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ORIGINAL RESEARCH PAPER

Induction of axenic culture of *Arthrospira* (*Spirulina*) *platensis* based on antibiotic sensitivity of contaminating bacteria

Gang-Guk Choi · Myong-Sook Bae · Chi-Yong Ahn · Hee-Mock Oh

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Abstract *Arthrospira platensis* SAG 21.99 and the isolated bacteria (*Halomonas* spp., *Staphylococcus* sp., etc.) from the culture of *A. platensis* SAG 21.99 were treated with five antibiotics to determine the minimal lethal concentrations. The combination of a washing step and a consecutive treatment with antibiotics, imipenem (100 μg ml^{-1}), neomycin (100 μg ml^{-1}) and cycloheximide (20 μg ml^{-1}), treatment step was highly effective in eliminating bacteria. An axenic culture of *A. platensis* SAG 21.99 could be induced within 3 days using this method. This technique is a simple and rapid method for obtaining axenic cultures of filamentous cyanobacteria.

Keywords Antibiotics · *Arthrospira platensis* · Axenic culture · Cyanobacteria · Imipenem · Neomycin

G.-G. Choi · H.-M. Oh
Biological Resource Center, Korea
Research Institute of Bioscience and Biotechnology,
Daejeon 305-806, Korea

M.-S. Bae · C.-Y. Ahn · H.-M. Oh (✉)
Environmental Biotechnology Research Center,
Korea Research Institute of Bioscience and
Biotechnology, 52 Eoeun-dong, Yuseong-gu,
Daejeon 305-806, Republic of Korea
e-mail: heemock@kribb.re.kr

Introduction

Arthrospira (*Spirulina*) *platensis* is a commercially important filamentous cyanobacterium, with an annual production estimated to be over 3,000 tons per year, the largest among microalgae (Pulz and Gross 2004). It is used as a food additive and feed for fish, poultry, and farm animals worldwide, since it is a rich source of proteins, minerals, vitamin B_{12}, β-carotene, and essential fatty acids, such as γ-linolenic acid (Vonshak 1997).

Axenic cultures are required for physiological, genetic, and taxonomic studies. Thus, axenic (bacteria-free) cultures of microscopic algae are usually prepared by single-cell isolation and density gradient centrifugation, rinsing (Vaara et al. 1979; Bolch and Blackburn 1996), UV irradiation, filtration, or treatment with antibiotics (Rippka 1988), and other germicidal chemicals (Kim et al. 1999). A particularly frustrating aspect of dealing with cyanobacteria is the laborious and time-consuming work involved in attempting to produce axenic cultures, especially when considering the low rate of success. The common factor related to the success of all these methods is that the researcher must be able to identify bacteria-free colonies, which requires practice and experience. Also these methods are only applicable to purification of unicellular cyanobacteria, not filamentous strains such as *Arthrospira*. In addition, problems created by the diversity of growth characteristics and environmental tolerances means that the

success of any one method of purification is by no means guaranteed with all strains.

The conventional standard plate method for the induction of axenic culture is time-consuming as the colonies can only be observed after 2 weeks of incubation. The main objective of this study was to identification of contaminated bacteria in the culture of *A. platensis* using molecular method, to determine the concentration and type of antibiotics for application, and to study the influence of treated antibiotics on *A. platensis*. Accordingly, a simple and rapid method to induce an axenic culture of *A. platensis* was developed, based on the antibiotic sensitivity of the associated bacteria.

Materials and methods

Strains and culture conditions

Arthrospira platensis SAG 21.99 was obtained from the Sammlung von Algenkulturen der Universität Göttingen (SAG), Germany. The organism was maintained an SOT medium (Ogawa and Terui 1970). During subsequent experiments in which antibiotics were added to eliminate bacteria, the medium was supplemented with 0.1% glucose and various concentrations antibiotics. The cultivation was carried out in 250 ml Erlenmeyer flasks containing 100 ml SOT medium under continuous light conditions, with an illumination of 100 μmol photons m^{-2} s^{-1} provided by cool white fluorescent lamps at 30°C and 150 rpm. The initial pH was 9.0. The cyanobacterial biomass was monitored via its optical density.

Isolation of contaminating bacteria and phylogenetic analyses

To count and isolate the contaminating bacteria, 100 μl aliquots of serially diluted *A. platensis* cultures were spread on the surface of an R2A agar (Difco Lab., USA) plate. Each test plate was allowed to incubate at 30°C for 1 week. The numbers of colony-forming units (c.f.u.) on three suitable dilutions were counted and averaged. In addition, based on their morphological characteristics of the gross appearance of the colonies on the agar plate, 9 colonies were isolated from the bacterial counting plates.

The genomic DNA of the isolated bacteria was extracted from pure cultured in R2A broth by using Genomic DNA isolation kit (Qiagen, Germany). The PCR amplification of the bacterial 16S rDNA was performed using two bacterial universal primers, 27F and 1492R (Cho and Giovannoni 2003). The PCR products were then purified using a QIAquick PCR purification column (Qiagen, Germany) and sequenced by the chain-termination method on an ABI377 automated sequencer. The nearly complete sequences of the 16S rRNA gene were initially compared with sequences available in GenBank using BLAST network services to determine their approximate phylogenetic affiliations. The sequences were then aligned using the ARB software package (Ludwig et al. 1998). Thereafter, phylogenetic trees were inferred by neighbour-joining using the Kimura two-parameter model (Saitou and Nei 1987), and the resulting neighbour-joining trees evaluated by bootstrap analyses based on 1,000 resamplings. The sequences of the isolated bacterial 16S rDNA used in the phylogenetic analyses have been deposited in GenBank under accession numbers DQ644493 - DQ644501.

Antibiotics selection

The antibiotic susceptibility tests of *A. platensis* and the isolated bacteria were performed using cefoxitin, chloramphenicol, erythromycin, imipenem, and neomycin. All the antibiotics, except for imipenem, were purchased from Sigma imipenem was obtained as a pure powder donated by Eulji University Hospital, Daejeon, Korea. The minimal lethal concentration (MLC) of the agents was determined according to non-inhibition surrounding the test-disk-added antibiotics using a modified disk diffusion method (NCCLS 2001).

Eliminating heterotrophic bacteria in cultures

The contaminating bacteria in the culture of *A. platensis* were eliminated using a two-step process. The first step (washing step) involved centrifugation (3,000*g*, 15 min) during 5 days cultivation, after

which the precipitate was picked out using a micropipette, transferred to a fresh SOT broth medium, and disaggregated. This process was carried out 3 times. The second step (antibiotic treatment step) then involved treatment with two antibiotic mixtures. After centrifugation (3,000*g*, 15 min), the pellets were resuspended in an SOT medium containing 0.1% glucose and treated with a mixture of imipenem (100 μg ml^{-1}) and cycloheximide (20 μg ml^{-1}). The specimens were then incubated for 1 day at 30°C and 150 rpm in a shaking incubator in the dark. Thereafter, the washing step was repeated three more times, and the pellets resuspended in an SOT medium containing 0.1% glucose and treated with a mixture of neomycin (100 μg ml^{-1}) and cycloheximide (20 μg ml^{-1}). The specimens were then incubated for 1 day at 30°C and 150 rpm in a shaking incubator under dark conditions. After every step taken to eliminate the contaminating bacteria, the samples were stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) (Porter and Feig 1980) and observed under an epifluorescence microscope.

The established presumptive axenic clones were inoculated into 250-ml Erlenmeyer flasks containing 100 ml SOT medium with 0.1% glucose, sealed with cotton wool bungs, and grown at 30°C for 1 day. The cultures were then inoculated into several liquid test media (LB, R2A, and 1/10 NA medium) and the same solid media. The solid test media were examined visually and microscopically for evidence of macroscopic and microscopic colonies of bacteria. In the absence of evidence of bacteria in any of the test media for 2 days, the cultures were judged to be axenic.

Results

Population and isolation of contaminating bacteria and phylogenetic analyses

The population density of contaminating heterotrophic bacteria in the *A. platensis* culture was 3.7 (±0.1) × 10^6 c.f.u. ml^{-1}. This population size was relatively small, as the SOT medium is a preserving medium and produces oligotrophic conditions with an elevated pH of 11 ∼12 during long-term preservation. Nine colonies were isolated from the R2A agar plates, based on the colony size, color, and morphology. The isolated nine bacterial strains were routinely maintained on R2A agar plate.

To identify the isolated bacteria, phylogenetic analyses were performed using the 16S rDNA sequences. A total of 1,444 nucleotides (nt) were determined in the 16S rDNA gene sequences of 9 isolated bacterial strains, and the BLAST search results are shown in Table 1. Sequence comparisons with validly published bacteria indicated that the Ap-1 strain was most closely related to the Glacial ice bacterium SB12K-2-1 (GenBank no. AF479358), albeit with a low similarity value (95%). The Ap-2 strain was most closely related to *Aeromicrobium alkaliterrae* (AY822044) with a 99% similarity value. The Ap-3 strain was most closely related to *Halomonas desiderata* (X92417) with a 98% similarity value. Ap-4 and Ap-5 were most closely related to *Halomonas nitritophilus* with 98% (DQ289006) and 99% (AJ309564) similarity values, respectively. The others (Ap-6–Ap-9) were most closely related to *Staphylococcus saprophyticus* (AJ008934) with a

Table 1 Relationship of 16S rDNA sequences of isolated bacteria from *Arthrospira platensis* SAG 21.99 culture with other sequences in GenBank database

Bacterial strain	Similarity (%)	Closest relative	Accession number	Taxonomic description
Ap-1	1180/1240 (95%)	Glacial ice bacterium SB12K-2-1	AF479358	actinobacteria
Ap-2	656/661 (99%)	*Aeromicrobium alkaliterrae*	AY822044	actinobacteria
Ap-3	1035/1053 (98%)	*Halomonas desiderata*	X92417	γ-proteobacteria
Ap-4	1035/1051 (98%)	*Halomonas nitritophilus*	DQ289066	γ-proteobacteria
Ap-5	1485/1499 (99%)	*Halomonas nitritophilus*	AJ309564	γ-proteobacteria
Ap-6	1542/1545 (99%)	*Staphylococcus saprophyticus*	AP008934	Firmicutes
Ap-7	1518/1526 (99%)	*Staphylococcus saprophyticus*	AP008934	Firmicutes
Ap-8	1538/1546 (99%)	*Staphylococcus saprophyticus*	AP008934	Firmicutes
Ap-9	1543/1545 (99%)	*Staphylococcus saprophyticus*	AP008934	Firmicutes

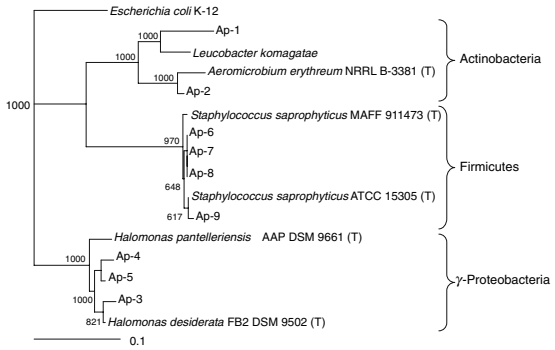

Fig. 1 Neighbour-joining tree based on nearly complete 16S rDNA sequences, showing relationships between isolated bacteria from *Arthrospira platensis* SAG 21.99 culture and other species. Percentage bootstrap values (>50%, 1,000 resamplings) are given at branch points. *Escherichia coli* K-12 was used as an outgroup. Bar, 0.1 nucleotide substitutions per position

99% similarity value. Preliminary sequence analyses using the BLAST network service and subsequent phylogenetic analyses showed that the strains belonged to γ-Proteobacteria (Ap-3–Ap-5), Actinobacteria (Ap-1 and Ap-2), and Firmicutes (Ap-6–Ap-9) (Fig. 1). As shown in the neighbour-joining tree, the Ap-9 strain was significantly unassociated with 3 strains (Ap-6–Ap-8), with a weak bootstrap support (64%).

Antibiotic selection

To determine the optimal concentration of antibiotic treatment for *A. platensis*, five different agents were tested against the cell survival of *A. platensis*. Erythromycin and chloramphenicol caused the lowest MLC (<10 μg ml^{-1}) for *A. platensis*, cefoxitin an intermediate MLC (50 μg ml^{-1}), and neomycin and imipenem the highest MLC (>300 μg ml^{-1}) (Fig. 2). *A. platensis* was resistant to two out of the 5 antibiotics tested. To optimize antibiotic selection as regards the isolated bacteria, cefoxitin showed a higher MLC for Ap-5 and Ap-9, imipenem a higher MLC for Ap-9, neomycin a higher MLC for A-5 and Ap-8, chloramphenicol a higher MLC for Ap-6, and erythromycin a higher MLC for Ap-5, Ap-6, and Ap-9. Among the 9 isolated bacteria, *Halomonas* sp. (Ap-5) and *Staphylococcus* sp. (Ap-6, Ap-8, and Ap-9) were both resistant to all the antibiotics tested. Ap-5 was resistant to neomycin, erythromycin, and cefoxitin, yet sensitive to imipenem. Ap-8 was resistant to neomycin, yet sensitive to imipenem. Ap-9 was resistant to imipenem, yet sensitive to neomycin.

After 7 days of incubation, the biomass of *A. platensis* SAG 21.99, which was grown in an SOT medium supplied with imipenem (150 μg ml^{-1} and 300 μg ml^{-1}) and imipenem and neomycin (each 150 μg ml^{-1}), was less than 89, 86, and 69% of that with no antibiotics, respectively (Fig. 3). These results showed that neomycin (150 μg ml^{-1}) was more toxic to *A. platensis* SAG 21.99, while it was resistant to imipenem (up to 300 μg ml^{-1}).

Eliminating heterotrophic bacteria in cultures

The washing and antibiotic treatment used to eliminate bacteria from the *A. platensis* culture grown for 7 days in an SOT medium. The bacterial concentration was reduced to about 1/100 by the washing step (data not shown). The microphotographs show a reduction in the contaminating bacteria after the antibiotic treatment (Fig. 4), where the final photograph (Fig. 4C) shows a clear background, implying no contaminating bacteria.

For effect of antibiotics, it was determined that the growth rate and photosynthetic efficiency of axenic strain. In autotrophic cultivation, the growth of the non-axenic and axenic strains for 5 days was similar. However, the growth of the non-axenic strain

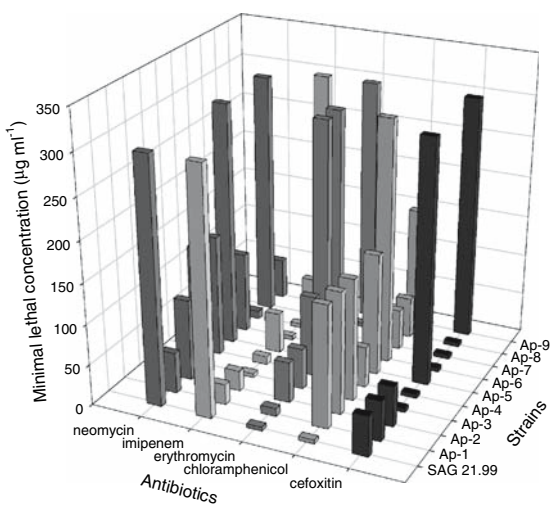

Fig. 2 Minimum lethal concentration of several antibiotics against *Arthrospira platensis* and bacteria isolated from its culture

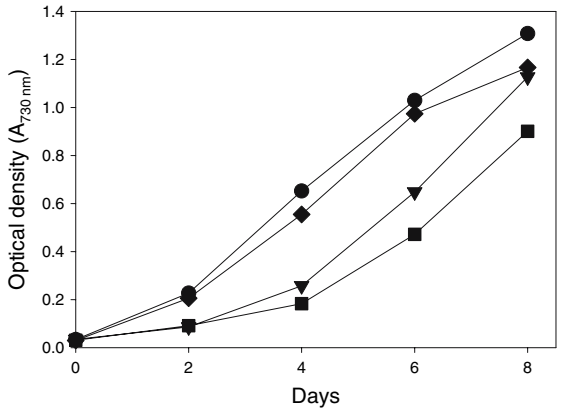

Fig. 3 Growth curve of *Arthrospira platensis* in SOT medium containing antibiotics. Cultures were incubated at 30°C and exposed to 100 μmol photons m^{-2} s^{-1}. (●, no antibiotics; □, imipenem 150 μg ml^{-1}; ▼, imipenem 300 μg ml^{-1}; ■, imipenem 150 μg ml^{-1} + neomycin 150 μg ml^{-1})

decreased after 5 days, and the standard deviation of the biomass concentration of the non-axenic strain increased. It is possible that non-axenic strain was affected by the contaminating bacteria. When measuring the photosynthetic activity using the ^{14}C technique at a 64 μmol photons m^{-2} s^{-1} light intensity following 30 min of incubation, the ^{14}C uptake was found to be linear over the incubation period, plus the maximum photosynthetic rate on a cell C basis for the non-axenic and axenic strains was 0.135 h^{-1} and 0.139 h^{-1}, respectively.

Discussion

The diversity of the bacteria isolated from the *A. platensis* cultures was relatively simple, essentially due to the long term preservation of the *A. platensis* culture in the culture collection. Among the contaminating bacteria, *Leucobacter* sp., *Aeromicrobium* sp., and *Staphylococcus* spp. all grow in human skin. Thus, it is possible that this contamination occurred during the subculturing process. In contrast, *Halomonas* spp., included γ-proteobacteria, grow in alkaline ponds. Therefore, this contamination possibly resulted from the collected water sample.

Arthrospira platensis was resistant to imipenem and neomycin but isolated 9 bacteria were sensitive to imipenem and/or neomycin. Therefore, imipenem and neomycin were finally selected out of the five tested antibiotics to eliminate the contaminating bacteria.

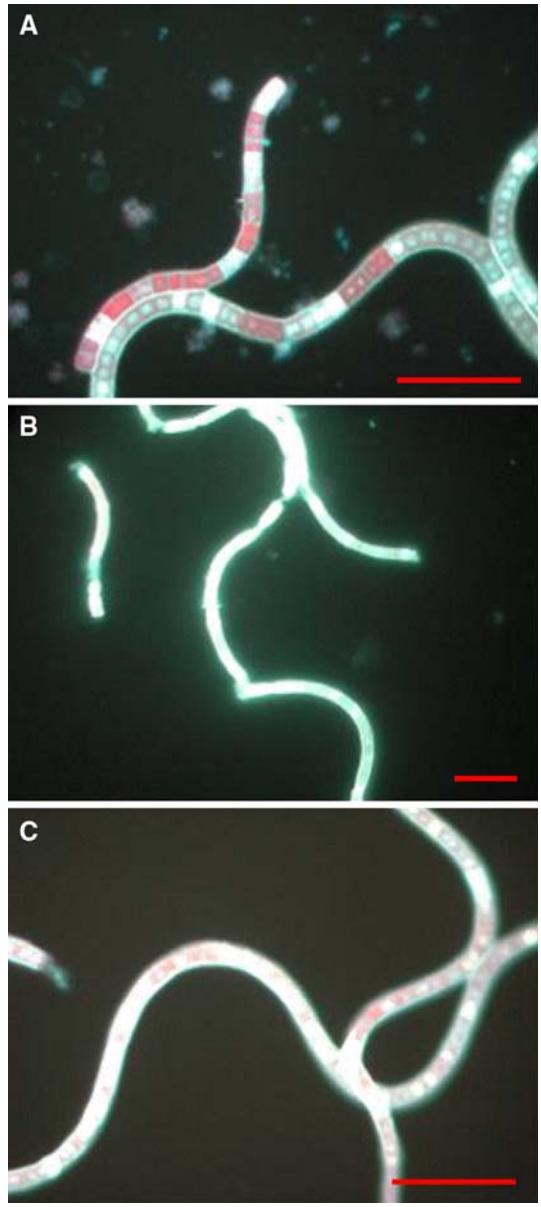

Fig. 4 Fluorescence microphotographs of DAPI-stained *Arthrospira platensis* culture after washing and antibiotic treatment (**A** and **C**; **B**). Scale bars, 20 μm. (**A**, after washing step; **B**, after treatment with mixture of imipenem [100 μg ml^{-1}] and cycloheximide [20 μg ml^{-1}]; **C**, after treatment with mixture of neomycin [100 μg ml^{-1}] and cycloheximide [20 μg ml^{-1}])

Previous report of various antibiotic treatment showed that neomycin, gentamycin, and kanamycin treatment increased bacteria numbers to 130–145% of the concentration in cultures grown without antibiotics (Cottrell and Suttle 1993). The reduction in bacterial numbers after incubation with imipenem (2.5 orders of

magnitude in 24 h) was consistent with the data of Ferris and Hirsch (1991). Bolch and Blackburn (1996) also showed that 48 h incubation produced a much more considerable reduction of bacterial contamination (4.5 orders of magnitude).

After centrifugation, the *A. platensis* was separated from the bacteria in the lower layer, due to the gas-vesicles in the cytoplasm. The only precipitate of *A. platensis* was picked out using a micropipette easily.

When the antibiotic treatment was performed in the light condition, *Arthrospira* spp. strains were killed. The relative imipenem resistance of *Arthrospira* spp. in dark condition was thus due to their inability to grow in darkness in SOT medium containing 0.1% glucose. During the subsequent experiments in which antibiotics were added to eliminate the contaminating bacteria, the medium was supplemented with 0.1% glucose, as modified by Cottrell and Suttle (1993), to stimulate the bacterial growth and antibiotic susceptibility. The conventional process used for axenic culture induction is finally based on a plated method (Walsby 1981), yet this is time-consuming, taking more than 2 weeks. In contrast, the proposed method is more appropriate than a solid medium and only requires 3 days. Furthermore, axenic strains have also been induced from 5 strains of *A. platensis* and 1 strain of *A. maxima* for 3 days using the proposed washing and antibiotic treatment method (data not shown).

No significant damage was caused to the *A. platensis* by the antibiotic treatment. However, the axenic strain induced by the antibiotic treatment may have lost its gas-vacuolation, while retained by the non-axenic parent. This phenomenon was previously reported with isolated axenic *Microcystis* strains (Bolch and Blackburn 1996).

To verify the axenicity of the cyanobacterial strain, a test was performed using an SOT agar medium enriched with 0.1% glucose, and another test carried out with an LB, R2A, and 1/10 NA agar. The axenic cultures were incubated in an SOT medium containing glucose for 2 days, followed by DAPI staining and epi-fluorescence microscopy. Purity verification of axenic *A. platensis* was confirmed for 1 week by test agar plate. While plating or inoculating old cultures onto test media is a convenient and rapid way to assess contamination, the examination of presumptively axenic cultures by DAPI staining under an epi-fluorescence microscopy is a more reliable test.

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