacteria [23].

Here, we report on the genomic content of five bacterial strains isolated from *Microcystis* aggregates in western Lake Erie in August 2017, focusing on the genetic potential for interaction with *Microcystis*. Access to the genomic information of *Microcystis*-associated heterotrophic bacteria has provided new insight into the potential microbial interactions and metabolic pathways that occur within *Microcystis* blooms, specifically that nutrient exchange may occur in the *Microcystis* phycosphere. To demonstrate the ecological relevance of these strains, we surveyed available metatranscriptomic data from *Microcystis* spp. blooms in North America and China. These observations show the potential for bidirectional, mutualistic interactions in the *Microcystis* phycosphere which could serve as a future target for cHAB mitigation.

Results and discussion

Sample collection and environmental conditions

Surface samples were collected from four stations in the Western Basin of Lake Erie in August 2017 (WE02, WE04, WE13, and MB18) using 20 μm and 80 μm mesh plankton nets. These pore sizes were chosen to exclude free-living bacteria and enrich for those bacteria that are

associated with *Microcystis* aggregates. Previous studies have characterized *Microcystis*-associated bacteria in size fractions ranging from ≥ 3 μm up to 100 μm. Environmental conditions at the time of sample collection were reported by Boedecker et al. [24].

Isolate characteristics. Five isolates were targeted for full genome sequencing from a library of over 100 individual isolates generated from the Lake Erie bloom samples. These isolates were selected based on their N-utilization and pigment production capabilities. The five isolates selected to be sequenced were identified at the genus level *via* 16S rRNA and *rpoB* gene sequences from genomic data as an *Exiguobacterium* sp. (JMULE1), an *Enterobacter* sp. (JMULE2), a *Deinococcus* sp. (JMULE3), a *Paenibacillus* sp. (JMULE4), and an *Acidovorax* sp. (JMULE5) (**S1-S5 Figs in S1 File**). *Exiguobacterium* sp. JMULE1 is a gram-positive, rod-shaped, motile bacterium that produces orange pigmented colonies. Members of this genus have previously been shown to impact colony formation in individual strains of *Microcystis*, both positively [25] and negatively [26], depending on the strains tested. *Enterobacter* sp. JMULE2 is a gram-negative, rod-shaped bacterium from the class Gammaproteobacteria. Multiple *Enterobacter* strains have been found to have microcystin degradation capabilities and induce cell aggregation by *Microcystis* [21, 27, 28]. *Deinococcus* sp. JMULE3 is a gram-positive rod-shaped bacterium belonging to the class Deinococci that produces pink-orange pigmented colonies; members of the Deinococcus-Thermus phylum have been previously identified in *Microcystis* metagenomes [13, 29]. *Paenibacillus* sp. JMULE4 is a gram-negative, rod-shaped bacterium in the class Bacilli that produces endospores, and members of this genus have been previously identified in cyanobacterial bloom communities [30]. Isolates of *Paenibacillus* are commonly applied as algal bioflocculants, as they induce algal cell aggregation [31, 32]. *Acidovorax* sp. JMULE5 is a gram-negative, rod-shaped bacterium belonging to the class Betaproteobacteria. Several strains of this genus have been isolated from samples of *Microcystis*, both in culture [33] and from the environment [34].

The microbiome of freshwater lakes and rivers is often dominated by members of the phylum Actinobacteria. While none of the bacteria isolated for this study were members of the Actinobacteria phylum, this is consistent with recent work demonstrating that this phylum is significantly depleted in populations closely associated with the *Microcystis* phycosphere [35]. In fact, members of the phyla Proteobacteria and Firmicutes are enriched in *Microcystis* aggregate samples compared to free water bloom samples in several studies [22, 25, 35, 36]. It has been hypothesized that Actinobacteria likely do not rely on *Microcystis*-derived carbon due to actinorhodopsin activity [35, 37]. Furthermore, one benefit to phycosphere bacteria may be protection from predation by zooplankton, to which ultramicrobacterial Actinobacteria are not as vulnerable [38, 39].

Sequencing output, assembly, and annotation

The number of raw reads ranged from 197,286 (*Deinococcus* sp. JMULE3) to 455,299 reads (*Enterobacter* sp. JMULE2) (**S1 Table in S1 File**). Read correction done within the PacBio *de novo* assembly pipeline resulted in 27,959 (*Deinococcus* sp. JMULE3) to 69,289 reads (*Enterobacter* sp. JMULE2) (**S1 Table in S1 File**). Genome completeness was assessed with the PATRIC Genome Assembly tool and ranged from 98.2% (*Paenibacillus* sp. JMULE4) to 100% (*Enterobacter* sp. JMULE2 and *Acidovorax* sp. JMULE5) (**Table 1**).

Acidovorax sp. JMULE5 was the only isolate for which a single contig was obtained (**Table 1**). Its closest sequenced relative, *Acidovorax* sp. KKS102 was originally isolated from soil and has been shown to degrade polychlorobiphenyl (PCB) (**Table 2** [40]). Recently, a strain of *Acidovorax* was isolated from a *Microcystis* bloom in Korea, but genomic information is not currently available for this isolate [33]. At 5,742,593 bp, the genome of *Enterobacter* sp.

Table 1. Characteristics of genome assemblies obtained from the PacBio *de novo* assembly pipeline in the CLC Genome Finishing Module.

Isolate	# of Contigs	N50 (Mbp)	GC %	Total Length (Mbp)	Coding Sequences	Genome Completeness
Exiguobacterium sp. JMULE1	2	3.11	47.13	3.15	3,289	99.5%
Enterobacter sp. JMULE2	20	0.61	54.79	5.74	5,736	100%
Deinococcus sp. JMULE3	5	3.28	69.75	4.22	4,208	99.5%
Paenibacillus sp. JMULE4	19	0.39	49.95	5.40	5,888	98.2%
Acidovorax sp. JMULE5	1	5.45	64.48	5.45	5,101	100%

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JMULE2 is comparable to the most closely related isolate based on *rpoB* identity, *Enterobacter asburiae* sp. L1 (~5.4 Mbp; **Table 2** [41]). Based on *rpoB* identity and the two-way average nucleotide identity between the two genomes [42], it is unlikely that *E. asburiae* sp. L1 and the JMULE2 isolate are the same species (**Table 2**) [43]. However, *Exiguobacterium* sp. JMULE1 is likely the same species as its most closely related sequence isolate, *Exiguobacterium* sp. MH3, with a two-way ANI score > 95% (**Table 2**) [43]. *Exiguobacterium* sp. MH3 was isolated from the rhizosphere of duckweed (*Lemna minor*) and has both growth promoting and stress alleviating effects on its freshwater eukaryotic host [44, 45]. *Paenibacillus napthalovorans* sp. 32-OY is likely also the same species as *Paenibacillus* JMULE4, with an ANI score > 95% (**Table 2**). *P. napthalovorans* sp. 32-OY was originally isolated from soil and can metabolize dibenzothiophene, an organosulfur compound [46, 47]. The ability to degrade high molecular weight compounds is a signature of bacteria associated with *Microcystis* aggregates and may indicate an important role in the transformation of algal-derived organic compounds in bloom communities [22, 48].

Functional annotation

To better understand the dominant metabolic pathways encoded by each Lake Erie isolate, the protein families of each genome were annotated using the Subsystems (SEED) approach [49, 50]. Overall, the five genomes contain the highest number of subsystems related to the Carbohydrates, Amino Acids and Derivatives, and Protein Metabolism categories (**Fig 1**). The genome of *Paenibacillus* sp. JMULE4 contained the greatest number of pathways related to Carbohydrates, while *Exiguobacterium* sp. JMULE1 and *Deinococcus* sp. JMULE3 contained a proportionately greater number of pathways related to Protein Metabolism (**Fig 1**). As isolates of *Paenibacillus* have been shown to be capable of degrading complex carbohydrates, this may be indicative of similar capabilities for the Lake Erie isolate of this genus [51, 52] and bacteria associated with *Microcystis* aggregates [22, 36]. The *Paenibacillus* sp. JMULE4 genome also contained the most genes related to Dormancy and Sporulation, and this is the only isolate of the five observed to produce endospores (**Fig 1**).

Table 2. Closest sequenced relatives of each isolate based on *rpoB* identity and ANI score.

Lake Erie Isolate	Closest sequenced relative	Genome Size (Mbp)	GC %	Genes	*rpoB* % Identity	Two-Way ANI Score (%)	Citation
Exiguobacterium sp. JMULE1	*Exiguobacterium* sp. Strain MH3	3.16	47.2	3,273	99.8	98.2	Tang et al., 2013
Enterobacter sp. JMULE2	*Enterobacter asburiae* sp. L1	4.56	56.1	4,426	98.5	90.0	Lau *et al.* 2014
Deinococcus sp. JMULE3	*Deinococcus soli* N5	3.24	70.2	3,146	97.0	92.4	Joo et al., 2015
Paenibacillus sp. JMULE4	*Paenibacillus napthalenovorans*32O-Y	5.20	49.7	5,103	99.5	99.3	Butler et al., 2016)
Acidovorax sp. JMULE5	*Acidovorax* sp. KKS102	5.20	64.9	4,883	92.5	87.3	Ohtsubo et al., 2012

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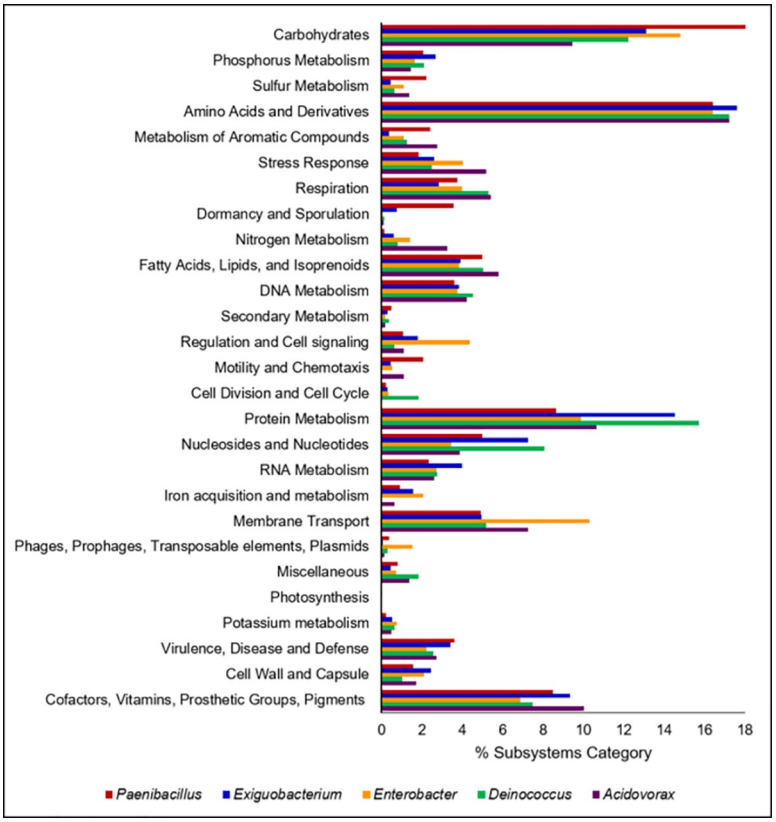

Fig 1. Percent of annotated genes in each Subsystems (SEED) category. Subsystem coverage for each of the isolates was 30% for *Exiguobacterium* sp. JMULE1, 33% for *Enterobacter* sp. JMULE2, 23% for *Deinococcus* sp. JMULE3, 26% for *Paenibacillus* sp. JMULE4, and 31% for *Acidovorax* sp. JMULE5.

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Nitrogen utilization

Bacterial heterobionts are thought to be a source of nitrogen (N) to algae in the phycosphere [35, 36, 53]. In many ways this is self-evident, as respiration of biological materials from phytoplankton results in the loss of C (as CO_2) and residual, excess N [54, 55]. The presence of several different N-transformation genes in the bacterial genomes we examined suggests that these bacteria have the capability to act as an external source of ammonium for *Microcystis*. All the genomes except *Exiguobacterium* sp. JMULE1 contain genes for the reduction of nitrate and nitrite to ammonium (**Table 3; S2-S6 Tables in S1 File**). Enrichment for this function has been previously identified in metagenomes generated from *Microcystis* aggregates in Lake Erie compared to whole water samples [35]. The denitrifying reductase gene clusters in the *Acidovorax* sp. JMULE5 genome includes genes for nitric and nitrous oxide reductase as well as cyanate hydrolysis (**Table 3; S2 Table in S1 File**). Cyanate is a by-product of the urea cycle and produces bicarbonate and ammonium ions upon hydrolysis via the enzyme cyanase (*cynS*) [56]. *Microcystis* populations have been shown to upregulate transcription of *cynS* in response to urea additions [57], indicating *Microcystis* has the genetic capability to use cyanate derived from associated bacterial populations in systems such as Lake Erie during periods of N limitation.

While previous work has identified diazotrophic bacterial constituents of *Microcystis* blooms and culture consortia, none of the five Lake Erie isolates have the genetic capacity to produce nitrogenases [20, 23, 58]. Members of the genus *Paenibacillus* have the ability to fix

Table 3. Nitrogen genes called by RAST and PGAP and their roles in the bacterial isolates.

Gene	Role	Isolate(s)
nirD	Nitrate reductase small subunit	JMULE2, JMULE3, JMULE4, JMULE5
nirB	Nitrate reductase large subunit	JMULE2, JMULE3, JMULE4, JMULE5
narG	Respiratory nitrate reductase alpha chain	JMULE2, JMULE5
narH	Respiratory nitrate reductase beta chain	JMULE2, JMULE5
narI	Respiratory nitrate reductase gamma chain	JMULE2, JMULE5
narJ	Respiratory nitrate reductase delta chain	JMULE2
norR	Anaerobic nitric oxide reductase transcription regulator	JMULE2, JMULE5
nsrR	Nitrite-sensitive transcriptional repressor	JMULE1, JMULE2
gltB	Glutamate synthase large chain	JMULE1, JMULE3 JMULE5
gltD	Glutamate synthase small chain	JMULE1, JMULE3 JMULE5
glnN	Glutamine synthetase type III	JMULE3
cynS	Cyanate hydratase	JMULE5
cynR	Cyn operon transcriptional activator	JMULE5
nosF nosR, nosY	Nitrous oxide reductase maturation protein	JMULE5
norB	Nitric-oxide reductase subunit B	JMULE5

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N_2 [59, 60], however, the JMULE4 isolate only encodes a NifU-like protein, which is a nonessential protein for nitrogen fixation in organisms such as *Dolichospermum* (*Anabaena*) [61]. The role of these isolates in potentially providing reduced N to *Microcystis* likely comes from the breakdown other exogenous N sources, such as urea and nitrate.

Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide (CO_2) [62]. In addition to serving as an N source for freshwater cyanobacteria, including *Microcystis*, the CO_2 released during urea hydrolysis also can act as a carbon source for *Microcystis* during periods of high biomass [63, 64]. As the pH increases during bloom events, it becomes increasingly difficult for additional CO_2 to dissolve in the water. CO_2 availability can be impacted by static conditions (no aeration), which can cause *Microcystis* to stop growing [65]. The genomes of *Enterobacter*, *Paenibacillus*, and *Acidovorax* spp. contain genes encoding the alpha (*ureC*), beta (*ureB*), and gamma (*ureA*) subunits of the urease enzyme complex. The *Deinococcus* sp. JMULE3 genome contains genes for the alpha (*ureC*) and gamma (*ureA*) urease subunits. The *Deinococcus* sp. JMULE3 and *Acidovorax* sp. JMULE5 genomes contain all of the urease accessory genes (*ureEFGD*), while the *Enterobacter* sp. JMULE2 and *Paenibacillus* sp. JMULE4 genomes encode a subset of these accessory genes (*ureEFD* and *ureFGD* respectively). *Enterobacter* sp. JMULE2, *Deinococcus* sp. JMULE3, and *Acidovorax* sp. JMULE5 were all confirmed to be ureolytic by inducing a color change on urea slants and can grow with urea as their sole N source. Urease is a metalloenzyme that binds to and requires nickel to function [66, 67]. The genomes of *Enterobacter* sp. JMULE2 and *Acidovorax* sp. JMULE5 contain the nickel-binding accessory genes *ureJ* and *hupE*. Genes for nickel incorporation proteins (*hypAB*) were identified in the *Enterobacter* sp. JMULE2 and *Deinococcus* sp. JMULE3 genomes. Due to its potential as a source of CO_2, the breakdown of urea by heterotrophic bacteria in the phycosphere could act as a dual source of both carbon and N for *Microcystis*, as has been shown in marine systems [68].

Carbon utilization

The monosaccharide composition of *Microcystis* extracellular polysaccharides (EPS) has been extensively characterized, and multiple species of bacteria can use components of the *Microcystis* EPS as a sole carbon source [69–71]. The genomes of *Paenibacillus* sp. JMULE4 and

Enterobacter sp. JMULE2 contain genes for xylose utilization, a key monosaccharide in the EPS of *Microcystis* [69, 70]. In both freshwater and marine phycosphere communities, xylose is a known low molecular weight (LMW) carbohydrate source for associated bacteria [72–74]. *Paenibacillus* sp. JMULE4 genome contains genes for xylose isomerase, transporters, and binding components, while *Enterobacter* sp. JMULE2 has genes encoding XylFGHR proteins that allow for transcriptional regulation, xylose transport, and ATP binding (**S2-S6 Tables in S1 File**). All five of the isolates' genomes also contain genes for mannose utilization (**S2-S6 Tables in S1 File**), another component of the *Microcystis* EPS [69, 70]. All of the isolates have the genetic capacity to produce mannose-6-phosphate (M6P) isomerase (**S2-S6 Tables in S1 File**), which converts M6P to fructose-6-phosphate (F6P), an intermediate of glycolysis.

The genomes of *Deinococcus* sp. JMULE3, *Paenibacillus* sp. JMULE4, and *Acidovorax* sp. JMULE5 contain the *glcD* gene for glycolate dehydrogenase, indicating they likely have the ability to use algal-derived glycolate as a carbon source (**S2-S6 Tables in S1 File**). Glycolate is an organic carbon source produced from the oxygenase activity of RuBisCO during photorespiration by phytoplankton, with rates of excretion dependent upon the form of N available [73, 75]. The potential utilization of glycolate by heterotrophic bacteria is indicated by the presence of the *glcD* gene, which encodes glycolate oxidase; this gene is now considered a biomarker for the ability to consume algal-derived carbon [76, 77]. Bacterial utilization of glycolate released by phytoplankton has been examined in both marine systems and lakes [77, 78]. To determine whether these organisms actively attempt to access glycolate pools during bloom events, we examined the genetic potential of bloom communities to use this C source in metagenomes from Lake Greenfield (Iowa, USA) and the active transcription of *glcD* in a set of transcriptomes from Lake Erie (North America) and Lake Tai (*Taihu*) and (**Table 4**). Signatures of *Paenibacillus* or *Deinococcus glcD* expression are largely non-existent (**Table 4**), indicating they were not actively using (or capable of using) glycolate during the bloom events sampled. While overall few reads recruited to *glcD* from the bloom metatranscriptomes, the greatest number of reads recruited to the *Acidovorax* JMULE5 *glcD* gene, indicating that there is some active transcription of this gene during bloom events by this species and other members of the Comamonadaceae during bloom events in Taihu and Lake Erie (**Table 4**). The increased recruitment of reads from the Greenfield metagenomes is likely a function of samples being DNA rather than RNA, indicating the potential of these organisms to use glycolate, rather than active transcription. The low number of reads which recruited to the *glcD* sequences in these bloom libraries is likely a function of the sample collection, as these were all whole water samples rather than *Microcystis*-aggregates. Furthermore, little is known about seasonality of bacterial interactions in the phycosphere. There may be a specific bloom stage during which phycosphere bacteria may actively consume algal-derived carbon such as glycolate. These organisms are members of phyla that are significantly reduced in whole water samples compared to aggregates [14, 35], where Actinobacteria are universally dominant in freshwater systems [14, 79, 80]. Unfortunately, few if any datasets exist that measure gene expression specific to bacteria within *Microcystis* aggregates, although several recent studies

Table 4. Metatranscriptome and metagenome reads recruited to the *glcD* gene of *Acidovorax* JMULE5, *Deinococcus* JMULE3, and *Paenibacillus* JMULE4. Libraries from Lake Erie metatranscriptomes (Steffen et al., 2017; Stough et al., 2019), Taihu metatranscriptomes (Stough et al., 2019), and Greenfield metagenomes were recruited to each *glcD* gene.

Lake	*Acidovorax* (Comamonadaceae)	*Deinococcus* (Denococcaceae)	*Paenibacillus* (Paenibacillaceae)
Taihu	12 (128)	0 (0)	0 (0)
Erie	38 (252)	0 (0)	0 (0)
Greenfield	3,205 (21,536)	0 (0)	4 (0)

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have reconstructed bacterial functional potential within aggregates using metagenomics [22, 25, 35, 36]. Furthermore, it is likely that the type of interactions between *Microcystis* and its associated bacteria may vary between synergistic or mutualistic and antagonistic depending on the stage of bloom development [74]. Taken together, the content of these isolates' genomes likely indicates carbon exchange in the *Microcystis* phycosphere.

Iron utilization

Iron deprivation affects phytopigment production and photosynthetic efficiency of *Microcystis* spp. [81]. *Enterobacter* sp. JMULE2, *Paenibacillus* sp. JMULE4, and *Deinococcus* sp. JMULE3 genomes all contain genes encoding various siderophore transporters, biosynthetic pathways, and utilization proteins (**S3 Table in S1 File**).

The *Enterobacter* sp. JMULE2 genome contains genes encoding FepBCDEG proteins for the transport of ferric enterobactin (**S3 Table in S1 File**). Genes encoding the enterobactin biosynthesis pathway proteins EntBSH are also present (**S3 Table in S1 File**). Enterobactin siderophores are characteristic of the Enterobacteriaceae family and are amongst the strongest siderophores with a high affinity for iron [82]. The genomes of 115 *Microcystis aeruginosa* isolates in Genbank do not contain a gene encoding enterobactin esterase (*fes*), the enzyme necessary to remove iron from the enterobactin siderophore. If *Microcystis* spp. cannot use enterobactin, it is possible that iron scavenging by phycosphere bacteria could be competitive with their cyanobacterial host during specific phases of bloom development [74]. The *Enterobacter* sp. JMULE2 genome also contains *iucA-D* genes necessary for aerobactin synthesis (**S3 Table in S1 File**). Aerobactin siderophores do not have as strong an affinity for iron as enterobactins, but have an advantage for bacterial growth in iron-limited conditions [83]. Members of the bloom-forming genus *Dolichospermum* (formerly *Anabaena*) can use ferric aerobactin in culture, although it is not considered a robust iron donor for cyanobacteria [84].

The genome of *Deinococcus* sp. JMULE3 contains genes for isochorismate synthase (**S3 Table in S1 File**), a precursor of siderophores including enterobactin [85]. Isochorismate synthase is necessary for the synthesis of salicylic acid for plant defense [86]. The *Paenibacillus* sp. JMULE4 genome contains genes related to bacillibactin and anthrachelin siderophores (**S3 Table in S1 File**). The genome also contains genes for Feu A-C proteins for Fe-bacillibactin transport [87]. Bacillibactins are catechol-based siderophores that are structurally like enterobactin siderophores which are produced by different members of the *Bacillus* genus, including *Bacillus anthracis* [88]. These siderophores have also been described in a *Paenibacillus* honeybee pathogen [89]. *Paenibacillus* sp. JMULE4 genome contains genes for anthrachelin uptake transporters (**S3 Table in S1 File**).

The Ton and Tol transport systems are used to transport ferric-siderophore complexes and vitamin B_{12} across the cell membrane [90]. The genomes of *Acidovorax* sp. JMULE5, *Deinococcus* sp. JMULE3, and *Enterobacter* sp. JMULE2 contain genes involved in the Ton and Tol transport systems. All three organisms also have genes for TonB-dependent receptors (**S3 Table in S1 File**). *Enterobacter* sp. JMULE2 and *Acidovorax* sp. JMULE5 have genes for the TolA protein (**S3 Table in S1 File**). The *Enterobacter* sp. JMULE2 genome contains the gene encoding aerobactin siderophore receptor, *iutA* (**S3 Table in S1 File**).

Production of auxins

Each of the five sequenced bacterial genomes encode genes for the biosynthesis of tryptophan, and four encode homologues of *ipdC*, the gene which encodes indole-3-pyruvate decarboxylase (**S2-S5 Tables in S1 File; Fig 2**). Sequences of *ipdC* fall into four clusters based on similarity to sequences of known function; *Enterobacter* sp. JMULE2 belongs to cluster I, encoding

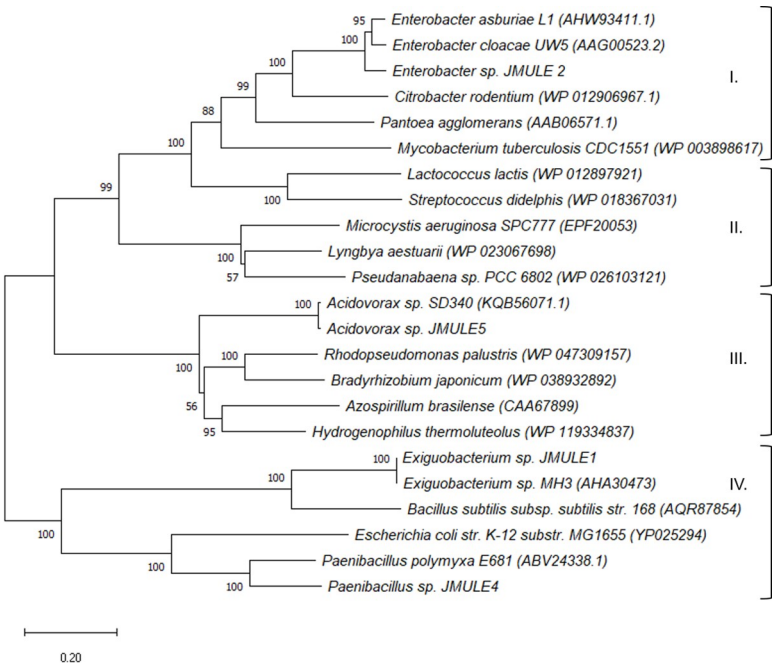

Fig 2. Phylogenetic tree of bacterial homologues of indolepyruvate decarboxylase (IpdC). Clusters are based on similarity to sequences of known function, specifically indolepyruvate decarboxylase (Group I), α-keto decarboxylase (Group II), acetolactate synthase (Group III), and phenylpyruvate decarboxylase (Group IV). Sequence alignment (527 amino acids) was performed using T-coffee (Notredame *et al*., 2000; Di Tomasso *et al*., 2011) and the Neighborhood-Joining phylogenetic tree was generated in Mega X (Kumar *et al*., 2018) with a bootstrap test of phylogeny (1000).

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indolepyruvate decarboxylase, along with other members of the *Enterobacter* genus and close relatives such as *Citrobacter* (Fig 2). *Acidovorax* sp. JMULE5 falls in cluster II, whose members encode an α-keto decarboxylase, while *Exiguobacterium* sp. JMULE1 and *Paenibacillus* sp. JMULE 4 belong to cluster IV, which encodes a phenylpyruvate decarboxylase (Fig 2). IpdC and its homologues catalyze the second reaction in the indole-3-pyruvic acid (IPA) IAA synthesis pathway, converting IPA to indole-3-acetaldehyde (IAAld) (Fig 2). When supplemented with 5 mM tryptophan, all five isolates were confirmed to produce IAA via colorimetric assay, with a range of 3.3 μM *(Exiguobacterium* sp. JMULE1) to 47.3 μM (*Acidovorax* sp. JMULE5) after a 24-hour incubation (**S6 Table in S1 File**). The closest relative of JMULE1, *Exiguobacterium* sp. MH3, produces auxins that are hypothesized to play a role in growth-promoting activity in its aquatic plant host [44]. Production of auxins by native bacteria increases cell density of freshwater eukaryotic microalgae [91]. Tryptophan is an important precursor for indole-3-acetic acid (IAA), the main auxin that occurs in plants. Amin et al. (2015) found that a bacterial consortium promoted diatom cell division due to the secretion of an auxin synthesized from diatom-derived tryptophan. *Microcystis aeruginosa* NIES 843 has the genetic capability to produce tryptophan and could therefore serve in a similar role [16, 92]. The production of auxins like IAA by these isolates suggests a possible important growth-promoting effect of the bacteria on *Microcystis* and other freshwater cHAB formers.

Quorum sensing and signaling

The *Enterobacter* sp. JMULE2 genome contains genes for AI-2 transport and processing. Quorum sensing, or cell-to-cell communication in bacteria, relies on the production of signaling

molecules known as autoinducers [93]. Autoinducer 2 (AI-2) is an autoinducer produced by many different bacterial species. This *lsrACDBFGE* operon has also been described in *Salmonella enterica* and *Escherichia coli* [94, 95], both members of the Enterobacteriaceae family with *Enterobacter*. In these organisms, the *lsrACDB* genes encode AI-2 transporter components while the rest of the genes in the operon are needed from processing AI-2 once it is internalized [95]. Its closest relative (based on *rpoB* identity), *E. asburiae* sp. L1 (**Table 3**) is known to produce an array of quorum sensing molecules, including AHLs [41].

Vitamin production

Many algae, including some cyanobacteria [96–98], require vitamin B_{12} for growth yet are unable to produce it and must rely on exogenous B_{12} [97]. *Microcystis* requires vitamin B_{12} for the methionine biosynthesis pathway. This pathway requires B_{12} as a cofactor for a type-II MetH enzyme [48, 97]. Microalgae can obtain vitamin B_{12} directly from bacterial interactions [97]. *Paenibacillus* sp. JMULE4, *Acidovorax* sp. JMULE5, and *Deinococcus* sp. JMULE3 genomes all contain genes for cobalamin (vitamin B_{12}) synthesis (MTR, *cobY*, *cobU*, *cobQ*, *bluB*) (**S1-S5 Tables in S1 File**). Vitamin B_7 (biotin) is a cofactor that is essential for carboxylase enzymes, including acetyl coenzyme A (CoA) carboxylase which is used in the production of fatty acids. As with vitamin B_{12}, cultures of *Microcystis* are supplemented with B_7 in the growth medium. All five of the bacterial genomes contain genes for biotin biosynthesis (**S1-S5 Tables in S1 File**). In marine systems, 22% of HAB forming organisms are vitamin B_1 (thiamine) auxotrophs [99]. The *Enterobacter* sp. JMULE2, *Paenibacillus* sp. JMULE4, and *Acidovorax* sp. JMULE5 genomes contain genes for vitamin B_1 synthesis (**S1-S5 Tables in S1 File**) and could provide *Microcystis* with this essential nutrient in natural populations.

Potential for interaction in the *Microcystis* physcosphere

The reductionist approach to understanding the dynamics of HABs is shifting toward a more dynamic model. No organism lives in isolation, including the phytoplankton which form HABs. The phycosphere, a potential hotbed for interactions between algae and their heterotrophic bacterial microbiome, can be considered a counterpart of the terrestrial rhizosphere. Within this microenvironment, exchange of nutrients and other compounds drive the mutualistic relationships between phytoplankton like *Microcystis* and their associated bacteria. Genomic analysis of five bacterial isolates from a 2017 *Microcystis* bloom in Lake Erie indicate these bacteria have the genetic potential for bidirectional exchange of nutrients and other growth-promoting compounds such as vitamins and hormones with their photosynthetic partner. The carbon-rich EPS produced by *Microcystis* contains the sugars mannose and xylose, which can be taken up and utilized by all five of the Lake Erie isolates (**Fig 3**). For decades, it has been posited that the bacteria associated with the *Microcystis* mucilage likely benefit from access to these various forms of carbon [38, 100]. Less is known, however, about how *Microcystis* may benefit from this close association with heterotrophic bacteria. One potentially important mechanism of exchange may be the bidirectional exchange of carbon. Bacteria respire CO_2, as well as produce it as a byproduct of the hydrolysis of urea. During peak bloom conditions, *Microcystis* populations in Lake Erie have increased transcription of genes involved in carbon concentration, suggesting the potential for CO_2 limitation in dense bloom populations [101]. Respiration by associated bacteria could provide a supplemental source of CO_2 for *Microcystis* (**Fig 3**) [102]. Many phytoplankton, including *Microcystis* require an exogenous source of vitamins B_{12}, B_1, and B_7 [97, 99]. Phycosphere bacteria have been identified as potential sources of these vitamins for marine bacteria, and four of the five Lake Erie isolates may serve in this capacity during *Microcystis* blooms (**Fig 3**). In addition to providing vitamins, these bacteria

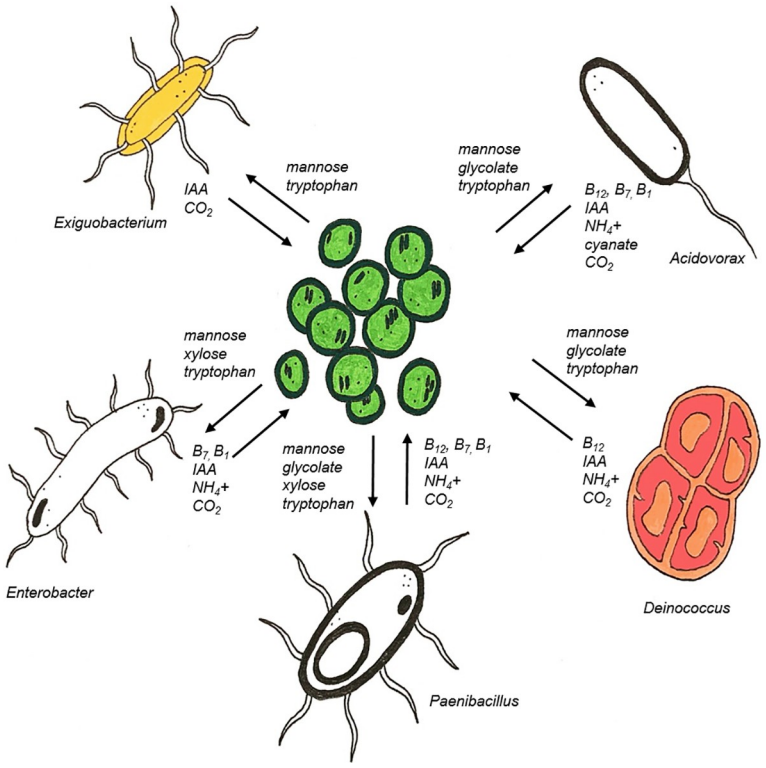

Fig 3. Proposed mechanisms of interaction in the *Microcystis* phycosphere. The genomes of the five sequenced Lake Erie isolates indicate the genetic potential for bidirectional exchange of nutrients and other compounds with *Microcystis* colonies.

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may also provide reduced N to *Microcystis* during periods of N stress, which is common in systems such as Lake Erie during peak bloom conditions (Fig 3). Interestingly, all five of the sequenced Lake Erie isolates can produce the plant hormone IAA when supplemented with tryptophan. IAA has been shown to have growth promoting effects on both freshwater and marine algae [16, 91], and may have an important role in the mutualistic exchange that occurs between *Microcystis* and its associated bacteria (Fig 3). The genetic capacity of these bacteria to provide critical vitamins and other nutrients provides new insight into the role that biotic interactions may have in the development of *Microcystis* blooms. Further culture studies will reveal the mechanisms which underlie these hypothesized multidimensional interactions illustrated in the genomic potential of these five bacterial isolates.

Methods

Sample collection, isolation, and identification

Water samples were collected from Lake Erie on August 9, 2017 from the Ohio State University Stone Lab R/V Gilbraltar III. Samples were taken from four different stations: WE02 (N 41˚ 45.777', W 83˚ 12.931'), WE04 (N 41˚ 49.634', W 83˚ 11.659'), WE13 (N 41˚ 44.619', W 83˚ 08.081'), and MB18 (N 41˚ 44.886', W 83˚ 24.061'). 20 μm and 80 μm mesh plankton nets were used to collect samples from each site to ensure only those bacteria tightly associated with the *Microcystis* colonies would be isolated. 150 mL were collected and stored on ice until transport back to the laboratory.

Both general (LB agar) and selective (CT medium with 100 μM urea agar) media were used for the isolation of bacterial samples [63]. Bacterial isolates are maintained on CT-TY, CT medium with 1 g/L tryptone and 1 g/L yeast extract [103]. Roughly 500 μL of each water sample was plated onto each medium and incubated at 26˚C and 32˚C, for 48 hours (LB/CT-TY) or seven days (CT-urea). Single colonies were re-streaked onto new plates of the respective media until the isolates were pure as confirmed by microscopy.

Isolates that were grown on urea-supplemented CT were tested for urea utilization capabilities. Briefly, the isolates were inoculated into urea broth and urea agar slants (Hardy Diagnostics) and observed for color change to indicate urease activity *via* ammonia production. To extract DNA for *ureC* screening, turbid overnight cultures were pelleted at 17949 x *g*. The pellet was resuspended in 500 μL of sterile water and heated at 95˚C in a dry bath for 15 minutes. After heating, the tubes were centrifuged again for one minute. The supernatant containing the DNA template was used for PCR amplification of the *ureC* gene using primers IGKAGNP-forward (`5′ATHGGIAARGCIGGIAAYCC3′`) and HEDWGA-reverse (`5′IGYICCCCART CYTCRTG 3′`) (modified from Collier et al. [104]). The PCR program was as follows: 94˚C for one minute, 25 cycles of 94˚C for 30 seconds, 53˚C for 30 seconds, and 72˚C for 45 seconds, with a final extension of 72˚C for 10 minutes.

DNA extraction and sequencing

Isolates were grown from a single colony in CT-TY broth for 48 hours at 26˚C or 32˚C (JMULE4). The DNeasy UltraClean Microbial Kit (Qiagen) was used for DNA extraction according to manufacturer's instructions. A NanoVue Plus (GE Healthcare) was used to check the quantity and purity of the DNA. The genomic DNA was sent to Genewiz (South Plainfield, NJ, USA) for sequencing on the PacBio Sequel System. The PacBio SMRTbell library was prepared according to the manufacturer's instructions. The SMRTbell libraries were then sequenced with the PacBio Sequel System.

The SMRTLink suite was used to demultiplex the sequence libraries that were generated from the PacBio Sequel platform. These demultiplexed sequence files (BAM and FASTQ) were then provided by the GENEWIZ sequencing facility for assembly and annotation.

Genome assembly and annotation

The genomes were assembled using the PacBio *de novo* assembly pipeline on the CLC Genomics Workbench plugin CLC Genome Finishing Module (Qiagen) using default parameters. First, the raw reads were imported into CLC Genomics Workbench and corrected for sequencing errors and untrimmed adapters. The error-corrected reads were then assembled into contigs with the "*de novo* Assemble PacBio Reads" tool. The corrected reads were then mapped to the contigs to close gaps and join contigs and subsequently mapped to the larger contigs.

The genomes were annotated with the NCBI prokaryotic genome annotation pipeline (PGAP) [105] and RAST [106, 107]. The annotated genomes were viewed on the RAST SEED Viewer [50]. Genome assemblies and annotations are available through NCBI at BioProject PRJNA521711 and RAST (6666666.419766—*Exiguobacterium* sp. JMULE1, 6666666.419768—*Enterobacter* sp. JMULE2, 6666666.419773—*Deinococcus* sp. JMULE3, 6666666.419775—*Paenibacillus* sp. JMULE4, 6666666.419779—*Acidovorax* sp. JMULE5). Average nucleotide identity (ANI) was calculated using the calculator at http://enve-omics.ce.gatech.edu/ani/ [42]. Amino acid alignments were generated using T-coffee (http://tcoffee.crg.cat/apps/tcoffee/) and phylogeny was calculated using the Maximum-Likelihood method with a Bootstrap test of phylogeny (1000 iterations) in Mega X [108–110].

Confirmation of IAA production

Bacterial isolates were tested for the capability to produce the auxin indole-3-acetic acid (IAA). The isolates were inoculated in 1mL of CT-TY broth fortified with 5 mM L-tryptophan and incubated while shaking at 26˚C, with the exception of 32˚C for *Paenibacillus*, for 48 hours. 200 μL of axenic *Microcystis* and 2 μL of broth containing each isolate were added to a 96-well plate in triplicate. 200 μL of the isolate alone were also added in triplicate to the 96-well plate. The well plate was then left to incubate for 24 hours at 26˚C. 150 μL of Salkowski's reagent (0.5 M $FeCl_3$ and 70% perchloric acid) were added to each well in the dark [102]. The plate was incubated in the dark for 30 minutes before measuring absorbance at 530 nm to observe color change and compare to a standard curve.

Prevalence in environmental data

To determine whether these isolates are present during bloom conditions, we analyzed sequence libraries from blooms that occurred in three locations: Taihu (China) [103], Lake Erie (North America) [4, 103], and Lake Greenfield (North America). Recruitments were performed in CLC Genomics Workbench with a similarity fraction of 0.8 and a length fraction of 0.5 to capture closely related organisms [104]. All recruited reads were then classified via blastn in CLC Genomics Workbench, and those that did not match each isolate at the family- or genus-level based on e-value were excluded. The Lake Greenfield samples were collected on 20 July 2018 from the dock and a second site at a drainage pipe. The samples were collected during a period when the town of Greenfield detected microcystin in the drinking water supply. One liter of water was filtered through Sterivex units and kept on ice until frozen at -20˚C (~2 hours). Samples were extracted using the DNEasy® PowerWater® (Qiagen) extraction kit [105]. Genomic DNA was sent to GeneWIZ® for library preparation and sequencing on the Illumina HiSeq4000 platform to generate 150 bp paired end reads. Reads are available at the NCBI SRA under BioProject PRJNA610583.

Supporting information

S1 File.
(DOCX)

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