Isolation and characterization of algicidal bacteria and its effect on a musty odor-producing cyanobacterium Dolichospermum crassum in a reservoir

Taketoshi Shimizu, Takuya Oda, Hiroyuki Ito and Ichiro Imai

ABSTRACT

Algicidal bacteria that attack Dolichospermum crassum were isolated from the Karasuhara Reservoir in October 2010. Phytoplankton monitoring was performed from April 2010 to March 2011, and D. crassum was detected from August to November. At its peak frequency (in early October), it accounted for 23% of all phytoplankton cells. Heterotrophic bacteria were isolated from the surface water, and an algicidal assay was conducted. As a result, 3 out of 47 bacterial strains showed strong algicidal activity, and they completely destroyed the trichomes of D. crassum. An initial inoculation dose of only $1.0 \times 10^2$ cells ml$^{-1}$ of these strains was enough to digest D. crassum. These strains were identified as Rheinheimera spp. according to 16S rDNA sequence analyses. This is the first report about algicidal bacteria that attack D. crassum. Algicidal bacteria could be key agents for controlling D. crassum in reservoirs.

Key words | algicidal bacteria, Anabaena crassa, Dolichospermum crassum, geosmin, musty odor, Rheinheimera spp

INTRODUCTION

Musty odor is a worldwide problem for drinking water supplies. In Japan, the odorous compounds geosmin and 2-methylisoborneol are included in the quality standards for drinking water (their concentrations should be <10 ng/L), and water suppliers are struggling to provide good-tasting drinking water. Dolichospermum crassum (Lemmermann) Wacklin, Hoffmann et Komárek (syn.: Anabaena crassa (Lemmermann) Komárková-Legnóvá et Cronberg), which belongs to the Cyanophyceae, is the major producer of geosmin in eutrophic lakes in temperate areas of the world. The water in Kobe City has also been suffering from a musty odor, mainly due to geosmin produced by D. crassum, since the mid-1990s. Chemicals such as copper sulfide have been widely used to kill such algal blooms. However, this is not a perfect solution. Several types of bacteria have been reported to demonstrate algicidal activity against harmful plankton in marine coastal systems (Imai et al. 1995; Harvey et al. 2016) and eutrophic lakes and ponds (Li et al. 2015). Such bacteria are found at high densities in the biofilms attached to seaweed (Imai et al. 2006). Therefore, algicidal bacteria have potential as an environmentally friendly tool for controlling cyanobacterial blooms.

In reservoirs, many bacteria have been found to attach to the trichomes of D. crassum, and we assume that some of them affect the growth of the plankton. However, so far there have not been any reports about algicidal bacteria that attack D. crassum. In 2010, we successfully isolated algicidal bacteria that attack D. crassum from the Karasuhara Reservoir, which is a water source in Kobe City. Here, we describe several properties of the isolated algicidal bacteria.
MATERIALS AND METHODS

Sampling

Water samples were collected at the Karasuhara Reservoir (34°41′28.3″ N, 135°09′30.4″ E). The effective storage capacity of the reservoir is 1,350,000 m³, and its maximum depth is 19.2 m. Five aerators operate throughout the year to improve the reservoir’s dissolved oxygen concentration. Surface water samples were collected every month from April 2010 to March 2011. To isolate algicidal bacteria, surface water was collected using sterilized glass bottles on 26 October 2010, when the frequency of *D. crassum* was in its decreasing phase.

Identification and counting of phytoplankton

The surface water samples were transferred to optical plastic plankton counters (MPC-200, MATSUNAMI, Japan), and the numbers of phytoplankton were counted using a microscope (BX-50, OLYMPUS, Japan) at magnifications of 100 to 200×.

Isolation of heterotrophic bacteria

One to 1,000-fold dilutions of the surface water samples were prepared using R2A liquid medium (Reasoner & Geldreich 1985). Then, 1 ml of each water sample was inoculated into R2A agar medium (DAIGO, Nihon Pharmaceutical Co., Ltd. Japan), which was then incubated at 20°C for 14 days. Then, pure cultures of heterotrophic bacteria were obtained by inoculating each colony onto R2A agar medium.

Algicidal assay

For the algicidal assay, axenic *D. crassum* (1.2 × 10⁴ cells ml⁻¹) was prepared using CT medium (Watanabe & Ichimura 1977). Each colony of isolated heterotrophic bacteria was collected and inoculated into 3 ml of the *D. crassum* culture in sterilized glass tubes. The tubes were then incubated at 25°C under a photon flux density of 21 μmol m⁻² s⁻¹ and a 16 h light-8 h dark photo cycle for 7 days. The tubes in which *D. crassum* was destroyed were considered to be algicidal-positive.

DNA extraction from algicidal bacteria

Colonies of bacteria were collected from R2A agar plates and added to 180 μl of ATL buffer (DNeasy Tissue Kit, Qiagen, Tokyo, Japan) in polymerase chain reaction (PCR) tubes. They were then subjected to five cycles of freezing at −80°C for 3 min and thawing at 37°C for 1 min. After that, DNA was extracted with the DNeasy tissue kit according to the manufacturer’s recommended procedures.

PCR amplification of algicidal bacteria DNA

To analyze the phylogenetic classification of algicidal bacteria, full-length 16S rDNA was amplified with the 27f and 1492r primer set (Table 1) and 100 ml of a PCR mixture containing 10 μl of 10× Ex Taq buffer, 8 μl of dNTP mixture, 50 pmol of each primer, 0.5 μl of TaKaRa Ex Taq polymerase (Takara, Otsu, Japan), and 5 μl of extracted DNA. The amplification conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension step of 72°C for 8 min. The amplicons were electrophoresed on 1.5% (wt/vol) agarose gel containing ethidium bromide (final conc.: 0.5 μg ml⁻¹).

Sequencing and phylogenetic analysis

The amplified DNA fragments were sequenced via the dideoxy method using an ABI 3730xl DNA analyzer (Applied Biosystems, Tokyo, Japan). The full-length 16S rDNA sequences of the algicidal bacteria were determined by overlapping

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>27f</td>
<td>5′-AGA GTT TGA TCC TGG CTC AG-3′</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td>517r</td>
<td>5′-ATT ACC GCG GCT GCT GG-3′</td>
<td>Muyzer et al. (1993)</td>
</tr>
<tr>
<td>785f</td>
<td>5′-GGA TTA GAT ACC CTG GTA-3′</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td>805r</td>
<td>5′-GAC TAC CAG GGT ATC TAA TC-3′</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td>1492r</td>
<td>5′-GGT TAC TTT GTT ACG ACT T-3′</td>
<td>Weisburg et al. (1991)</td>
</tr>
</tbody>
</table>

Table 1 | Sequences of the primers used for the PCR amplification
sequencing reactions involving complementary DNA strands and the primers listed in Table 1. Then, their sequences were analyzed via a BLAST search of the National Center for Biotechnology Information (NCBI) database (available at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=Blast-Search). Phylogenetic trees were constructed using the neighbor-joining algorithm of ClustalW via the DNA Data Bank of Japan (DDBJ) (http://clustalw.ddbj.nig.ac.jp/).

**Nucleotide sequence accession numbers**

The 16S rDNA sequences of the algicidal bacteria were deposited in the GenBank database under the following accession numbers: K-12, LC054835; K-28, LC054836; K-44, LC054837.

**Effects of bacterial density on algicidal activity**

The algicidal bacteria (strain K-44) were incubated in R2A liquid medium at 20°C. Cell suspensions of algicidal bacteria in the logarithmic growth phase were inoculated into 30 ml of the *D. crassum* cultures (8.7 × 10⁴ cells ml⁻¹) in plastic flasks at initial densities of 1.0 × 10² and 1.0 × 10⁴ cells ml⁻¹. Cultures that had not been inoculated with the bacteria served as controls. Then, the cultures were incubated in the same conditions as described in the algicidal assay. The assay was conducted in triplicate. The densities of *D. crassum* and the algicidal bacteria were monitored for 7 days via microscopic observation. The cell densities of the bacteria were determined by SYBR GREEN I staining-based direct counting with epifluorescence microscopy. The bacterial solutions were stained using SYBR GREEN I (1:10,000 dilution) (Molecular Probes, Eugene, USA) in 1.5-ml microtubes for 30 minutes. Then, they were filtered through black polycarbonate membranes (pore size: 0.2 μm; Whatman, USA) and subjected to microscopic examinations.

**RESULTS AND DISCUSSION**

**Growth of phytoplankton**

Figure 1 shows the surface water temperature of the reservoir. The water temperature increased from April to September, when it peaked at 31.1°C, and then decreased to a minimum of 3.1°C in February. The total number of phytoplankton cells changed markedly from 270 to 330,000 cells ml⁻¹ during the observation period, and these changes were water temperature-dependent (Figure 2). Two peaks were observed, the first was in July (330,000 cells ml⁻¹), and the second was in October (120,000 cells ml⁻¹). Cyanophyceae dominated from July to December, and algal blooms occurred throughout the season. *D. crassum* was detected from August to November. Its frequency increased to 2.8 × 10⁴ cells mL⁻¹ on October 4, when it accounted for 23% of all phytoplankton cells. After that, the frequency of *D. crassum* decreased rapidly, and it had disappeared by December. The musty odor of the reservoir became stronger with the growth of *D. crassum*, and a distinct smell was present around the reservoir.

![Figure 1](https://iwaponline.com/ws/article-pdf/17/3/792/409896/ws017030792.pdf)
when *D. crassum* bloomed. Hashimoto (1986) reported that the occurrence of musty odor increased when the annual mean total nitrogen and phosphorus levels of water environments exceeded 0.6 mg l\(^{-1}\) and 0.02 mg l\(^{-1}\), respectively. The Karasuhara Reservoir was classified as a eutrophic pond according to the Organisation for Economic Co-operation and Development (OECD) criteria (1982). The annual mean total nitrogen and phosphorus levels of the reservoir’s surface water were 0.62 mg l\(^{-1}\) and 0.042 mg l\(^{-1}\), respectively. These nutritional conditions presumably promoted the dominance of Cyanophyceae from July to December and the growth of *D. crassum* in the reservoir.

In late October, the frequency of *D. crassum* decreased rapidly. In contrast, the number of bacteria attached to the sheaths of *D. crassum* increased (Figure 3). No other predators, such as protozoa or fungi, were observed during the period when the frequency of *D. crassum* fell, and so we suspect that bacteria contributed to killing the *D. crassum* blooms in the reservoir. In marine environments, the number of algicidal bacteria was found to increase along with the number of phytoplankton, which cause red tide, and these bacteria played a major role in the reduction in the number of harmful plankton (Imai *et al.* 1998; Kim *et al.* 1998).

**Isolation of algicidal bacteria**

Heterotrophic bacteria were isolated from the surface water after the number of *D. crassum* had started to decrease. The number of heterotrophic bacteria was 3.9 \(\times\) 10\(^3\) colony-forming units ml\(^{-1}\). Three strains out of the 47 isolates showed clear algicidal activity (K-12, 28, and 44). *D. crassum* was digested within a few days when it was inoculated with these strains. The algal culture became clear after the digestion procedure. During microscopic examinations, many bacteria were found to have attached to the mucilaginous sheathes of *D. crassum* (Figure 4). They gradually surrounded the *D. crassum* cells and eventually digested entire trichomes. These bacteria digested not only the vegetative cells, but also akinetes. The trichomes of *D. crassum* contracted and eventually disintegrated during the digestive process. In addition, the trichomes became smaller in diameter, and each coil came closer. This phenomenon was also observed in the reservoir when the size of the *D. crassum* population decreased. Several types of phytoplankton exhibit morphological defense mechanisms that are induced by substances released from predators (Hessen & Van Donk 1993; Von Elert & Franck 1999).
probably protects *D. crassum* to some extent from bacterial attack by reducing its surface area. Thus, we suggest that the amount of attached bacteria and trichome morphology are useful indicators for predicting the dynamics of *D. crassum* in water.

**Phylogenetic analysis**

Sequencing of the full-length 16S rDNA molecule revealed that the three algicidal bacterial strains all belonged to the genus *Rheinheimera* (Chromatiaceae, Gammaproteobacteria) (Figure 5). Strain K-12 showed 99.4% sequence similarity to *Rheinheimera texasensis* (NR 043133), strain K-28 demonstrated 98.5% sequence similarity to *Rheinheimera chironomi* (NR 043699), and strain K-44 displayed 99.7% sequence similarity to *Rheinheimera texasensis* (GQ284452). In the microscopic examinations, all of the bacteria were rod-shaped and motile, and their colonies were circular, smooth, and non-pigmented. The medium around the K-28 colonies became gray in color during their incubation on R2A plates.

*Rheinheimera* spp. ubiquitously inhabit various aquatic environments, such as marine (Romanenko *et al.* 2015) and fresh water environments (Merchant *et al.* 2007; Chen *et al.* 2010) and chironomid egg masses sampled from a river (Halpern *et al.* 2007). They are minor members of fresh water environments; Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia are the main microorganisms in fresh water (Zwart *et al.* 2005). However, *Rheinheimera* spp. proliferated in the blooms of Cyanophyceae (Berg *et al.* 2009) and in fresh water that had been treated with a degradable organic compound (Pinhassi & Berman 2003). Therefore, it is suggested that they play an important role in the cycling of organic carbon during the killing of cyanobacterial blooms in the Karasuhera Reservoir.

Some of them exhibit antimicrobial activity derived from the production of hydrogen peroxide (Chen *et al.* 2010) or colicin V (Gupta *et al.* 2011). *R. texasensis* displays algicidal activity against phytoplankton, including Chlorophyceae and Cyanophyceae, which is derived from the production of hydrogen peroxide (Chen *et al.* 2010). Based on the findings of the latter report, we performed a preliminary experiment to detect hydrogen peroxide production by the K-44 strain using a plating method based on the Prussian blue-forming reaction described by Chen *et al.* (2010), and we confirmed that this strain produced hydrogen peroxide. We suppose that this substance is related to algicidal activity.

![Figure 5](https://iwaponline.com/ws/article-pdf/17/3/792/409896/ws017030792.pdf)
activity. *R. chironomi* and *R. texasensis* are motile, as they possess flagella (Halpern et al. 2007; Merchant et al. 2007), and they are subsequently able to effectively reach and attack *D. crassum*.

### Effects of bacterial density on algicidal activity

Figure 6 shows the changes in the density of *D. crassum* after the inoculation of the algicidal bacterial strain K-44. When the inoculation was performed at an initial bacterial density of $1.0 \times 10^2$ cells ml$^{-1}$, the frequency of *D. crassum* was slightly increased at 1 day after the inoculation procedure (A), but had decreased by 4 days after the inoculation procedure. When the inoculation was performed at an initial density of $1.0 \times 10^4$ cells ml$^{-1}$, the frequency of *D. crassum* was markedly decreased at 2 days after the inoculation procedure (B). On the other hand, in the absence of the inoculation of algicidal bacteria the frequency of *D. crassum* continued to increase until the end of the experiment. The density of the algicidal bacteria increased rapidly after the inoculation procedure and peaked after 2 days in the experimental cultures, with initial densities of $1.0 \times 10^2$ cells ml$^{-1}$ and $1.0 \times 10^4$ cells ml$^{-1}$. The rate of reduction in the frequency of *D. crassum* accelerated as the initial bacterial density increased. The inoculation of a small amount of bacteria induced algicidal activity; hence, we postulate that the K-44 strain is an effective predator of *D. crassum*. It is suggested that algicidal bacteria have the potential to control *D. crassum* levels in water environments, and further investigations are needed to examine the seasonal dynamics of algicidal bacteria in reservoirs and the mechanisms responsible for algal bloom termination.

### CONCLUSION

We isolated 3 strains of algicidal bacteria that attack *D. crassum* from the Karasuhara Reservoir when the frequency of *D. crassum* was in its decreasing phase. These bacteria effectively killed and digested akinetes and trichomes. They were identified as *Rheinheimera chironomi* and *Rheinheimera texasensis* according to their 16S rDNA sequences. These bacteria have the potential to control *D. crassum* in reservoirs.

### ACKNOWLEDGEMENTS

We are very grateful to the reviewer for providing precious information about the production of hydrogen peroxide as an antimicrobial compound by the genus *Rheinheimera*.

### REFERENCES


First received 21 June 2016; accepted in revised form 26 October 2016. Available online 11 November 2016.