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## **Association of heterotrophic bacteria with aggregated *Arthrospira platensis* exopolysaccharides: implications in the induction of axenic cultures**

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*Abbreviations:* EPS, exopolysaccharides; cfu, colony forming unit; DAPI, 4',6-diamidino-2-phenylindole.

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## **Abstract**

Inducing an axenic culture of the edible cyanobacterium *Arthrospira (Spirulina) platensis* using differential filtration alone is never successful; thus, it has been thought that, in non-axenic cultures, a portion of contaminating bacteria is strongly associated with *Arthrospira* cells. However, examination of the behavior of these bacteria during filtration revealed that they were not associated with *Arthrospira* cells but with aggregates of exopolysaccharides (EPS) present in the medium away from the *Arthrospira* cells. Based on this finding, a rapid and reliable method for preparing axenic trichomes of *A. platensis* was established. After verifying the axenicity of the resulting trichomes on enriched agar plates, they were individually transferred to fresh sterile medium using a handmade tool, a microtrowel, to produce axenic cultures. With this technique, axenic cultures of various *A. platensis* strains were successfully produced. The technique described in this study is potentially applicable to a wider range of filamentous cyanobacteria.

**Key words:** cyanobacteria; *Arthrospira platensis*; exopolysaccharides; axenic cultures; Alcian blue

## Introduction

Edible cyanobacteria belonging to the genus *Arthrospira* (formerly known as *Spirulina*) are consumed worldwide as sources of food and food additives as well as animal and fish feed, because they are rich in proteins, minerals, vitamins, and other nutrients (e.g.,  $\beta$ -carotene).<sup>1-3)</sup> Phycobilin pigments extracted from these cyanobacteria are also widely used as natural colorants for foods and cosmetics.<sup>3,4)</sup> Because most strains of *Arthrospira* were living in and collected from alkaline lakes, they can be cultured under high pH conditions (up to pH 11) in which propagation of other microalgae is suppressed. This enables *Arthrospira* to be propagated as nearly single algal strains in open ponds, making them suitable for commercial large-scale production.<sup>1,2)</sup> Among the species belonging to the genus *Arthrospira*, *A. platensis* is one of the most commonly used for commercial culture. Because of its economic importance, it has been used by an increasing number of laboratories for both basic and applied studies.

In the study of microorganisms, pure cultures, or axenic cultures, are often important to avoid irrelevant microorganisms affecting experimental results. Usage of axenic cultures is in particular important for highly sensitive technologies such as genome sequencing, genome-wide transcriptome analysis and metabolome analysis, because results of these analyses are easily affected by contaminating microorganisms. Although high pH and high salt conditions of the growth medium of *A. platensis* suppress the propagation of other microalgae, there are a number of bacterial species that can tolerate these conditions. Thus, *Arthrospira* strains collected from natural habitats are generally non-axenic, as they contain a variety of heterotrophic bacteria. In order to obtain axenic cultures, efforts have been made to eliminate contaminating bacteria with eventual success. However, as noted below, the techniques used in previous studies included laborious and time-consuming steps.

It is particularly important in studies of *A. platensis* to establish a rapid and efficient method to eliminate contaminating bacteria, because there is no reliable method available for cryopreserving this cyanobacterium.<sup>5)</sup> Because strains of *A. platensis* are maintained by sub-culturing rather than cryopreservation, any axenic culture may eventually be lost once it is contaminated with bacteria that propagate in its growth medium. Although the alkaline and high salt conditions of the medium for *Arthrospira* may suppress the propagation of many heterotrophic bacteria, bacterial contamination still occurs. For example, examination of the heterotrophic bacteria in a non-axenic culture of *A. platensis* SAG21.99 showed that, in addition to the bacteria that grow in alkaline lakes, bacterial species that grow on human skin were also found in these cultures,<sup>6)</sup> suggesting that this contamination occurred during the maintenance of the culture. Bacterial contamination was also observed in our laboratory in a (previously axenic) culture of *A. platensis*. Thus, even after an axenic culture has been obtained, a rapid technique to eliminate contaminating bacteria is still required.

In unicellular bacteria, spreading or streaking a cell suspension on an agar plate may result in the isolation of axenic colonies. However, this is not the case for *Arthrospira*, which has a filamentous multicellular form that typically reaches more than several hundred micrometers in length. Spreading, streaking, or pouring aliquots of a non-axenic culture onto agar plates almost

always results in the growth of contaminating bacteria that are attached on the surfaces of these filaments, or trichomes. The gliding motility observed in many strains makes things worse. If contaminating bacteria are present on or near trichomes, they are smeared over the entire trichome surface as they crawl on agar plates.

The first report on inducing an axenic culture of *A. platensis* was by Ogawa and Terui (1970).<sup>7)</sup> They obtained axenic cultures by first filtering cell suspensions through silk cloth in which the interlacing yarn trapped *Arthrospira* trichomes while allowing the majority of contaminating bacteria to pass through. Because filtration alone was insufficient to completely eliminate contaminating bacteria, suspensions of the cells that were recovered from the silk cloth were irradiated with ultraviolet (UV) light to kill contaminating bacteria. It was thought that the mucilage surrounding *Arthrospira* cells may possibly shield these cells from a lethal UV-irradiation dose. After treatment with UV light, trichomes were individually transferred to 124 tubes containing culture medium, and three of the resulting cultures were determined to be axenic. This treatment with UV light was thought to have been essential to produce an axenic culture, as the number of contaminating bacteria after the filtration step was as many as  $10^2$ – $10^3$  cells per each trichome.<sup>7)</sup> These bacteria were thought to be cohabiting in the mucilage that surrounded *Arthrospira* cells.<sup>7,8)</sup>

UV irradiation poses a risk of inducing unwanted mutations. In order to avoid this, an alternative technique that employed antibiotics to eliminate contaminating bacteria was developed.<sup>8)</sup> After washing *Arthrospira* trichomes by filtering through a fast-flow filter paper, a mixture of antibiotics was added to the cell suspension, plated on a rich medium, and kept in the dark, where the propagation of *Arthrospira* was suppressed but that of heterotrophic bacteria was not. Because the antibiotics used in this technique inhibited cell wall synthesis, *Arthrospira* cells, which cease cell division while in the dark, were not killed by these antibiotics, whereas heterotrophic bacteria, which grow under these conditions, rupture because of the generated osmotic pressure as they grew. When employing this technique, five of 76 cultures generated from antibiotic-treated trichomes were axenic.<sup>8)</sup> This technique has also been employed by other researchers, with modifications to the combinations and concentrations of antibiotics.<sup>6,9)</sup> In this technique, careful adjustments of the concentrations of each antibiotic are necessary to selectively kill contaminating bacteria, because *Arthrospira* is also sensitive to these agents.

However, successful attempts that use no toxic agents have also been reported.<sup>10-12)</sup> For example, trichomes of *Arthrospira* were repeatedly transferred to fresh sterile medium for 20–25 times, and the resulting washed trichomes were individually cultured. Axenic cultures were obtained after 1 month of culture.<sup>12)</sup> This result radically conflicted with the view noted above that considerable numbers of bacteria cohabit with *Arthrospira* cells by being embedded in the mucilage that surrounds these cells. In contrast, this suggests the possibility that these bacteria are not tightly associated with the trichomes of *Arthrospira* and that the reason for the failure of previous attempts to filter out contaminating bacteria was simply because the filtration methods used were inefficient for removing them.

It was attempted also in our laboratory to produce axenic cultures of various *A. platensis* strains by serially transferring trichomes to droplets of fresh sterile medium to dilute

contaminating bacteria and was successful in many cases. However, because the nature of contaminating bacteria was not described in previous reports, with each washing the trichomes were incubated for a long period of approximately 30 min to overnight before transferring them to the next fresh medium, with the anticipation of this promoting the dissociation of contaminating bacteria from the trichomes. However, the cultures obtained were, in quite a few cases, found to be non-axenic after a long culture period of several weeks.

To improve the efficiency of removing contaminating bacteria, the efficiency of various filter media for removing contaminating bacteria was examined in the present work. The behavior of contaminating bacteria during filtration was also investigated, and this showed that a portion of these bacteria were associated with aggregates of exopolysaccharides (EPS) that were in the medium away from the *Arthrospira* cells. Based on this finding, a rapid technique to eliminate contaminating bacteria was developed. Using this technique, contaminating bacteria can be easily eliminated by simple filtration and rapid washing. In addition, the time required for the determination of the axenicity status is greatly reduced using a technique of single-trichome manipulation with a handmade tool, a microtrowel. With this technique, multiple axenic trichomes can be obtained in less than 30 min, and the axenicity status of individual trichomes can be determined in less than 1 day.

## Materials and Methods

*Strains and cultivation conditions.* A non-axenic culture of *A. platensis* UTEX 1926 was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX). Non-axenic cultures of *A. platensis* SAG 21.99 and SAG 85.79 were obtained from Sammlung von Algenkulturen der Universität Göttingen (SAG). Axenic cultures of *A. platensis* NIES-39 and NIES-46 were obtained from Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba (MCC-NIES). A spontaneous *A. platensis* variant with a straight morphology was isolated from *A. platensis* NIES-39. Till date, this variant has been maintained for more than 2 years, and no phenotype reversion has been observed. Thus, it is likely to be a mutant. During the maintenance of its culture by serial transfer, it became non-axenic, as it was contaminated with some heterotrophic bacteria. *A. platensis* strains were cultured at 30°C on a 12-h light/12-h dark cycle in SOT medium<sup>7,13)</sup> that was prepared as described,<sup>14)</sup> except that the medium for *A. platensis* UTEX 1926 was 0.5× the strength of the standard medium.<sup>15)</sup> The photosynthetic photon flux density during the light period was approximately 70  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  unless otherwise stated. To determine the colony-forming units (cfu) of contaminating bacteria, samples that were serially diluted with the SOT medium were spread on enriched SOT plates (agar plates made with the SOT medium supplemented with 0.2% (w/v) yeast extract),<sup>7)</sup> and the numbers of colonies were counted after incubation at 30°C for 2 days. When preparing the enriched SOT plates, a melted agar solution in water containing yeast extract was mixed with other components after autoclaving.

*Filter media and filtration.* Nylon mesh with 20  $\mu\text{m}$  openings (Cat. No. N-NO. 508S) was purchased from NBC Meshtec (Tokyo). It was attached to bottoms of polypropylene tubes to make handmade sieves, or filtration tubes (see below). Polycarbonate membranes with pores of a defined size (Nuclepore track-etched membrane, 8.0  $\mu\text{m}$  pore size) were purchased from GE Healthcare Japan (Tokyo). Silk fabric *habutaé* used as a liner for Japanese *kimono* clothes was purchased from a local clothing shop. Fast-flow quantitative filter paper with a particle retention size of 20  $\mu\text{m}$  (grade 41) was from GE Healthcare Japan. Polycarbonate membranes, pieces of the silk cloth, and filter papers were placed in polycarbonate filter holders (13 mm Pop-Top Filter Holder, GE Healthcare Japan) and were sterilized by autoclaving at 121°C for 15 min. After washing the trichomes on these filters with 50 mL of SOT medium, filter holders were disassembled, and the washed trichomes on these filters were recovered in a small volume of the same medium (typically <1 mL).

*Filtration tubes.* Handmade nylon sieves, or filtration tubes, were used for filtration with a nylon mesh. These were made with 15-mL polypropylene conical centrifuge tubes that were cut with a saw at a cross-section approximately 2 cm from the bottom. The cut end was heated with a Bunsen burner until it was slightly melted. This end was then quickly pressed on a piece of nylon mesh placed on a flat surface and kept in place by pressing until it cooled and hardened. Then, the unnecessary regions of the nylon mesh were cut off with scissors. The filtration tubes prepared in this way could be repeatedly used after autoclaving. Since solutions applied on it freely drop down through the nylon mesh due to the gravity, solutions were continuously poured into the filtration tube when trichomes were washed with a large volume of medium.

*Histological Staining.* For Alcian blue staining to detect acidic polysaccharides, trichomes from 5 mL of culture were collected with a filtration tube with 20- $\mu\text{m}$  openings, washed on the mesh with 50 mL of SOT medium, and then suspended in 0.5 mL of the same medium. An aliquot (50  $\mu\text{L}$ ) of the suspension was placed on a MAS-coated glass slide (Matsunami Glass Ind., Ltd., Osaka), and as much medium as possible was removed using a micropipette. For the trichomes on the glass slide, 80  $\mu\text{L}$  of 3% acetic acid was added, and it was kept for 10 min at room temperature. As much of the acetic acid solution as possible was then removed with a micropipette. Equilibration using 3% acetic acid was repeated once. Alcian blue staining solution (pH 2.5; Nakalai Tesque, Kyoto) was then added to the trichome preparation on a glass slide. After 10 min at room temperature, the excess solution was removed, and the sample on the glass slide was carefully washed twice with 100- $\mu\text{L}$  each of 3% acetic acid and then three times with 100  $\mu\text{L}$  of distilled water. After placing a cover slip on this preparation, it was observed using the All-in-One Microscope BZ-9000 (Keyence, Osaka). For double staining with Alcian blue and Hoechst 33342, an Alcian blue-stained sample on the glass slide was incubated with Hoechst 33342 (2  $\mu\text{g}/\text{mL}$  in distilled water) at room temperature for 10 min, washed 3 times with 100  $\mu\text{L}$  of water, and then observed using the All-in-One Microscope BZ-9000.

*Microtrowel.* A microtrowel was used to manipulate individual *A. platensis* trichomes. This was made from a platinum wire of approximately 0.5 mm in thickness and 7 cm in length. The tip of the wire (approximately 3 mm) was flattened by repeated hitting with a hammer on a Jeweler's steel bench block. The upper edge was sharpened by sanding with P1000-grit sandpaper. Then, while observing under the dissecting microscope, the tip was bent using fine-tipped forceps that are used for ophthalmologic surgery so that its scooping action became easy. The other end was set into a holder for the platinum loop commonly used in microbiology experiments. The tip of the microtrowel was sterilized intermittently before use with a gas lighter or a Bunsen burner.

*Induction of axenic culture.* The following protocol was used routinely to induce axenic cultures. A non-axenic culture was diluted to 1/20 with fresh medium and cultured at 30°C. When the culture was in the mid- to late-log phase (absorbance at 730 nm of 0.2–0.8), 1 mL of this was mixed with 19 mL of SOT medium, transferred to a 50-mL conical tube, and agitated vigorously for 2 min using a Vortex mixer. This was then applied on a filtration tube with 20- $\mu$ m openings. After filtration, the medium that remained at the bottom of the tube was removed by blotting with a clean Kimwipe (Nippon Paper Crexia, Tokyo). The trichomes that were trapped on the mesh of the filtration tube were washed with 50 mL of fresh sterile SOT medium. After washing, the medium that remained at the bottom of the filtration tube was completely removed by blotting. The trichomes on the mesh were recovered by suspending them in 1 mL of the SOT medium, and then transferred into a sterile Petri dish. The suspension of the washed trichomes was diluted with 10 mL of SOT medium. One trichome was retrieved from the suspension with 1  $\mu$ L of medium under a dissecting microscope using a micropipette (Pipetman P20, Gilson). Then, this was transferred to a 0.3-mL droplet of fresh *Arthrospira* medium placed on a Petri dish. After stirring the medium with a plastic tip of the micropipette, the trichome with 1  $\mu$ L of medium was transferred to another 0.3-mL droplet of fresh SOT medium. The trichome washing was repeated once, after which it was transferred using a micropipette to a plate of a solidified, enriched SOT medium. The trichome processing was repeated until a desired number of trichomes had been placed on this plate. The trichomes on the plate were incubated at 30°C under reduced light intensity (the photosynthetic photon flux density was approximately 40  $\mu$ mol m<sup>-2</sup> sec<sup>-1</sup>). After 20–24h, they were observed under the dissecting microscope, and those that were verified to be axenic were individually transferred to sterile SOT medium using the microtrowel. These were then propagated to produce axenic cultures.

*Examination of the axenicity status of cultures.* After propagating the trichomes for 1 month at 25°C under 12-h light/12-h dark cycle under reduced light conditions (the photosynthetic photon flux density during light period was approximately 40  $\mu$ mol m<sup>-2</sup> sec<sup>-1</sup>), the axenicity of cultures was tested by spreading 0.2 mL of the culture on enriched SOT plates, R2A plates,<sup>16)</sup> and LB plates<sup>17)</sup> Cultures were also spread on agar plates made of the SOT medium that contained 0.1% (w/v) peptone and 10 mM methylamine hydrochloride to detect any

methylaminotrophs.<sup>18)</sup> Samples spread on these plates were incubated at 30°C for up to 2 weeks with occasional inspection. Also, to detect any bacteria including unculturable ones, bacteria in the cultures were collected using track-etched polycarbonate membranes (0.2  $\mu\text{m}$  pore size) as described,<sup>19)</sup> with some modifications. The track-etched membranes (13 mm diameter; GE Healthcare Japan) used in this experiment were prestained with Sudan Black B to prevent background fluorescence.<sup>20,21)</sup> Cultures were prefiltered through track-etched membranes of 8  $\mu\text{m}$  pore size to remove *Arthrospira* trichomes, because when present in the samples they interfered with the detection of heterotrophic bacteria on membranes, covering entire membrane surfaces. Heterotrophic bacteria collected on the prestained track-etched membranes (0.2  $\mu\text{m}$  pore size) by filtration were fixed for 30 min at room temperature with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) by applying 2 mL of the fixation solution on the membranes. The membranes were then washed with 2 mL of Tris-buffered saline (50 mM Tris-Cl (pH 7.5), 150 mM NaCl). Bacterial cells on the membranes were stained with DAPI (50  $\mu\text{g}/\mu\text{L}$  in Tris-buffered saline) for 10 min at room temperature and observed using the All-in-One Microscope BZ-9000.

## Results

### *Trichome sizes and dimensions of Arthrospira strains*

The trichomes of wild-type *Arthrospira* strains display filamentous helical structures.<sup>22)</sup> However, their overall appearance were slightly different from one another (Supplemental Fig. 1). Even among individuals of a single wild-type strain, the lengths and dimensions of trichomes were different from one another to some extent. Before performing filtration experiments, the size distribution of the trichomes of various strains was determined, because this was the most important factor that affected whether they would be retained on particular filters. A spontaneous variant of *A. platensis* NIES-39 with a straight morphology was also included in this analysis. Examination of the size distribution of these strains showed that length of the majority of trichomes distributed between 200 and 800  $\mu\text{m}$ , and the width of helices of wild-type strains distributed between approximately 40-70  $\mu\text{m}$  (Supplemental Fig. 2). Filament thickness was approximately 8  $\mu\text{m}$  for all strains except *A. platensis* SAG 85.79, which had filament thickness of approximately 11  $\mu\text{m}$ . These trichomes appeared sufficiently large to be separated from unicellular bacteria by differential filtration.

### *Evaluation of filters suitable for the removal of contaminating bacteria*

The following filter media were compared: A Japanese silk fabric *habutaé*; a quantitative filter paper with a particle retention size of 20  $\mu\text{m}$ ; a track-etched polycarbonate membrane with pores of 8  $\mu\text{m}$ ; and a nylon mesh with openings of 20  $\mu\text{m}$ . The silk fabric *habutaé* was the one used in the pretreatment in a former experiment that employed UV-irradiation to kill contaminating bacteria.<sup>7)</sup> The quantitative filter paper was used in a former experiment that employed antibiotics to kill contaminating bacteria.<sup>8)</sup> The track-etched polycarbonate membrane

was shown to efficiently remove contaminating bacteria from cultures of various microalgae,<sup>23)</sup> although its application to an *Arthrospira* culture had not been previously reported. The nylon mesh had never been used in the induction of axenic cultures of *Arthrospira*, but it was expected to efficiently remove contaminating bacteria because it had a high number of homogeneous openings that were sufficiently large to allow unicellular bacteria to freely pass through.

To determine which was the most effective in removing contaminating bacteria, a non-axenic culture of *A. platensis* UTEX 1926 was filtered on these filter media. After washing trichomes on them with fresh medium, the cfu of heterotrophic bacteria remaining in the resulting trichome preparations were determined by spreading diluted samples on solidified SOT medium supplemented with 0.2% yeast extract. This enriched medium supports the propagation of heterotrophic bacteria in non-axenic cultures of *A. platensis*.<sup>7)</sup> The filtration experiment showed that the nylon mesh was the most effective in removing contaminating bacteria, as the number was reduced by an average of  $5.5 \times 10^{-4}$  compared with that in the original culture (Supplemental Fig. 3). This was better than the average obtained with the track-etched polycarbonate membrane ( $2.1 \times 10^{-3}$ ). Silk cloth and the filter paper that had been used in previous reports were less effective than these filter media.

Although filtration on nylon mesh was effective in removing contaminating bacteria, repeated filtration did not significantly reduce the number of contaminating bacteria; it was reduced by more than 3 orders of magnitude after a single round of washing, but second and third rounds of washing reduced it only by, on average, 24% and 21% respectively (Supplemental Fig. 3). This result was consistent with the previous notion that a portion of contaminating bacteria is associated with trichomes and never completely removed by filtration. However, since slight decreases in the numbers of bacteria were observed after the second and third filtrations, it was possible that these reflected the dissociation of contaminating bacteria from the trichomes over time. Thus, the time-course for the dissociation of contaminating bacteria from the washed trichomes was examined next.

#### *Contaminating bacteria are not associated with trichomes*

After *A. platensis* UTEX 1926 trichomes were washed by filtering through the nylon mesh, they were suspended in fresh medium. Then, the time-course for the release of bacteria from these trichomes was monitored by carefully removing small aliquots of the medium from the suspension so that they contained no trichomes. The rationale for this experiment was that if contaminating bacteria associated with trichomes were released over time, then the number of bacteria in the aqueous portion of the suspension would increase with time. To determine the contribution of cell propagation during incubation, it was also necessary to monitor the propagation of contaminating bacteria during this time period. This was achieved by monitoring the increase in cfu in trichome-free filtrates prepared by filtering a non-axenic culture through a track-etched polycarbonate membrane that allowed free-floating, contaminating bacteria to pass through but not the trichomes and their fragments. In addition, to detect trichome-associated bacteria, individual trichomes were retrieved from the suspension

along with minimal volumes of medium (approximately 1  $\mu\text{L}$ ) using a micropipette under a dissecting microscope. Each of these trichomes was rapidly washed by consecutive transfers to three separate droplets (0.3 mL each) of fresh sterile medium. Each washed trichome was then placed on an agar plate of enriched medium and incubated at 30°C to detect any heterotrophic bacterial growth associated with the trichome.

As shown in Figure 1A, an increase in contaminating bacteria in the trichome-free filtrate was not detected during the incubation period. This was understandable because the carbon source required for heterotrophic bacterial growth was not included in the medium of *A. platensis*. The experiment to detect the release of bacteria from trichomes (Fig. 1B) indicated that considerable numbers of contaminating bacteria were present in the aqueous part of the suspension from the beginning of incubation (0 h) and that this number remained nearly constant during the incubation period. This result was unexpected. Because the trichomes used in this experiment had been thoroughly washed on the nylon mesh, contaminating bacteria that remained after the washing should have been associated with relatively large materials that barely passed through this mesh. These contaminants were most likely associated with *Arthrospira* trichomes, because such large aggregates of contaminating bacteria were not detected by the visual inspection of the suspension under a microscope. However, the results in Figure 1B indicated that a considerable number of contaminating bacteria was in the aqueous part of the suspension from the beginning and had been separated from the *Arthrospira* trichomes.

The result of the rapid washing of individual trichomes (Fig. 1C) was also unexpected but was consistent with the experimental results shown in Figure 1B. Incubating individual trichomes on enriched agar plates showed that those trichomes that had been washed on nylon mesh were free of heterotrophic bacteria from the beginning of incubation (Fig. 1C). Overall, the results shown in Figure 4 indicated that the heterotrophic bacteria that remained after the differential filtration were apparently in the aqueous part of the trichome suspension rather than being associated with trichomes.

#### *Extracellularly released materials in A. platensis cultures*

The materials that contaminating bacteria were associated with may have been larger than 20  $\mu\text{m}$ , because they did not pass through the nylon mesh of this size. However, no such materials were found in the trichome suspension under a bright-field microscope (Fig. 2A). Thus, they appeared transparent. In this regard, many cyanobacteria have been reported to produce extracellularly secreted polysaccharide substances,<sup>24,25)</sup> which are referred by different names such as extracellular polymeric substances, exocellular polysaccharide substances, exocellular polysaccharides and exopolysaccharides, and are commonly abbreviated as EPS. For some cyanobacterial species, a fraction of the EPS form aggregates in culture medium and can be visualized by histological staining with dyes (*e.g.*, Alcian blue) that bind to acidic mucopolysaccharides.<sup>26)</sup> To determine whether any aggregates of the EPS were in the *Arthrospira* preparations, the trichome preparation after washing on the nylon mesh was stained with Alcian blue.

As shown in Figure 2B, a number of amorphous materials were in the samples stained with Alcian blue and were larger than the size of the openings of the nylon mesh (20  $\mu\text{m}$ ). Many of these appeared to be present away from the trichomes (although some of these appeared to be associated with trichomes in the microscopic image, they were easily separated from trichomes by gentle stirring). Thus, the reason that contaminating bacteria were not completely removed by filtration may be that a portion of bacterial cells was associated with these amorphous materials. These materials were not unique to non-axenic cultures, as similar Alcian blue-stainable materials were also observed in axenic cultures of *A. platensis* NIES-39 and *A. platensis* NIES-46 (Fig. 2C and D). The presence of these materials in axenic cultures indicated that they were produced by *Arthrospira* cells themselves rather than by contaminating bacteria.

To determine whether contaminating bacteria were actually associated with these amorphous materials, trichome preparations were double-stained with Alcian blue and the fluorescent dye Hoechst 33342. Because Hoechst 33342 stains DNA, the genomic DNA of any contaminating bacteria should be detectable. As shown in Figure 3B, a number of fluorescent signals associated with the Alcian blue-stained materials were detected with Hoechst 33342 staining when samples from a non-axenic culture were used. In contrast, no such signals were detected when samples were prepared from an axenic culture of the same strain (Fig. 3E). Thus, these fluorescent signals associated with the amorphous materials were certainly derived from contaminating bacteria. This clearly showed that, in a non-axenic culture, a number of contaminating bacteria were associated with the Alcian blue-stainable amorphous material. Because many of these were larger than the filter openings, they were not eliminated by filtration. However, as demonstrated in the experimental results shown in Figure 1C, they could be readily removed by washing individual trichomes through droplets of fresh medium, as they were not tightly bound to trichomes.

#### *Protocol for eliminating contaminating bacteria is applicable to various strains of A. platensis*

For the experimental results in Figure 1C, a total of 36 trichomes (six trichomes each from 6 different time points) were retrieved from the trichome suspension, and none gave rise to colonies of heterotrophic bacteria. Thus, they seemed to be axenic. However, it is known that unculturable bacteria are often present in algal cultures obtained from their natural habitats. To detect any heterotrophic bacteria, trichome preparations prepared in this way were observed using a fluorescence microscope after staining them with Hoechst 33342. Fluorescent signals of heterotrophic bacteria were observed neither in the medium nor on the trichome surfaces. This suggested that contaminating bacteria were not present in this preparation.

The steps used to obtain these trichomes were as follows: (1) washing trichomes on a nylon mesh and (2) rapid washing of individual trichomes through three droplets of sterile medium. Because it took less than 20 min to complete these steps and an additional 10 min to obtain multiple trichomes, this protocol can be useful to obtain axenic trichomes if it was applicable to other strains of *A. platensis*. Because the initial series of experiments described thus far were conducted using only *A. platensis* UTEX 1926, it was next determined whether this protocol was applicable to other strains.

As shown in Table 1, the vast majority of trichomes did not give rise to heterotrophic bacterial colonies when this protocol was used for non-axenic culture of *A. paltensis* SAG 21.99, *A. platensis* SAG 85.79, and a straight variant of *A. platensis* NIES-39. It is worth noting that, of 16 trichomes, bacterial growth was observed during the preparation of one in the experiment with *A. platensis* SAG 85.79 (Table 1). This indicated that, although this protocol was efficient, experimental verification was necessary to establish that a particular trichome was free of contaminating bacteria.

It is also worth noting that, for the experiment in Table 1, track-etched polycarbonate membrane with 8  $\mu\text{m}$  pores was used for the straight variant of *A. platensis* NIES-39. The nylon mesh with 20- $\mu\text{m}$  openings that was used for other strains could not be applied to this variant, as almost all trichomes of this strain passed through it. The successful isolation of axenic trichomes with the track-etched membrane indicated that, although it was slightly less efficient than the nylon mesh, it was still sufficiently efficient and could be used as an alternative filter for variants with thinner morphology than typical wild-type strains.

#### *Preparing axenic cultures by single-trichome manipulation*

Most of the trichomes obtained in the experiments shown in Table 1 were considered to be free of contaminating bacteria, because heterotrophic bacterial growth was not observed from them on enriched agar plates. If these trichomes were transferred and propagated in fresh medium, axenic cultures would be obtained. However, *A. platensis* trichomes tend to have reduced viability when subjected to mechanical damage during transfer, as with many other filamentous cyanobacteria.<sup>27,28)</sup> To manipulate individual trichomes on agar plates efficiently without subjecting them to any physical damage, a specially shaped handmade tool, or microtrowel, was made using a platinum wire. This tool had a miniature trowel-like structure at the tip that aided in scooping up a single trichome of *Arthrospira* along with a thin-layer of solid medium beneath it, thus minimizing any damage to it (Supplemental Fig. 4 and Supplemental Video).

By using this tool, five trichomes of each of the four strains listed in Table 1 were individually transferred to fresh medium. After propagating these for 1 month under reduced light, the axenicity of individual cultures was examined by spreading a portion of these cultures on agar plates of various media. All cultures did not give rise to colonies of bacteria. Also, to detect any heterotrophic bacteria including unculturable ones, particulate materials in the cultures were collected on track-etched polycarbonate membranes (0.2  $\mu\text{m}$  pore size) and stained with a fluorescent dye DAPI to detect bacterial cells. No signals of heterotrophic bacteria were detected in these cultures, whereas many bacterial cells were observed in control samples prepared from non-axenic cultures (Figure 4). In addition, microscopic observation of the obtained cultures using fluorescence microscope did not detect any bacteria other than *A. platensis* (see Fig. 3E for an example of an axenic culture of *A. platensis* UTEX 1926 obtained in this study). These analyses indicated that all these cultures were axenic.

## Discussion

It has been thought by many researchers that a considerable number of contaminating bacteria are associated with *Arthrospira* cells in non-axenic cultures, as contaminating bacteria are never completely eliminated by differential filtration.<sup>7,8)</sup> However, in this study, a careful examination of the behavior of contaminating bacteria revealed that they were not associated with *Arthrospira* cells but with aggregates of EPS present in these cultures away from the *Arthrospira* cells. This finding provided a rationale to establish a rapid, reliable method for producing axenic cultures.

The combination of filters to remove free-floating bacteria and rapid washing of trichomes to eliminate EPS-associated bacteria provided nearly instantaneous removal of contaminating bacteria. Beginning with a freshly growing non-axenic culture, multiple washed trichomes could be obtained in less than 30 min, and most of these were axenic. The absence of contaminating bacteria was confirmed by incubating them on plates of a solidified, enriched medium. Axenic cultures were then produced by transferring and culturing these trichomes in fresh sterile medium. This last process was greatly facilitated by a technique of single-trichome manipulation performed with a microtrowel (Supplemental Fig. 4 and Supplemental video). Although this technique for inducing axenic culture was initially developed using *A. platensis* UTEX 1926, it was also successfully applied to other strains such as *A. platensis* SAG 21.99 and *A. platensis* SAG 85.79. It was also successfully applied to a non-axenic culture of a morphology variant of *A. platensis* NIES-39. These results indicated that this technique was applicable to most, if not all, strains of *A. platensis*.

It was found in this study that *Arthrospira* produced aggregated EPS in the growth medium, with which a portion of contaminating bacteria was associated. EPS have been detected in the cultures of many cyanobacterial species<sup>24,25)</sup> as well as in *A. platensis* cultures.<sup>29-31)</sup> Like EPS of other cyanobacteria, those of *A. platensis* contain sulfate groups and uronic acids in addition to neutral sugars.<sup>29,31,32)</sup> Fractionating EPS from *Arthrospira* showed that they contained at least three different classes of molecules that are separable by anion exchange chromatography,<sup>30)</sup> although their localization in culture had not been determined because they were isolated from the total biomass of *A. platensis*. Some of these polysaccharide macromolecules appear to be released into the medium during *A. platensis* growth and aggregate to form the Alcian blue-stainable amorphous materials detected in this study. As mentioned above, EPS with similar chemical compositions have been found in many different cyanobacterial species. Therefore, when attempting to produce their axenic cultures, it would also be important to remove their EPS that are probably associated with heterotrophic bacteria. In this regard, the technique described in this study was successfully applied to a straight variant of *A. platensis*, whose morphology was very similar to other filamentous cyanobacterial species. This suggests that the technique described in this study is potentially applicable to many other filamentous cyanobacteria.

Further studies are needed to address questions regarding the aggregated EPS of *A. platensis*. *Arthrospira* cells have been reported to produce polysaccharide macromolecules that exhibit

potentially valuable pharmaceutical activities. For example, a fraction of polysaccharide macromolecules, calcium spirulan (Ca-SP), was shown to have antiviral activity against various enveloped viruses<sup>33,34)</sup> as well as activity to inhibit tumor invasion and metastasis when added to cultured mammalian cells.<sup>35)</sup> Also, a polysaccharide preparation (Immulina) from *A. platensis* has been reported to exhibit immunostimulatory activity *in vitro*<sup>36)</sup> and *in vivo*<sup>37)</sup>. These molecules appear to be related to EPS, as they are polysaccharide macromolecules that contain uronic acids and sulfate groups.<sup>35,36,38)</sup> Their localizations in culture are, however, yet to be determined because they were identified in and were purified from total extracts prepared from freeze-dried powder of *A. platensis*.<sup>33,36)</sup> The relationship between these molecules and the aggregated EPS detected in this study is of interest and will need to be determined by additional studies. The physiological roles of the aggregated EPS on the growth and survival of *A. platensis* are also unknown. Previous studies on EPS of other cyanobacteria suggested many possible roles, such as protecting cells from UV light and dehydration, protecting cells from salt and metal stresses, accumulating sparse amounts of metal ions around cells, operating cell sedimentation, promoting cell adhesion to solid substrates, and promoting cell-cell interactions that may lead to biofilm formation.<sup>24,25,39,40)</sup> However, in order to exert these activities, EPS must be present in close proximity to cyanobacterial cells. Because the aggregated EPS found in this study were present in the medium away from *Arthrospira* cells, it is difficult to attribute any of these functions to these aggregates when *A. platensis* propagates in liquid medium. It is possible that these aggregated EPS play more pronounced roles when *Arthrospira* propagates on solid substrates rather than in liquid media, because secreted EPS can directly interact with *Arthrospira* cells on the solid surfaces.

In addition to the finding that heterotrophic bacteria were readily eliminated from trichome preparations, this study provides an additional significant improvement in the production of axenic cultures. In previous efforts to produce axenic cultures, the most time-consuming step involved verifying the axenicity of resultant trichome preparations, because verification is performed after culturing trichomes for several weeks in liquid medium to allow propagation of heterotrophic bacteria possibly present in the preparation. The time required for this step was greatly reduced in the techniques described in this study by introducing the technique to incubate individual trichomes on an enriched agar plate rather than in a liquid medium. After determining the axenicity status of trichomes on an enriched agar plate, they were transferred to a liquid medium. All the resultant cultures were axenic when examined after trichome propagation, because the trichomes inoculated into the liquid medium were those that had already been verified to be free of contaminating bacteria.

This technique was facilitated by the introduction of a handmade tool, a microtrowel, which enabled efficient manipulation of individual trichomes on agar plates. This tool was a modification of a microspade, which is a small spatula-like tool that is used to cut out agar blocks along with bacteria.<sup>28,41)</sup> Unlike the microspade, the shape of the microtrowel was designed so that it was suitable for slicing out small thin pieces of agar from an agar surface. The alteration of its shape enabled the smooth transfer of individual trichomes together with thin layers of agar beneath them without subjecting the trichomes to any physical damage.

Using this tool in studies of *Arthrospira* would not be limited to the production of axenic cultures. For example, in genetic studies of *Arthrospira*, gliding motility exhibited by many strains has been an obstacle that impeded the isolation of clones selected for specific phenotypes on agar plates, because trichomes of motile strains that move around an agar surface do not form colonies. The microtrowel enables efficient manipulation of trichomes on agar plates, making it easy to individually transfer them to new agar plates or individual wells of microtiter plates where they can be propagated separately. This enables efficient isolation and propagation of clones that exhibit specific phenotypes on agar plates, which would greatly facilitate genetic studies and breeding improvements for this economically valuable cyanobacterium.

In conclusion, a rapid and reliable technique for producing axenic cultures of *A. platensis* was established based on the finding that a portion of contaminating bacteria was associated with aggregated EPS. With this technique, multiple axenic trichomes could be obtained only in less than 30 min, and determination of their axenicity status was completed on the following day. This rapid and reliable method for preparing axenic trichomes should serve to back up various kinds of studies on *A. platensis*.

### **Supplemental material**

Supplemental materials for this paper are available at [URL].

### **Acknowledgment**

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**Table 1.** Isolation of axenic trichomes of various *Arthrospira* strains

Strain	CFU /mL <sup>a)</sup>	Ratio of non-axenic trichomes <sup>b)</sup>
<i>A. platensis</i> UTEX 1926	$1.3 \times 10^7$	0% (0/16)
<i>A. platensis</i> SAG 21.99	$2.1 \times 10^6$	0% (0/16)
<i>A. platensis</i> SAG 85.79	$3.4 \times 10^6$	6% (1/16)
<i>A. platensis</i> , straight <sup>c)</sup>	$2.7 \times 10^6$	0% (0/16)

<sup>a)</sup>Colony-forming units of heterotrophic bacteria in 1 mL of initial non-axenic cultures.

<sup>b)</sup>Number of trichomes that were found to be non-axenic on enriched agar plates and the total number of examined trichomes are shown in parentheses.

<sup>c)</sup>For this strain, track-etched polycarbonate membrane with 8  $\mu$ m pores was used for filtration and washing, whereas nylon mesh with 20- $\mu$ m openings was used for other strains.

## Figure Legends

Fig. 1. Contaminating bacteria in sub-fractions of non-axenic cultures. (A) Growth of contaminating bacteria. (B) Contaminating bacteria in the aqueous part of the suspension of washed trichomes. (C) Contaminating bacteria associated with trichomes.

Notes: To determine growth of contaminating bacteria, trichomes were removed from a non-axenic culture (1 mL) of *A. platensis* UTEX 1926 ( $1.8 \times 10^3$  trichomes/mL) by filtering through a track-etched polycarbonate membrane with  $8 \mu\text{m}$  openings. This filtrate containing free-floating bacteria was incubated at  $30^\circ\text{C}$ . At the indicated time points, samples were withdrawn, and colony-forming units of heterotrophic bacteria were determined. The data in (A) represent the averages of five measurements, and error bars indicate standard errors of the means. In the experiment in (B), to determine the cfu of contaminating bacteria in the aqueous part of the suspension of washed trichomes, a non-axenic culture (1 mL) was applied on a nylon mesh with  $20 \mu\text{m}$  openings, and the trichomes on the mesh were washed with 50 mL of sterile SOT medium. Washed trichomes were then suspended in 1 mL of the same medium and incubated at  $30^\circ\text{C}$ . At the indicated time points,  $5 \mu\text{L}$  aliquots of the medium were withdrawn with a micropipette so as to not contain trichomes, and cfu were determined. The data in (B) represent the averages of five independent filtration experiments beginning with a single non-axenic culture, and error bars indicate the standard errors of the means. In the experiment in (C), to detect contaminating bacteria associated with trichomes, at the indicated time points, six trichomes with  $1 \mu\text{L}$  each of medium were withdrawn using a micropipette from the trichome suspension prepared in the experiment in (B), and each of these was individually washed by serial transfer to three droplets ( $0.3 \text{ mL}$  each) of sterile SOT medium. After washing, trichomes in  $1 \mu\text{L}$  of the medium were placed on a solidified SOT medium supplemented with yeast extract. Trichomes on the agar plates were incubated at  $30^\circ\text{C}$  for 72 h with occasional observation under a dissecting microscope to detect the growth of any contaminating bacteria around the trichomes.

Fig. 2. Alcian blue staining for the preparations of *Arthrospira* trichomes. (A) Bright-field view of a trichome preparation from a non-axenic culture of *A. platensis* UTEX 1926. (B) Alcian blue-stained non-axenic culture of *A. platensis* UTEX 1926. (C) Alcian blue-stained axenic culture of *A. platensis* NIES-39. (D) Alcian blue-stained axenic culture of *A. platensis* NIES-46.

Notes: Scale bars represent  $100 \mu\text{m}$ .

Fig. 3. Association of bacteria with aggregates of EPS in non-axenic cultures.

Notes: Preparations of trichomes from non-axenic and axenic cultures of *A. platensis* UTEX 1926 were double-stained with Alcian blue and Hoechst 33342. (A–C) Preparation from a non-axenic culture. (D–F) Preparation from an axenic culture. (A) and (D) Bright-field views. (B) and (E) Fluorescence images. (C) and (F) Chlorophyll autofluorescence. Scale bars represent 20  $\mu\text{m}$ .

Fig. 4. Heterotrophic bacteria in the cultures prepared in this study and in original non-axenic cultures.

Notes: Cultures of *A. platensis* UTEX 1926, *A. platensis* SAG 21.99, *A. platensis* SAG 85.79 and a straight variant of *A. platensis* NIES-39 were filtered through track-etched membranes with 8  $\mu\text{m}$  pores to eliminate *Arthrospira* trichomes. Heterotrophic bacteria in the filtrates were collected on track-etched membranes with 0.2  $\mu\text{m}$  pores and stained with DAPI. Materials from 5 mL of  $\text{Arthrospira}$  cultures were collected for the preparations obtained in this study (Axenic), whereas those from 0.2 mL were collected from non-axenic cultures (Non-axenic). Scale bars represent 5  $\mu\text{m}$ .

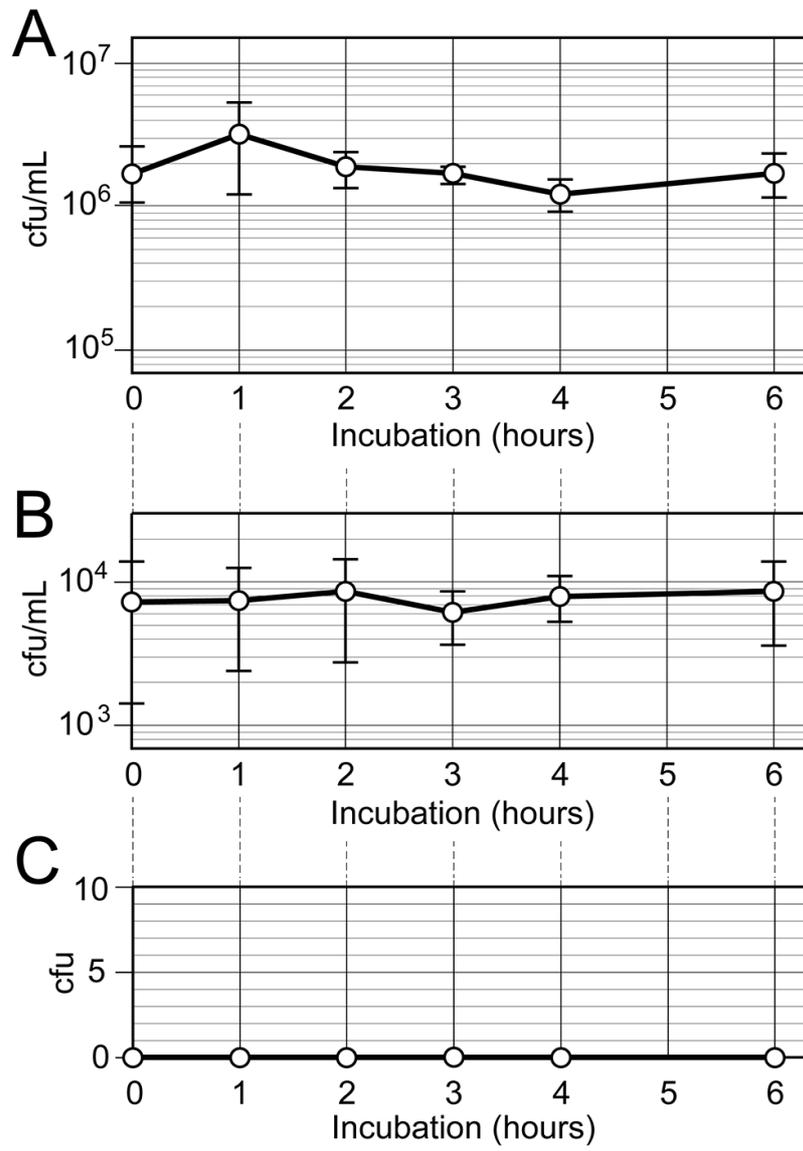


Figure 1

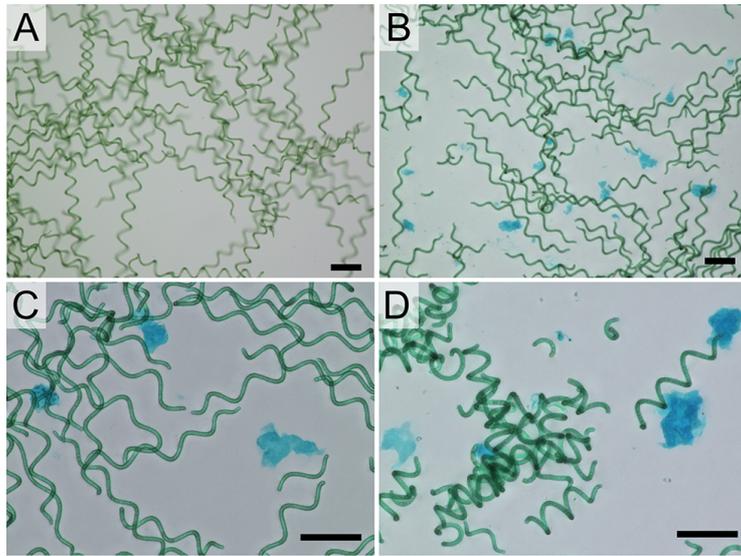


Figure 2

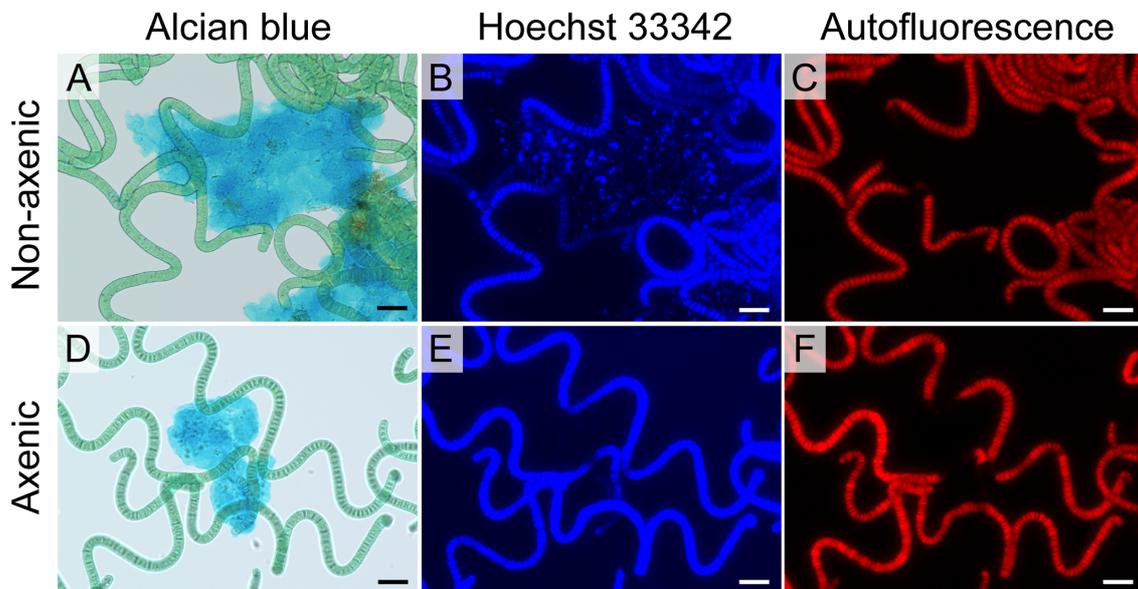


Figure 3

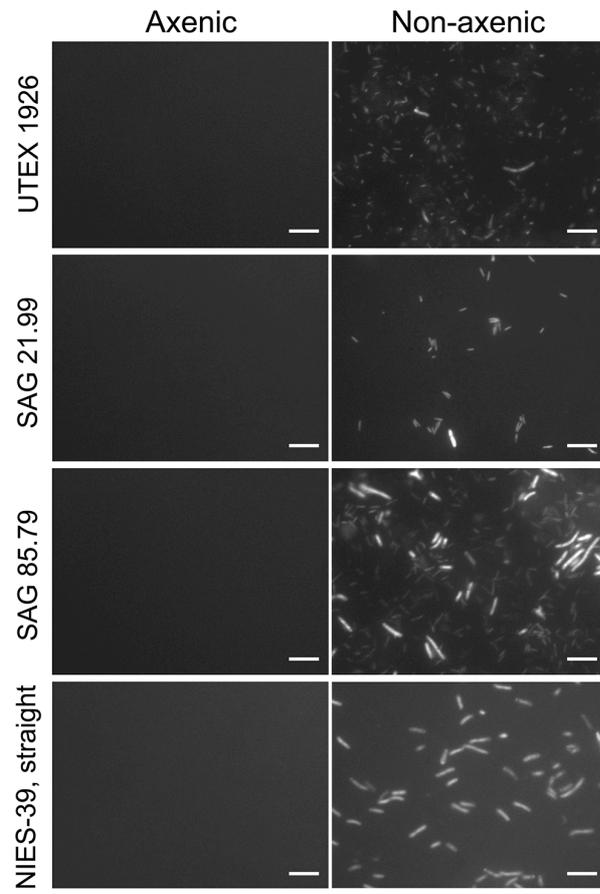
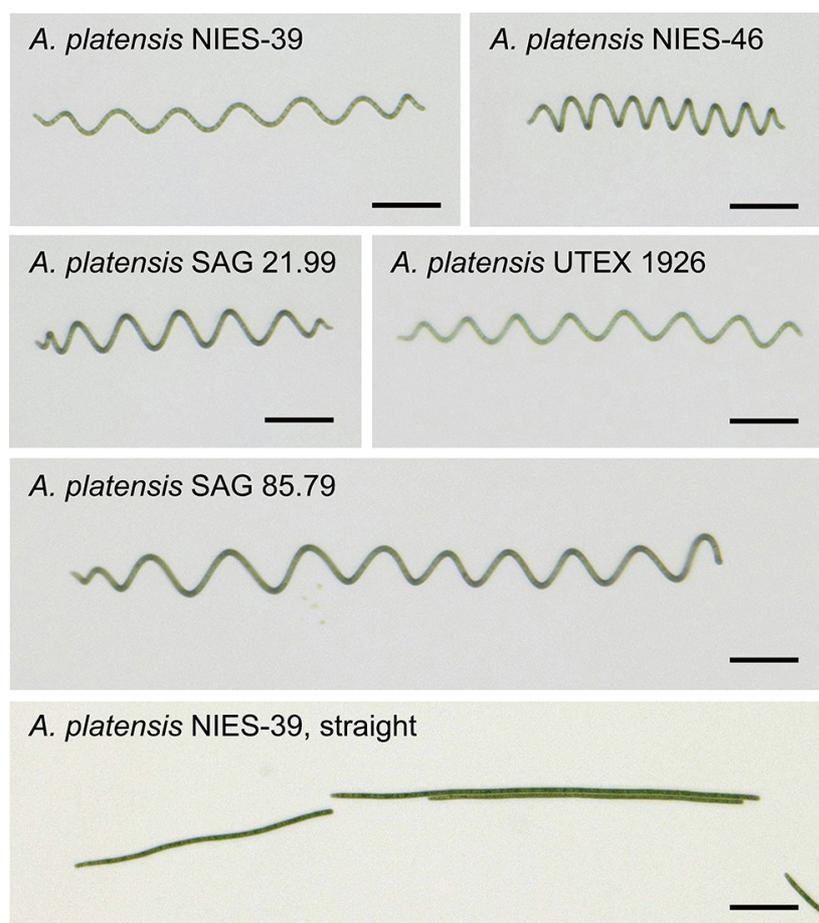


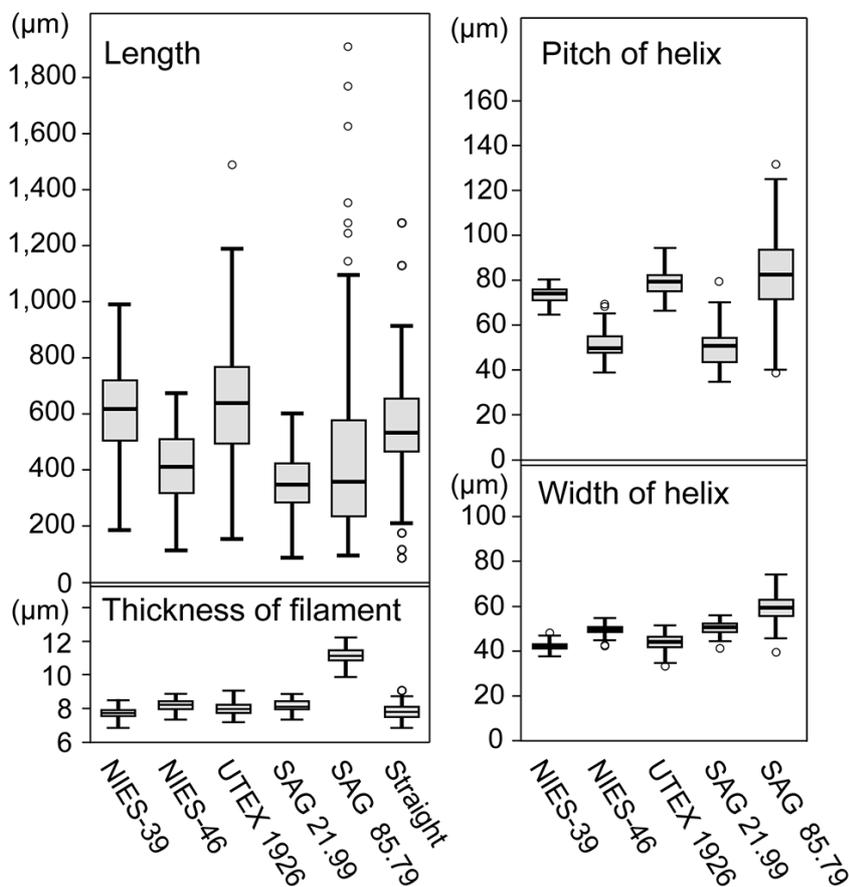
Figure 4

## Supplemental materials



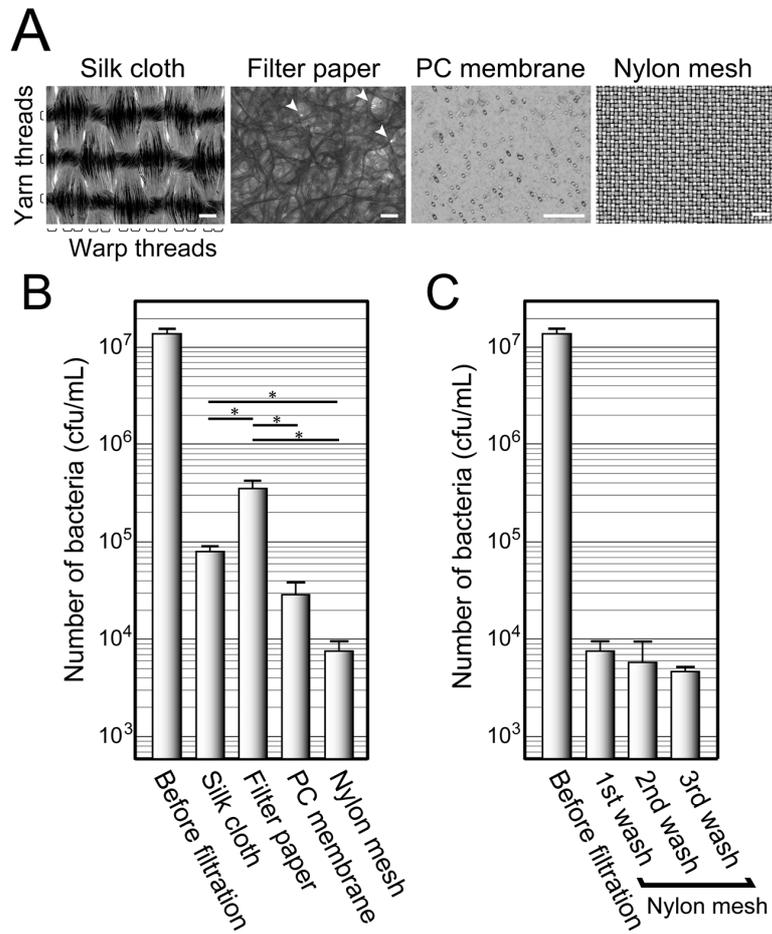
**Supplemental Fig. 1** *Arthrospira* strains used in this study.

Notes: Scale bars represent 100  $\mu\text{m}$ .



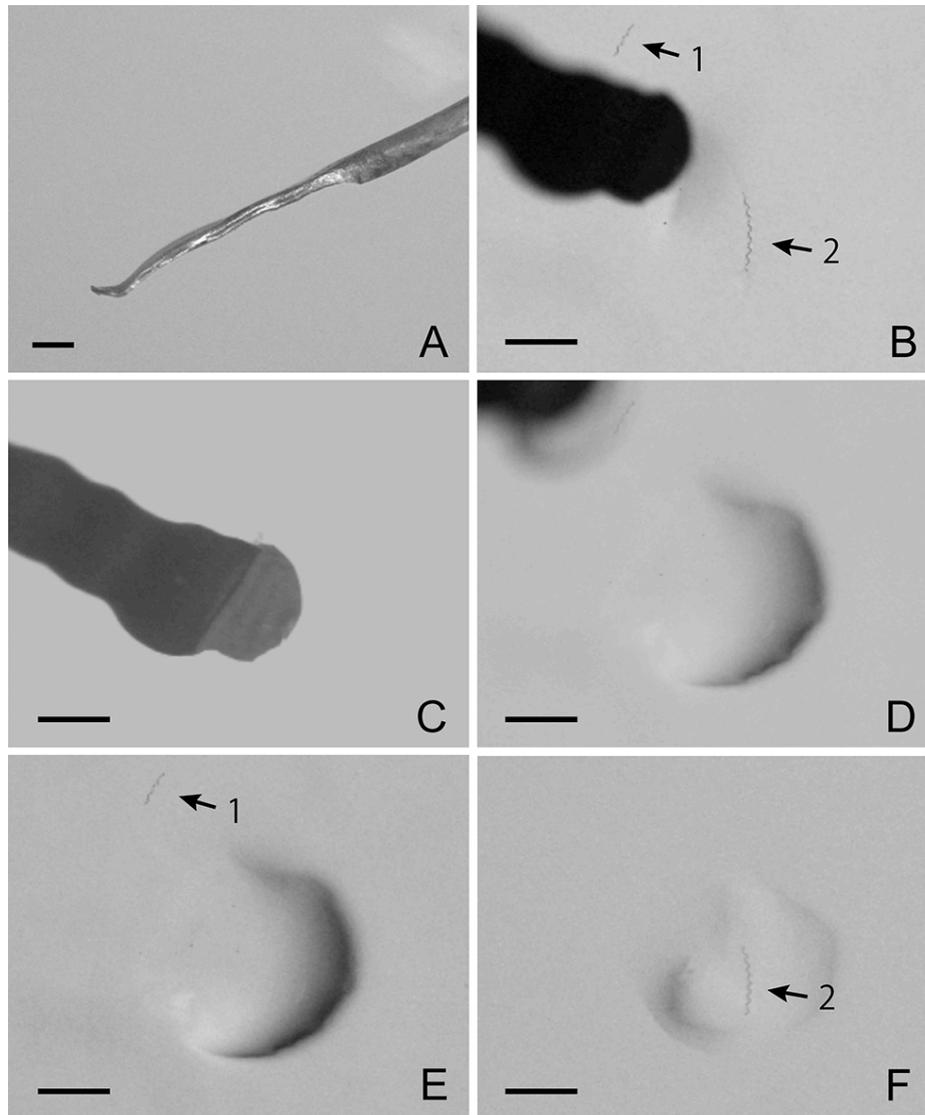
**Supplemental Fig. 2 Trichome sizes and dimensions of various *Arthrospira* strains.**

Notes: To determine the sizes and dimensions of trichomes, cell suspensions in the late log phase (culture optical density was 0.5–0.8 at 730 nm) were placed on a solidified SOT medium, and photographs were taken using the Digital Microscope VHX-2000 (Keyence, Osaka) equipped with a VH-Z50L zoom lens (Keyence) before the fluid was completely absorbed into the solidified medium. This prevented the structure deformation of trichomes while keeping them in a horizontal position. The length along the axis, width, and average pitch of the helices of individual trichomes were determined from these digital images. Trichomes shorter than two turns of helices were not used for these measurements, because it was impossible to properly measure their lengths and widths in many cases. To calculate the pitch of the strain SAG 21.99, 1 turn of a helix from each end, which often has an extremely small pitch, was excluded from these measurements. Filament thicknesses were measured on the digital images acquired using the bright-field mode of an All-in-One Microscope BZ-9000 (Keyence). Box plots show medians (lines in the boxes), interquartile ranges (boxes), largest and smallest values that are not outliers (whiskers), and outliers (circles). The numbers of trichomes subjected to the measurement of the length, pitch, and width were as follows: *A. platensis* NIES-39, N = 104; *A. platensis* NIES-46, N = 106; *A. platensis* UTEX 1926, N = 103; *A. platensis* SAG 21.99, N = 108; *A. platensis* SAG 85.79, N = 213; and the straight variant of *A. platensis* NIES-39, N = 171. The filament thickness of each strain was as follows (average  $\pm$  SD): NIES-39,  $7.6 \pm 0.4 \mu\text{m}$ ; NIES-46,  $8.2 \pm 0.4 \mu\text{m}$ ; UTEX 1926,  $8.0 \pm 0.4 \mu\text{m}$ ; SAG 21.99,  $8.1 \pm 0.3 \mu\text{m}$ ; SAG 85.79,  $11.0 \pm 0.4 \mu\text{m}$ ; and the straight variant of NIES-39,  $7.8 \pm 0.5 \mu\text{m}$ . The numbers of trichomes subjected to the measurement of filament thickness were 53, 61, 43, 60, 79, and 48, respectively, for each of the above strains.



**Supplemental Fig. 3 Differential filtration of non-axenic culture of *A. platensis* to remove contaminating bacteria.** (A) Filter media used. (B) Efficiency of the removal of contaminating bacteria with various filtration media. (C) Effect of repeated washing on nylon mesh.

Notes: Microscopic views of the following filter media are shown in A: silk cloth *habutaé* (Silk cloth); fast-flow quantitative filter paper with a particle retention size of 20  $\mu\text{m}$  (Filter paper); track-etched polycarbonate membrane with pores of 8  $\mu\text{m}$  (PC membrane); and nylon mesh with square openings of 20  $\mu\text{m}$  (Nylon mesh). Arrowheads indicate prominent pores in the filter paper. Bars represent 100  $\mu\text{m}$ . In the experiment in B, a portion of a non-axenic culture (1.2 mL) of *A. platensis* UTEX 1926 was filtered through these filtration media. Trichomes trapped on the filters were then washed with 50 mL of sterile SOT medium. The washed trichomes were resuspended in 1.2 mL of the same medium, and cfu of contaminating bacteria before and after the washing were determined. The data for filtered samples represent the averages of five independent filtration experiments beginning with a single non-axenic culture. Horizontal bars with asterisks indicate pairs of filtered samples with significant differences ( $p < 0.05$ , Steel-Dwass test). In the experiment in C, to determine the effect of repeated washing on nylon mesh, an aliquot (0.9 mL) of the trichome suspension recovered from the nylon mesh in the experiment in B (1st wash) was applied to a fresh nylon mesh, and the trichomes trapped on this mesh were washed with 50 mL of sterile medium. Washed trichomes were resuspended in 0.9 mL of the medium (2nd wash). An aliquot (0.6 mL) was taken from this suspension, and washing it on nylon mesh was repeated once to obtain 0.6 mL of a trichome suspension (3rd wash). Cfus in each suspension were then determined. The data for the repeated washing represent the averages of five independent filtration experiments beginning with a single non-axenic culture. Error bars indicate the standard errors of the means.



**Supplemental Fig. 4 Single-trichome manipulation with a microtrowel.** (A) Microtrowel made by shaping the end of a platinum wire into a miniature trowel-like structure. (B–E) Serial images that show the scooping action with the microtrowel. (F) Trichome of *A. platensis* UTEX 1926 placed on a new solid medium after scooping up in B–E.

Notes: Trichomes 1 and 2 in (B) correspond to trichome 1 in (E) and trichome 2 in (F), respectively. Scale bars represent 500  $\mu\text{m}$ .

### **Legend to Supplemental Video**

**Single-trichome manipulation with a microtrowel.** A trichome is scooped up from an agar plate using a microtrowel (00:05–00:12). After exchanging the plate with a new one (00:15–00:25), the trichome is placed on it (00:30–00:35).