

## Growth Inhibition of *Microcystis aeruginosa* by a Glycolipid-Type Compound from *Bacillus subtilis* C1

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**We attempted to identify the compound responsible for the growth inhibition of *Microcystis aeruginosa* occurring when a culture broth of *Bacillus subtilis* C1 was added to the medium. The active compound was purified from *B. subtilis* C1 culture broth by adsorption chromatography and HPLC, and was identified as a type of glycolipid based on <sup>1</sup>H NMR and MS analyses. The purified active compound completely inhibited the growth of *M. aeruginosa* at a concentration of 10 µg/ml. This is the first report of a glycolipid produced by a *Bacillus* strain that has potential as an agent for the selective control of bloom-forming *M. aeruginosa*.**

**Keywords:** *Bacillus subtilis*, glycolipid, growth inhibition, *Microcystis aeruginosa*

*Microcystis* spp. have been identified as a cause of algal blooms in eutrophic lakes and reservoirs, and are related to the production of hepatotoxins such as microcystins [5] that can poison livestock and wild mammals [13]. As such, *Microcystis* spp. currently represent a global environmental problem. Many studies have investigated ways of eliminating or controlling bloom-forming *Microcystis* spp., including ultrasonic radiation [2], antialgal compounds [3, 8, 10, 15], and microorganisms [4, 14]. However, these methods are costly and time-consuming, and some even incur secondary pollution [12]. Therefore, development of an effective method is still needed.

Ahn *et al.* [1] previously reported that a culture broth of *B. subtilis* C1 inhibited the growth of *M. aeruginosa*. In an effort to develop an effective method for selectively controlling this bloom-forming cyanobacterium, we attempted to purify, identify, and characterize the active antialgal component(s) in a culture broth of *B. subtilis* C1 that inhibits the growth of *M. aeruginosa*.

*B. subtilis* C1 strain KCTC 10439BP was provided by the Korean Collection for Type Cultures. The basal medium used to grow the bacteria was Cooper's mineral salts medium modified by Kim *et al.* [7]. Small-scale batch fermentation was executed in a 3.0-l fermentor (KFC-5L; Korea Fermentor Co., Ltd., Korea). The culture was grown at 30°C, agitated at 200 rpm, and aerated at 0.5 vvm, while the pH was maintained at 6.8–7.2. The culture broth obtained in the stationary phase on day 5 was then used in the following experiments.

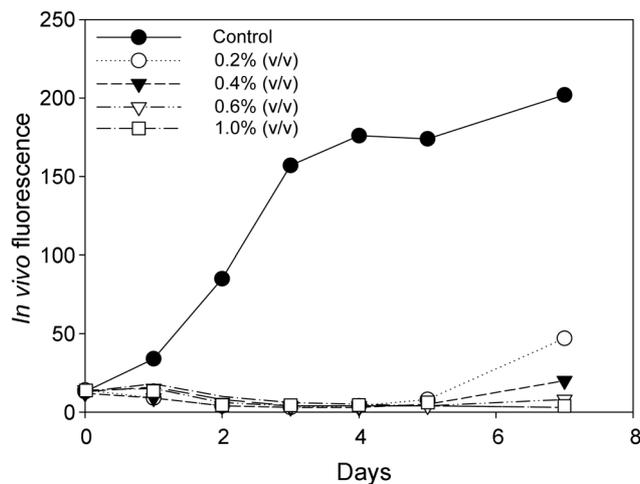
The culture supernatant of *B. subtilis* C1 was used directly in experiments after eliminating bacterial cells by centrifugation (13,000 ×g, 10 min). Various concentrations of cell-free culture broth [0.2 to 1.0% (v/v)] were added to Allen medium inoculated with *M. aeruginosa* UTEX 2388 (1 × 10<sup>6</sup> cells/ml). The algae were then incubated at 25°C and 100 rpm under illumination at 120–140 µmol/m<sup>2</sup>/s. After measuring the initial cell concentration of *M. aeruginosa*, the change in algal growth was measured over 7 days using *in vivo* fluorescence (Turner 450; Barnstead/Thermolyne, Dubuque, IA, U.S.A.).

Fig. 1 shows the effect of the concentration of *B. subtilis* C1 cell-free culture broth on the growth inhibition of *M. aeruginosa*. Growth was suppressed in all of the flasks containing the cell-free culture broth [0.2–1.0% (v/v)]. While the effectiveness of the inhibition decreased after 7 days with concentrations of 0.2–0.6% (v/v), algal growth was completely suppressed with a concentration above 1.0% (v/v).

To identify the active component(s) in the *B. subtilis* C1 cell-free culture broth, a bioassay-guided fractionation study was conducted. First, solvent partitioning of the filtered fermentation broth with ethyl acetate (EtOAc) (5 × 200 ml) provided an organic phase, which was then concentrated using a rotary evaporator to yield 4.5 g of a crude extract. Next, portions of the EtOAc- and water-soluble fractions were dissolved in 1% DMSO and tested for their ability to inhibit the growth of *M. aeruginosa*. The 1% DMSO solution by itself and the water-soluble fraction had no

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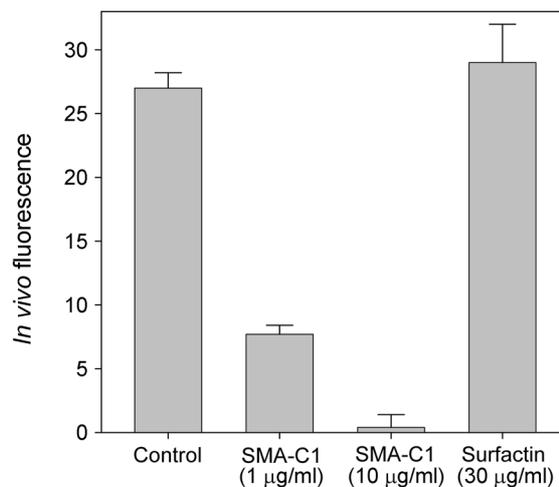


**Fig. 1.** Growth inhibition of *M. aeruginosa* by culture broth of *B. subtilis* C1 at various concentrations.

The cell-free culture broth was added to Allen medium inoculated with *M. aeruginosa* UTEX 2388. The change in growth was then measured over 7 days using *in vivo* fluorescence.

effect on the growth of *M. aeruginosa*; however, the EtOAc-soluble fraction exhibited growth suppression. Therefore, the latter fraction was subjected to  $C_{18}$  functionalized silica gel flash column chromatography (5.5×45 cm) and eluted with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) methanol (MeOH) in  $H_2O$ , followed by 1:1 MeOH- $CH_2Cl_2$  (500 ml each, 6 fractions). Since the fraction eluted with 40% MeOH (364 mg) showed inhibitory activity toward the growth of *M. aeruginosa* at a concentration of 10  $\mu\text{g/ml}$ , it was further subjected to semipreparative reversed-phase HPLC using a gradient of from 30% to 90%  $CH_3CN$  in  $H_2O$  (0.1% formic acid) over 70 min. The active compound, named SMA-C1 (suppressor of *M. aeruginosa*), was finally purified as a single peak on HPLC (23 mg) and stored at 4°C in the dark. Purified SMA-C1 exhibited complete growth inhibition of *M. aeruginosa* at 10  $\mu\text{g/ml}$  and about 70% growth inhibition of the control at 1  $\mu\text{g/ml}$  (Fig. 2). An authentic surfactin (Sigma, St. Louis, MO, U.S.A.) showed no inhibitory activity toward the growth of *M. aeruginosa* at 30  $\mu\text{g/ml}$ .

Proton nuclear magnetic resonance ( $^1H$  NMR) analysis was performed on a Varian UNITY-300 NMR spectrometer at 25°C in  $CD_3OD$  to analyze the chemical structure of the purified SMA-C1. Well-resolved signals were observed in the  $^1H$  NMR spectrum (Fig. 3), exhibiting a typical glycolipid-type structure with the characteristic resonances of an alkyl chain (0.8–2.8 ppm) and pattern of sugar signals (3.2–5.0 ppm), where the protons of the terminal methyl groups ( $-CH_3$ ) resonated at 0.90 ppm (triplet) and 1.2 ppm (singlet), the long methylene ( $-CH_2-$ ) groups in the alkyl chain at 1.3–1.8 ppm, and the hydroxylated methylene group at 2.3 ppm. The presence of oxygenated carbons was also assumed based on the observation of signals corresponding

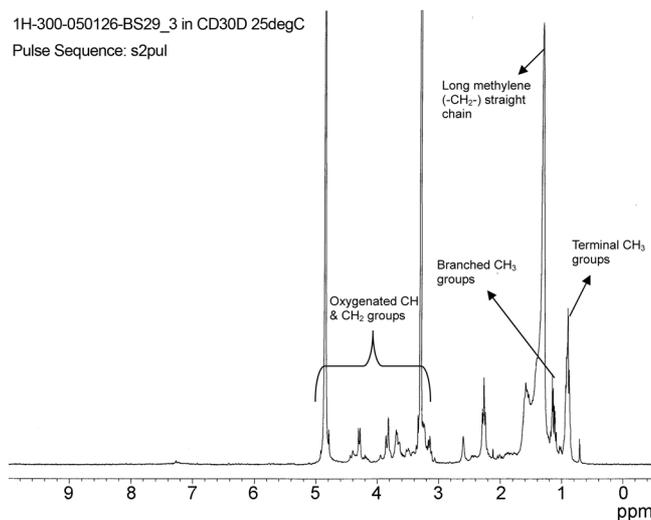


**Fig. 2.** Growth inhibition of *M. aeruginosa* by SMA-C1.

SMA-C1 (1 and 10  $\mu\text{g/ml}$ ) and surfactin (30  $\mu\text{g/ml}$ ) were added to Allen medium inoculated with *M. aeruginosa* UTEX 2388, and the change of *in vivo* fluorescence was measured after incubation for 24 h at 25°C.

to oxygenated methine or methylene groups in the spectrum. Thus, these data indicate that SMA-C1 is a glycolipid-type molecule containing a highly oxygenated portion. In addition, the molecular mass of SMA-C1 was estimated to be 578 Da, based on a negative and positive mode analysis of low-resolution electron spray ionization (ESI) mass spectrometry (data not shown).

The previous study by Ahn *et al.* [1] reported that a culture broth of *B. subtilis* C1 exhibited selective control of cyanobacteria, which is a promising result for bloom control. Since the mineral salts medium (Cooper's) used to culture *B. subtilis* C1 was shown to have no inhibitory effect on the growth of *M. aeruginosa*, we supposed that



**Fig. 3.**  $^1H$  NMR spectrum of SMA-C1 at 25°C in  $CD_3OD$ .

growth was suppressed by an active compound(s), possibly surfactin, produced during the cultivation of *B. subtilis* C1. It is well known that several strains of *B. subtilis* produce lipopeptides such as surfactin, which is a potent surface-active and antibiotic compound containing a  $\beta$ -hydroxy fatty acid with an ester peptide linkage to a seven-amino-acid cyclic peptide [6, 9]. *B. subtilis* C1 was also confirmed to produce surfactin, based on a TLC analysis (data not shown). However, we were unable to confirm the inhibitory effect of an authentic surfactin sample on the growth of *M. aeruginosa*, even at a concentration of 30  $\mu\text{g/ml}$  (Fig. 2), which is about five times the critical micelle concentration of the compound ( $6 \times 10^{-6}$  M). *M. aeruginosa* is also known to synthesize hepatotoxins such as microcystins, which consist of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (ADDA) and a seven-amino-acid cyclic peptide [11] with a chemical structure very similar to that of surfactin. Therefore, since *M. aeruginosa* produces a cyclic peptide, this would explain its tolerance to a surfactin containing such a peptide.

The active inhibitor of the growth of *M. aeruginosa* was successfully purified from the culture broth of *B. subtilis* C1 and identified as SMA-C1. This compound completely inhibited the growth of *M. aeruginosa* at a concentration of 10  $\mu\text{g/ml}$  (Fig. 2), and was confirmed to be a type of glycolipid, based on NMR analysis. Although further chemical structure analysis is still required, this is the first report of a glycolipid produced by a *Bacillus* strain that can inhibit the growth of *M. aeruginosa*.

In conclusion, an active compound extracellularly released by *B. subtilis* C1 that can inhibit the growth of *M. aeruginosa* was purified by adsorption chromatography and HPLC, and identified as a glycolipid based on  $^1\text{H}$  NMR and MS analyses. Thus, with its long-term goal of developing an effective method for controlling cyanobacterial blooms, this study took the first step in purifying and initially identifying the active glycolipid-type compound. Further studies will focus on the mechanism of growth inhibition of cyanobacteria by the glycolipid SMA-C1.

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