Journal of Visualized Experiments

Natural transformation, protein expression, and cryoconservation of the filamentous cyanobacterium Phormidium lacuna

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63470R2
Full Title:	Natural transformation, protein expression, and cryoconservation of the filamentous cyanobacterium Phormidium lacuna
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Karlsruhe, Germany
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TITLE:

Natural Transformation, Protein Expression, and Cryoconservation of the Filamentous Cyanobacterium *Phormidium lacuna*

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SUMMARY:

Phormidium lacuna is a filamentous cyanobacterium that was isolated from marine rockpools. This article describes the isolation of filaments from natural sources, DNA extraction, genome sequencing, natural transformation, expression of sfGFP, cryoconservation, and motility methods.

ABSTRACT:

Cyanobacteria are the focus of basic research and biotechnological projects in which solar energy is utilized for biomass production. *Phormidium lacuna* is a newly isolated filamentous cyanobacterium. This paper describes how new filamentous cyanobacteria can be isolated from marine rockpools. It also describes how DNA can be extracted from filaments and how the genomes can be sequenced. Although transformation is established for many single-celled species, it is less frequently reported for filamentous cyanobacteria. A simplified method for the natural transformation of *P. lacuna* is described here. *P. lacuna* is the only member of the order Oscillatoriales for which natural transformation is established. This paper also shows how natural transformation is used to express superfolder green fluorescent protein (sfGFP). An endogenous *cpcB* promoter induced approximately 5 times stronger expression than *cpc560*, *A2813,* or *psbA2*

promoters from *Synechocystis* sp. PCC6803. Further, a method for the cryopreservation of *P. lacuna* and *Synechocystis* sp. CPP 6803 was established, and methods for assessing motility in a liquid medium and on agar and plastic surfaces are described.

INTRODUCTION:

Cyanobacteria are prokaryotic organisms that utilize photosynthesis as an energy source[1,2]. Research is increasingly focused on cyanobacterial species. Several cyanobacteria can be transformed with DNA[3]. Genes can be knocked out or overexpressed in these species. However, transformation is restricted to a few species[4-11], and it can be difficult to establish transformation in strains from culture collections or the wild[8]. Strains of the filamentous species *Phormidium lacuna* (**Figure 1**) were isolated from marine rockpools, in which environmental conditions, such as salt concentrations or temperature, fluctuate over time. These filamentous cyanobacteria can be used as model organisms for the order Oscillatoriales[12] to which they belong.

During trials testing gene transfer by electroporation[13,14] for the use of *P. lacuna* for molecular studies and biotechnological projects, it was found that *P. lacuna* can be transformed by natural transformation[15]. In this process, DNA is taken up naturally by some cells. Compared to other methods of transformation[16,17], natural transformation has the advantage of not requiring additional tools that could complicate the procedure. For example, electroporation requires proper cuvettes, intact wires, and selection of the proper voltage. *P. lacuna* is presently the only Oscillatoriales member susceptible to natural transformation. Because the original protocol is based on electroporation protocols, it still included several washing steps that might be unnecessary. Different approaches were tested to simplify the protocol, leading to the transformation protocol presented here.

The genome sequence is essential for further molecular studies based on gene knockout or overexpression. Although genome sequences can be obtained with next-generation sequencing machines within short periods, the extraction of DNA can be difficult and depends on the species. With *P. lacuna*, several protocols were tested. A modified cetyl trimethyl ammonium bromide (CTAB)-based method was then established, resulting in acceptable purity of DNA and DNA yields of each purification cycle for continued work in the laboratory. The genome of five strains could be sequenced with this protocol. The next logical transformation step was to establish protein expression in *P. lacuna*.

The sfGFP used as a marker protein in this protocol can be detected with any fluorescence microscope. All promoters that were tested could be used for *P. lacuna* sfGFP expression. The increasing number of strains arising from transformation has resulted in the need for a method for storing the cultures. Such methods are established for *Escherichia coli* and many other bacteria[18]. In standard protocols, glycerol cultures are prepared, transferred in liquid nitrogen, and stored at -80 °C. This method requires only a few steps and is highly reliable for those species for which it is established. The standard protocol was not feasible for *P. lacuna* because living cells could not be recovered in all cases. Glycerol had to be removed after thawing the cells, and the cells survived after two washes in all trials. Simple methods are presented for the analysis of motility of *P. lacuna*, which can be combined with knockout mutagenesis to investigate type IV

pili or the role of photoreceptors. These assays are different from those of single-celled cyanobacteria[19-21] and can also be useful for other Oscillatoria.

PROTOCOL:

1. Isolation from the natural environment

NOTE: Green algae, diatoms, filamentous cyanobacteria, and other microalgae can be isolated. The protocol can be used for any microalga species from rockpools growing under laboratory conditions. Filamentous cyanobacteria that belong to Oscillatoriales can be easily recognized by their movement and filamentous shape. The species can be identified in a semipure state by genome sequencing or 16S rRNA sequencing.

1.1. Transfer liquid seawater samples from marine rockpools (i.e., cavities in the rocky coast) into 50 mL flasks. For each flask, note the exact place or coordinates of the natural source. If possible, filter the content through 50 µL nets to reduce the amounts of zooplankton. Store the samples at 4 °C until they can be subcultured.

1.2. Transfer 1 mL cultures to 10 cm Petri dishes containing 3% bacto-agar in f/2 medium[22,23] (see the **Table of Materials**). Prepare up to 20 plates. Cultivate under white light of 50 µmol m^{-2} s^{-1}.

NOTE: Higher light intensity may be used for cultivation. Intensity up to 400 µmol m^{-2} s^{-1} can be used for *P. lacuna*, although other species might be more light-sensitive.

1.3. After one week, transfer the desired cells to fresh agar plates using sterile forceps. Isolate the cells under a binocular microscope under sterile conditions. Store the old agar plate at 4 °C until cells appear and grow on the new agar plate.

1.4. Repeat this transfer step every week to eliminate contamination. Use the naked eye for detecting heavy contamination and a microscope with 400x magnification for additional checks for contamination.

1.5. If a sample seems free of contamination, test for bacterial or fungal contamination on agar plates. Transfer a fraction of the culture with an inoculation loop to an LB[24] agar plate (10 cm diameter), keep the plate at room temperature, and check for the growth of contaminants over 1–3 days.

1.6. If a sterile filamentous cyanobacterial species is obtained, use it for further culture work. Cultivate *P. lacuna* in liquid or on bacto-agar plates. Use 250 mL flasks with 50 mL of f/2 medium or $f/2^{+}$ medium for liquid culture.

2. DNA extraction

NOTE: This method is adopted from [25] [26]

2.1. Prepare two flasks with 50 mL of f/2 medium. Inoculate each with ~1 mL of *P. lacuna* filaments from other growing cultures. Keep the cultures for 7 days or longer under agitation (horizontal rotation) at 50 rpm under white light (50 µmol $m^{-2}s^{-1}$) at 25 °C.

2.2. Treat the culture with ultrasound (see the **Table of Materials**) for 2 min with full energy. Measure OD at 750 nm; check to ensure it is ~0.5. Continue to grow the cultures if the OD is too low.

2.3. Collect the filaments by 5,000 × *g*, 20 min centrifugation. Remove the supernatant. Transfer the filaments with residual liquid to the chamber of a French Press[27]. Set the pressure of the French Press to 20,000 psi and extract the cells.

NOTE: The French Press will lyse all the cells and release the DNA; strong shear forces will produce 1,500 bp DNA fragments.

2.4. Centrifuge the sample for 10 min at 10,000 × *g* and remove the supernatant.

2.5. Add 400 µL of lysis buffer (4 M urea, 0.1 M Tris/Cl, pH 7.4) and 50 µL of proteinase K (10 mg/mL) to the pellet. Heat the sample to 55 °C for 60 min with shaking at 550 rpm.

2.6. Add 1 mL of DNA extraction buffer (3% CTAB, 1.4 M NaCl, 10 mM EDTA, 0.1 M Tris/Cl, 1% Sarkosyl, 0.1 M DTT, pH 8) and incubate for 60 min at 55 °C and 550 rpm. Transfer the solutions to centrifugation tubes, and add two volumes of chloroform/isoamylalcohol (24/1).

2.7. After shaking, centrifuge the sample for 5 min at 9,000 × *g*. Transfer the upper, aqueous phase into reaction vials and add 1 mL of ice-cold ethanol and 50 µL of 3 M sodium acetate.

2.8. Vortex the sample and place it at -20 °C for 1 h or longer.

2.9. Centrifuge for 5 min at 10,000 × *g* (4 °C) and discard the supernatant. Wash the pellet with 70% ethanol.

2.10. Centrifuge the sample again. Remove the supernatant and dry the pellet overnight. Dissolve the DNA in nuclease-free water. Measure the DNA spectrum to check whether the OD 260 nm/OD 280 nm is between 1.6 and 1.9.

2.11. Analyze the size of the DNA on an agarose electrophoresis gel[28].

2.12. Sequence the genomic DNA by next-generation sequencing for 300 cycles, with a paired-end setting and read length of 150 bases (see the **Tables of Materials**).

2.13. Perform the assembly with the appropriate computer program; see the example given in the **Table of Materials**.

2.14. Submit the draft genome to the RAST server for annotation.

NOTE: Upload DNA sequences to obtain complete annotation within a few minutes.

3. Natural transformation and GFP expression

NOTE: Transformation is based on a plasmid vector propagated in *E. coli*; pGEM-T or pUC19 may be used as backbone vectors. Cloning techniques are established in many laboratories; see also standard protocols[28] and the articles on transformation vectors for *P. lacuna*[15,29]. Examples for vectors for sfGFP expression are described in the representative results section. Details of four yet unpublished vectors are provided in **Supplemental File 1**.

3.1. Perform all steps using sterile material under sterile laboratory conditions (clean bench, sterile glassware).

3.2. Inoculate 2 x 50 mL of f/2 liquid medium in two 250 mL flasks with 2 x 1 mL of *P. lacuna* filaments from a running culture. Cultivate in white light (50 µmol m^{-2} s^{-1}) under agitation (horizontal rotation, 50 rpm) for ~5 days at 25 °C.

3.3. Prepare ~200 µg of the transformation vector DNA using a midi prep kit (see the **Table of Materials**) according to the manufacturer's instructions.

3.4. Homogenize 100 mL of *P. lacuna* cell suspension (see the **Table of Materials**) at 10,000 rpm for 3 min. Measure OD at 750 nm (desired value = 0.35).

3.5. Centrifuge the cell suspension for 15 min at 6,000 × *g*. Remove the supernatant, and suspend the pellet in 800 µL (total volume including residual liquid and filaments) of the remaining liquid and additional $f/2^+$ medium.

3.6. Take eight $f/2^+$ bacto-agar plates (10 cm diameter) containing 120 µg/mL kanamycin. Pipette 10 µg of DNA into the middle of each agar plate. Immediately pipette 100 µL of cell suspension into the middle of each agar plate (on top of the DNA).

3.7. Keep the agar plate without a lid on the clean bench to allow the excess liquid to evaporate. Close the plate and cultivate it in white light at 25 °C for 2 days.

3.8. Distribute the filaments of each agar plate with an inoculation loop onto several fresh $f/2^+$ bacto-agar plates containing 120 µg/mL kanamycin. Cultivate the plates in white light at 25 °C and check the cultures regularly under a microscope.

3.9. Identify living, transformed filaments after 7–28 days under the microscope. Look for healthy, green filaments (**Figure 2**) that are different from other filaments must be visible. If these green filaments can be identified, continue with the next step; otherwise, keep the plate for

another 7 days.

3.10. Use forceps to transfer these identified living filaments into 50 mL of liquid f/2^{+} medium with 250 µg/mL kanamycin. Cultivate in white light at 25 °C on a shaker (horizontal rotation, 50 rpm). Observe growth for up to four weeks.

3.11. Transfer the filaments back to agar medium containing 250 µg/mL kanamycin and wait for the filaments to grow. After several days, transfer single filaments to a fresh agar plate with a higher concentration of kanamycin, e.g., 500 µg/mL. Keep the original plate.

3.12. Ensure that the filaments are propagated in a high concentration of kanamycin in liquid culture or on agar. Increase the kanamycin concentration again to speed up segregation.

NOTE: Transformed *P. lacuna* grows in up to 10,000 µg/mL kanamycin. Other species might not tolerate such high concentrations.

3.13. If resistant cells are grown and distributed broadly over a plate, test the integration of the insert into the genome of *P. lacuna* by performing PCR with outer and inner primers.

3.13.1. Use inner primers (e.g., those designed for cloning) to amplify the insert.

3.13.2. Ensure that the outer primers are 5' and 3' of the proposed insertion site on the genome of *P. lacuna* but outside the insertion.

3.13.3. Use the genome sequence outside the homologous ranges as a template for the design of these primers. Perform the PCR with the resistant line and the wild type.

NOTE: Inner primers indicate that the insert is present; outer primers show that the insert is inserted at the correct locus.

3.14. Place ~10 mg of the filaments directly in the PCR tubes and perform PCR according to standard protocols[24]. If no product is obtained, vary the annealing temperature and wash the filaments with water.

NOTE: Many different polymerases can be used in PCR. Standard polymerases, such as Taq polymerase, have a higher error rate than error-checking polymerases, which are more expensive. This analytical PCR does not require any error-checking polymerase. However, error-checking polymerase should be tested if no PCR product is obtained with a standard polymerase.

3.15. Analyze the PCR products of the resistant line on agarose electrophoresis[24].

3.15.1. Compare band positions with marker and compare the wild type and transformant. With inner **and** outer primers, look for a larger band for the transformant than the wild type (due to the insertion of the resistance cassette) or two bands for the transformant: one with the size of

the wild-type band and a larger one. As the latter case indicates incomplete segregation, continue cultivation with high kanamycin concentrations.

NOTE: For more details on PCR and electrophoresis, see[15,24] or other standard literature.

3.16. For GFP expression: observe single filaments with a fluorescence microscope (see the **Table of Materials**) at a magnification of the objective set at 40x or 63x. Capture a brightfield transmission image and a fluorescence image. Use the following settings for GFP: 470 nm bandpass for excitation, 525 nm bandpass for emission, and a 495 nm beam splitter, initial exposure time of 500 ms.

3.17. Adjust the exposure time for clear fluorescence signals, avoiding saturating intensities. Try to use the same setting for all samples.

3.18. As the wild-type filaments will also display fluorescence, capture images with the same settings as above for this background fluorescence.

NOTE: The strain expressing GFP must have a higher signal; otherwise, it is not expressing GFP.

3.19. Based on exposure times and the pixel intensities of the fluorescence images, calculate and compare the GFP content of the different filaments.

4. Cryoconservation

NOTE: *P. lacuna* and the single-celled cyanobacterium *Synechocystis sp*. PCC 6803 are used. The present method works better for *P. lacuna*.

4.1. Cultivate *P. lacuna* or *Synechocystis sp* PCC 6803 for at least 10 days in 10 mL of f/2^{+} or BG-11 medium, respectively, under white light (50 µmol m^{-2} s^{-1}) at 25 °C under agitation (horizontal rotations, 50 rpm).

4.2. Homogenize the *P. lacuna* culture (see the **Table of Materials**) at 10,000 rpm for 3 min or with an ultrasound device (see the **Table of Materials**) for 2 min at full energy. Determine OD 750 nm of either culture to check whether the value is between 1 and 7.

4.3. Collect the cells by centrifugation at 6,000 × *g* for 15 min. Remove the supernatant.

4.4. Suspend the cell pellet in 800 µL of f/2^{+} or BG-11 medium (final volume) and transfer to a 2 mL cryovial. Add 800 µL of a 50% glycerol solution to the cell suspension. Close the vial and mix by repeated inverting.

4.5. Transfer the cryovial to liquid nitrogen and store it in a cryobox in a -80 °C freezer. Note the position of the box within the freezer and the coordinates of the sample within the box.

4.6. For recovery of the cells, take out the cryovial and thaw the contents at room temperature. Transfer the contents to a 2 mL reaction tube.

4.7. Wash the sample twice. For the 1st wash, centrifuge at 6,000 × *g* for 5 min. Remove the supernatant, and resuspend the pellet in 2 mL of f/2+ or BG-11 medium. For the 2nd wash, recentrifuge at 6,000 × *g* for 5 min, remove the supernatant, and suspend the pellet in 2 mL of f/2+ or BG-11 medium.

4.8. To check the integrity of these cells that are ready for cultivation, transfer the pellet to 9 mL of medium and cultivate them in white light (50 µmol m^{-2} s^{-1}) under agitation (55 rpm). Compare the OD 750 nm of the culture on the first day and after 1 week.

5. Motility of *Phormidium lacuna*

NOTE: Three different assays will be described. The same culture is used in all cases.

5.1. Cultivate *P. lacuna* in f/2 medium under horizontal agitation (50 rpm) in white light (50 µmol m^{-2} s^{-1}) for ~5 days until the estimated OD 750 nm is 0.35. Store the sample at 4 °C until use.

5.2. Homogenize the filaments (see the **Table of Materials**) at 10,000 rpm for 3 min or with ultrasound (see the **Table of Materials**) for 1 min at maximum power and cycle of 1. Measure OD 750 nm. If above 0.35, dilute the fraction with f/2 medium. Use this solution in motility assays in steps 5.3, 5.4, and 5.5.

5.3. Assay for movement in liquid medium

5.3.1. For direct observation of motility, transfer 8 mL of medium containing *P. lacuna* (from step 5.2) into a 6 cm Petri dish. Wait a few minutes until the sample reaches room temperature. Cover the Petri dish with cellophane foil.

5.3.2. Place a microscope slide on the x–y table of a standard microscope with a camera. Switch on the microscope light. Ideally, always use the same electrical and optical settings for the lighting. Move a 4x or 10x objective into the path of the light.

5.3.3. Place the Petri dish on top of the slide. Adjust single filaments or filament bundles by x, y, and z movements of the table.

NOTE: Due to the three-dimensional arrangement, only a part of the relevant section can be in focus. The cellophane foil allows adjusting the focus without restriction.

5.3.4. Observe movements of single filaments or bundles. Ensure that the objective lens does not touch the liquid. Record the movements of filaments with a standard microscope camera (see **Supplemental Video S1**).

5.4. Assay for movement on the surface

5.4.1. For the observation of filament motility on agar surfaces, prepare 6 cm Petri dishes with f/2 bacto-agar. Ensure that the agar is high enough for the objective lens to get close to the agar surface. Alternatively, prepare a ~3 mm thick agar layer and record the filaments through the agar (keep the plate upside down or use an inverted microscope).

5.4.2. Pipette 0.5 mL of a solution containing *P. lacuna* (from step 5.2) on the bacto-agar surface of a 6 cm Petri dish. Allow the liquid to enter the surface. Close the Petri dish and observe the movement of the filaments on the surface using a 4x or 10x objective.

5.4.3. Ensure that the same electrical and optical settings of the microscope are used throughout the recording and in subsequent recordings.

5.4.4. Capture time-lapse recordings using an ocular camera and minicomputer system. Ensure that the time interval between subsequent images is 5 s–1 min. Program the Linux script of the minicomputer to control the time-lapse recording. See **Supplemental File 2** for an example script and **Supplemental Video S2** as an example.

5.5. Assay for phototaxis

5.5.1. For phototaxis experiments, prepare light-emitting diode (LED) holders (here, with a 3D printer) in which the selected 5 mm LEDs are mounted to irradiate an area of 20 mm^2 from below to above (**Figure 3**). If required, use many LED holders in parallel, connecting each LED electrically through a resistor and potentiometer to an adjustable power supply. Measure and adjust the LED intensities, depending on the experiment. Ensure that the whole setting is in a dark room or a closed dark container.

5.5.2. Place 8 mL of the medium containing *P. lacuna* (from step 5.2) into a 6 cm Petri dish. Adjust the light intensity of the LED. Close the Petri dish with the lid and place it on an LED holder so that the LED is in the center of the Petri dish.

5.5.3. After the desired duration (typically 2 days), capture an image of the Petri dish with a smartphone camera aimed directly at the position of the light treatment.

5.5.3.1. Use a white LED panel for irradiation of the specimen. Use the manual settings of the camera; avoid reflections of light; always adjust to get the same distance between the camera lens and the specimen. Ensure that the exposure settings give an image suitable for later analyses using ImageJ.

5.5.4. Quantify the diameter of the central circle of filaments using ImageJ software.

5.5.4.1. Open ImageJ, click on **File | Open**, select the desired file, and click **Enter**.

5.5.4.2. Select the **Straight** button (with a straight line). Press the left mouse button to draw a line from one end of the Petri dish to the opposite end. Ensure that the line passes through the center of the circle of filaments.

5.5.4.3. Press **Ctrl-K** on the keyboard or click **Analyze | Plot Profi**le in the ImageJ menu. Look for an x-y window with pixel intensities plotted versus distance—a 1D profile of the Petri dish. Ensure that the lowest pixel intensity is slightly above 0 and the highest value below 255.

5.5.4.4. Estimate an average value for the pixel intensity outside the circle and another average value for the pixel intensity in the circle. At the y- position between these values, estimate the x-values of both sides of the circle by pointing with the mouse on these positions. Note both values and calculate the difference.

5.5.4.5. Obtain the highest x-value by pointing the mouse at the y-axis on the right. Note that this value **e** represents the diameter of the Petri dish. If this diameter is 5 cm, calculate the **diameter of the central filament circle** as **d/e × 5 cm**.

REPRESENTATIVE RESULTS:

Following the above-mentioned methods, 5 different strains of *P. lacuna* were isolated from rockpools and sequenced (**Figure 1** and **Table 1**). All cultures were sterile after ~1 year of subculturing except *P. lacuna* HE10JO. This strain is still contaminated with *Marivirga atlantica*, a marine bacterium. During subsequent Helgoland excursions, other filamentous cyanobacteria were isolated from rock pools, which are different from *P. lacuna* and need to be characterized.

Several DNA extraction and purification methods were tested for *P. lacuna*. The best results were obtained with an optimized CTAB method as described above. DNA yields were 310 ± 50 µg/mL, OD 260 nm/OD 280 nm was 1.7 ± 0.03, and OD 260 nm/OD 230 nm was 0.78 ± 0.04 (n = 17). Genome sequencing showed that the DNA of all strains was slightly different, as expected (**Table 1**). Core protein sequences showed a maximum difference of 0.04% (**Table 2**). Although all draft genomes were incomplete, one can assume that >98% of the genome of HE10JO[30] was sequenced. This estimation is based on the number of incomplete open reading frames. Partial protein sequences could be easily identified after RAST annotation of HE10DO and HE10JO. In HE10JO, 60 proteins out of ~4,500 had a missing N- or C-terminal sequence.

Interestingly, strains of the same species were isolated from two islands, Helgoland and Giglio. The linear distance between both islands is 1,400 km. There must be a link between both places, e.g., by ships via the sea or, more likely, by migratory birds. Many bird species can be found on both islands, and many of them are migratory birds. The diversity within *P. lacuna* strains of one island was greater than between the closest Helgoland and Giglio strains (**Table 2**). This indicates an intense exchange between both places.

The natural transformation was tested with HE10DO as the major strain and with HE10JO. The present protocol is more straightforward than the protocol described earlier[12] because of the reduced number of washing steps and fewer transfer steps after transformation. This new

method is continuously used in the laboratory; ~15 successful transformations were achieved.

The KanR resistance cassette was usually integrated into the homologous site defined by the adjacent regions, as shown by PCR using inner and outer primers. Like most cyanobacteria, *P. lacuna* is polyploid. It can have more than 100 chromosomes per cell[12]. A PCR test with outer primers ~1 week after the transformation typically has 2 bands on the electrophoresis gel, one with the size of the wild-type band and one slower migrating band that indicates the insertion of the resistance cassette (**Figure 4**). The double band indicates that only a subfraction of the chromosomes contains the insertion. After 4 weeks of selection on kanamycin, segregation is usually complete, and only one large PCR band appears on gels. However, in the case of the transformation with pMH1 (see below), segregation was complete after more than 3 months.

The vectors pAK1, pAK2, pAK3, and pMH1 were constructed for tests on sfGFP expression. In pAK1, pAK2, and pAK3, the *sfGFP* gene is under the control of the *cpc560*, *A2813,* and *psbA2* promoters, respectively. These promoters are from *Synechocystis* sp. PCC 6803 or *Synechococcus* sp. PCC 7002[31]. For the construction of these vectors, the *sfGFP* promoter and terminator sequences were taken from vectors used for the transformation of *Synechococcus* sp. PCC 7002[31]. The relevant sequences were integrated into the homologous *chwA* (sc_7_37) site of pFN1 (or pFN_7_37_KanR[15]). The pMH1 expression vector was constructed by DNA synthesis using *P. lacuna* sequences as templates (**Supplemental File 3, Supplemental File 4, Supplemental File 5, Supplemental File 6,** and **Supplemental File 7**). The *cpcB–cpcA* (phycocyanin ß and phycocyanin α) sequences of *P. lacuna* are serially arranged. A 100 bp intergenic region separates both coding regions. The synthetic sequence contained this endogenous *cpcB–cpcA* sequence and the *cpcB* promoter. The sfGFP and KanR cassette is placed just 3' of the *cpcB* stop codon (5' of *cpcA*). The entire synthetic sequence with *cpcB* promoter, *cpcB*, *sfGFP*, *KanR*, *cpcA* (5' to 3') is cloned into pUC19. A map is shown in **Figure 5**. More details on the cloning of pAK1, pAK2, and pAK3 and the complete sequence of pMH1 are given in **Supplemental File 1**.

All 4 transformants (with pAK1, pAK2, pAK3, and pMH1) expressed GFP; all fluorescence levels were above the background fluorescence of wild-type filaments (**Figure 6**). The pMH1 transformants with incomplete segregation revealed a GFP signal that was very variable between the filaments. The fluorescence signal was evenly distributed when segregation was complete (**Figure 6E**). The microscope signals of pAK1, pAK2, and pAK3 transformants were similar but ~5x weaker than that of pMH1 (**Figure 6E**).

The established cryoconservation method is based on a method that was established for *E. coli*. When 2 washing steps were performed for glycerol removal after thawing, 15 out of 15 *P. lacuna* samples survived (**Table 3**). This protocol could also be used for *Synechocystis* PCC 6803, but only with 2 washing steps and not with 1 (**Table 3**).

Another feature of Oscillatoriales filaments is their motility: *P. lacuna* filaments move continuously on surfaces (**Figure 7**) and in a liquid medium (**Figure 8**). Both kinds of motion can be studied easily in Petri dishes without or with agar medium. Time-lapse recording is required because movement on agar is slow. Filaments move towards the light cone if a light beam comes

from below (**Figure 3**). The effects of light intensity, wavelength, and time can be easily studied with a simple setup. The photoreceptors of this effect are not yet clear. Possible candidates can be addressed with knockout mutants. The mechanism underlying how the filaments find the light is also unclear. For this question, an infrared system is required to record the filaments during their movement from darkness to light.

FIGURE AND TABLE LEGENDS:

Figure 1: Strains of *Phormidium lacuna* collected from Helgoland and Giglio. Filaments are propagated for 11 days on f/2 agar in 6 cm Petri dishes. (**A**) strain GI08AO; (**B**) strain GI08IO; (**C**) strain GI09CO; (**D**) strain HE10DO; (**E**) strain HE10JO; (**F**) strain HE15M2G1.

Figure 2: *Phormidium lacuna* filaments 5 weeks after transformation. The sfGFP expression vector pMH1 was used; selection occurred on f/2^{+} medium with 120 µg/mL kanamycin. The greenish filaments are resistant and alive; other filaments have died. Scale bar = 100 µm.

Figure 3: Phototaxis experiment. Left: LED holder with 4 red LEDs, connected to an adjustable power supply. On top of each LED, there is a 6 cm Petri dish with 8 mL of a *Phormidium lacuna* culture. Right: Petri dish with *P. lacuna* after 2 days on the red LED (15 µmol m^{-2} s^{-1}). Abbreviation: LED = light-emitting diode.

Figure 4: Integration and segregation of insert after transformation of *Phormidium lacuna* with pAK1. PCR with outer primers. The expected sizes of the product without and with insert are 2371 and 5016 bp, respectively. Left lane: marker, lanes 1, 2, 3, 4: PCR products of filaments 7 days, 11 days, 14 days, and 17 days after the isolation of a resistant filament (4 weeks after transformation), respectively. Lane 5: PCR product of wild-type (from a different gel). In the 7 day sample, the insert is present in a small fraction of the chromosomes. This fraction increases until 17 days, where no wild-type band is visible, i.e., segregation is complete.

Figure 5: Vector for sfGFP expression under the control of endogenous *cpcB* promoter. Orange: *Phormidium lacuna* homologous sequence, violet/blue: pUC-19 vector backbone, green: insert with sfGFP and KanR. Abbreviations: sfGFP = superfolder green fluorescent protein; KanR = kanamycin resistance.

Figure 6: Expression of sfGFP in *Phormidum lacuna*. Fluorescence images of *P. lacuna* wild-type filaments (**A**) and after transformation with pAK1 (**B**), pAK2 (**C**), pAK3 (**D**), and pMH1 (**E**). In pMH1, the *sfGFP* gene is placed 3' of the phycocyanin ß gene and therefore driven by the endogenous *cpcß* promoter; in the other cases, *sfGFP* is driven by *cpc560*, *A2813,* or *psbA2*s promoters from *Synechocystis* PCC 6803, respectively. The fluorescence settings are specific for GFP; all images were recorded with the same integration time and optical settings.

Figure 7: Merged image of *Phormidium lacuna* on agar surface at 4x magnification. The first image is presented in red, the second (taken 1 min later) in green. Note also the traces on the agar. Scale bar = 100 µm.

Figure 8: Merged image of *Phormidium lacuna* in liquid medium. The time interval between both images was 10 s. The first image is printed in red; the second is printed in green. Comparing both colors shows the movement within 10 s. Scale bar = 100 µm.

Table 1: *Phormidium lacuna* strains.

Table 2. Amino acid differences between strains in sequences of 20 core proteins with 10,876 amino acids.

Table 3: Cryoconservation trials with *Synechocystis* PCC 6803 and *Phormidium lacuna* HE10DO cyanobacteria. The first number shows the number of cultures that survived after freezing/thawing; the second number shows the total trials.

Supplemental Video S1: Movement of *Phormidium lacuna* filaments in liquid solution, without time-lapse.

Supplemental Video S2: Movement of *Phormidium lacuna* filaments on agar surface, with time-lapse.

Supplemental File 1: Cloning of vectors for transformation of *Phormidium lacuna.* List of transformation vectors; list of primers for cloning; sequence of pMH1 in gb format.

Supplemental File 2: Shell scripts (sh) for Raspberry Pi minicomputer.

Supplemental File 3: DNA sequence of HE152G1.

Supplemental File 4: DNA sequence of GI08AO.

Supplemental File 5: DNA sequence of GI09CO.

Supplemental File 6: DNA sequence of HE10DO.

Supplemental File 7: DNA sequence of HE10JO.

DISCUSSION:

Although many strains of cyanobacteria are available from culture collections[32-36], there is still a demand for new cyanobacteria from the wild because these species are adapted to specific properties. *P. lacuna* was collected from rockpools and is adapted to variations of salt concentrations and temperature[30]. Strains of this species were found during excursions in 2008, 2009, and 2010. With the procedure described here, 5 strains of *P. lacuna* were isolated, and 4 of these strains were sterile. The strain *P. lacuna* HE10JO is permanently contaminated with the bacterium *Marivirga atlantica*, a marine bacterium identified by rRNA and genome sequencing. This bacterium could not be separated from the cyanobacterium in spite of the application of

mechanical separation, growth at different temperatures, treatments with antibiotics, or chemical treatments. Despite the contamination, *P. lacuna* HE10JO can be cultivated similar to the other strains. In later excursions, other members of Oscillatoriales were found, which are yet not analyzed in detail. *P. lacuna* was not found again. It is not clear why *P. lacuna* was isolated in subsequent years and two different places but not found later. Its abundance is certainly dependent on nonpredictable conditions. Temperature, salt concentrations, and inorganic or organic nutrients are highly variable in rockpools. Therefore, the species composition could fluctuate over time in an unpredictable manner.

Natural transformation is established for different cyanobacteria, mostly single-celled species. The filamentous *P. lacuna* is the only species of the order Oscillatoriales for which natural transformation has been established. The transformation was almost always successful with the present protocol. In general, the numbers of resistant filaments after a transformation trial vary considerably, and sometimes transformation fails, resulting in the loss of valuable time. It is therefore advisable to perform several transformation projects in parallel. The time for complete segregation, usually 4 weeks after isolation of the resistant strain, can also vary. Because there is no guarantee for complete segregation after growth on kanamycin, it is crucial to perform the PCR tests using outer and inner primers.

Every vector must contain 2 x 500–1,000 bp homologous sequences, e.g., amplified from the host by PCR and interrupted by a resistance cassette (e.g., the kanamycin cassette KanR used here)[37,38]. For expression, a promoter, coding sequence (e.g., for sfGFP[39]), terminator, and resistance cassette must be cloned between the homologous sequences. The cloning strategies are species-specific and depend on the aim of the experiment.

This transformation method could be possible with other Oscillatoriales strains or other cyanobacteria as well: natural transformation is based on type IV pili, which are present in almost every other cyanobacterial genome[3,15,40]. Therefore, the present method could stimulate new trials with other species. Because type IV pili are also relevant for motility, it is important to check for conditions under which cyanobacteria are motile.

Gene insertion is based on homologous recombination and results in a disruption of the homologous sites. Therefore, transformation is often used for gene knockout. The expression of the inserted gene will be induced if an active promoter and a coding sequence are integrated into the homologous site. In *P. lacuna*, promoter activity was dependent on the species. The *cpc560*, *A2813,* and *psbA2* promoters of *Synechocystis* PCC sp 6803 or *Synechococcus* sp. PCC 7002 [31] and the *cpcB* promoter of *P. lacuna* could drive *sfGFP* expression. Of these constructs, the endogenous *cpcB* promoter induced the strongest expression, although the *sfGFP* gene is located 3' of the phycocyanin ß gene. This indicates a more general use of endogenous promoters in cyanobacterial expression.

The combination of gene knockout and motion studies will shed light on molecular mechanisms of motility and phototaxis. LED light sources can provide light for phototaxis experiments. Almost any wavelength is available, and light intensity can be modulated by an adjustable power supply

and potentiometers. LED holders can be built by 3D printers to easily realize combinations of different LEDs.

ACKNOWLEDGMENTS:
The work was supported by the Karlsruher Institute of Technology.

DISCLOSURES:
The authors have no conflicts of interest to disclose.

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Figure 1

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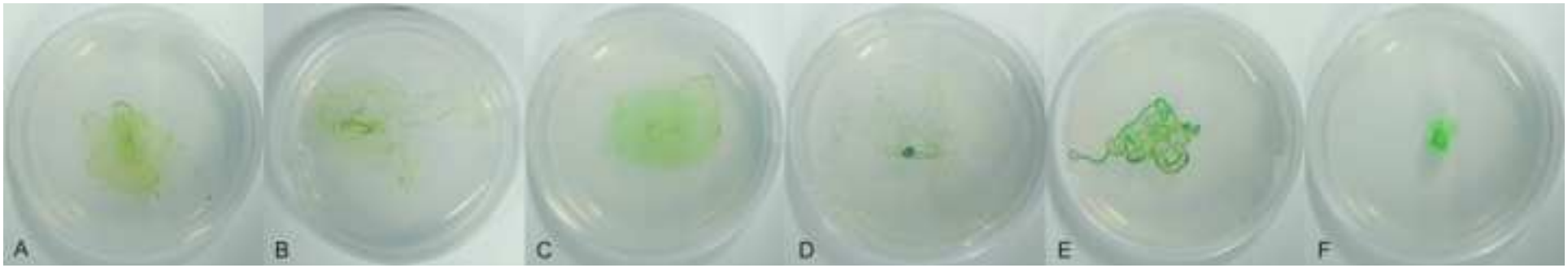

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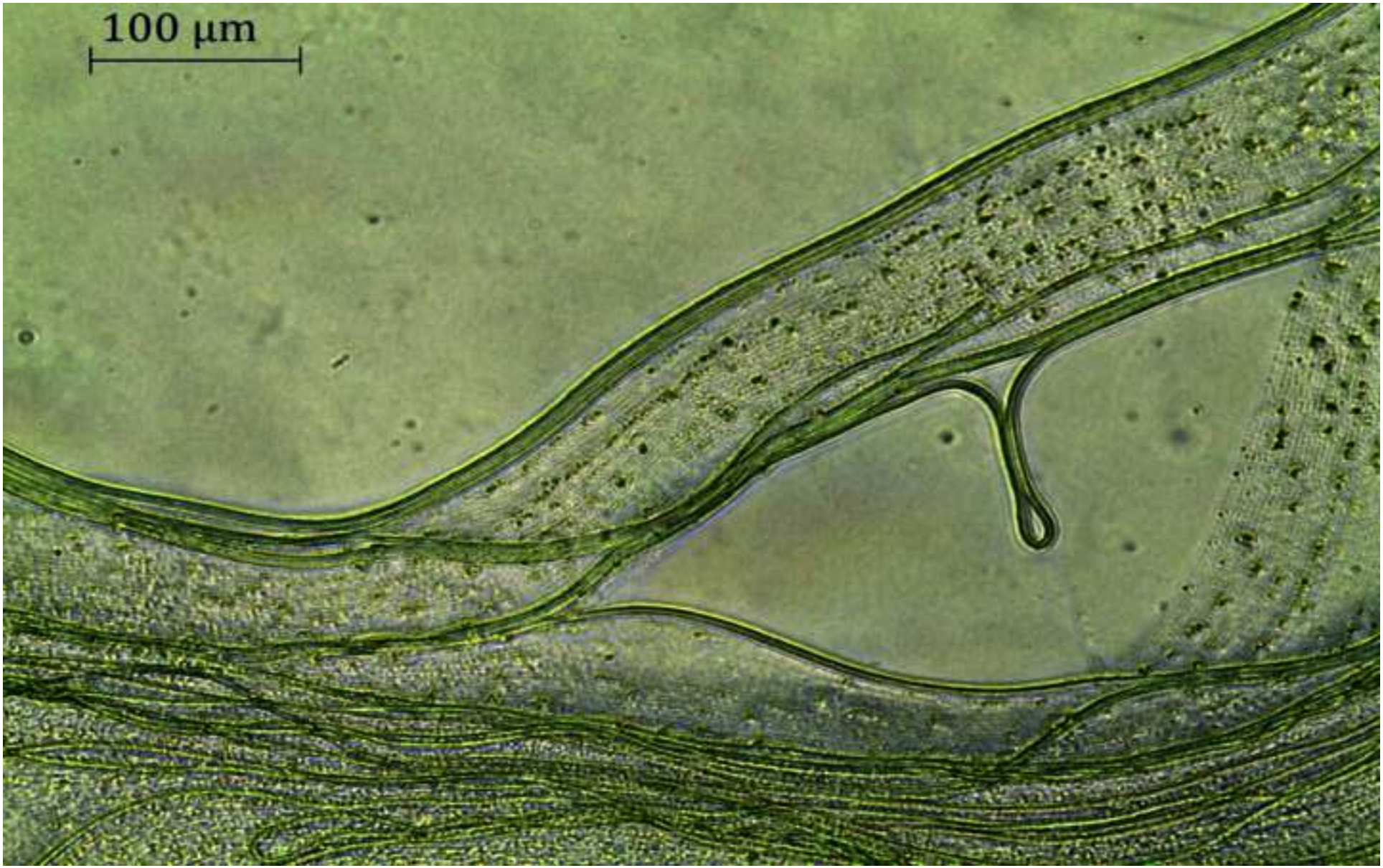

Figure

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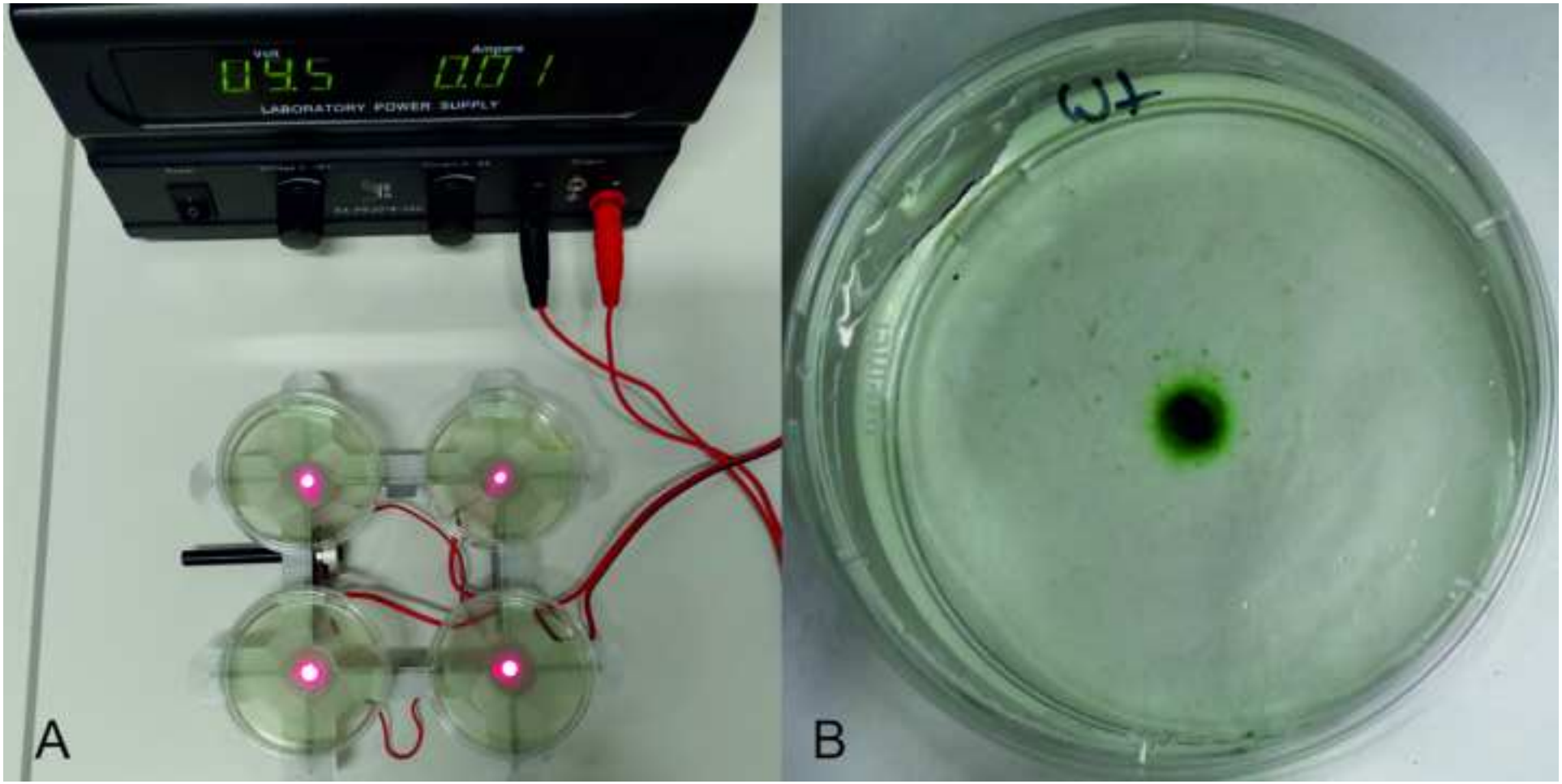

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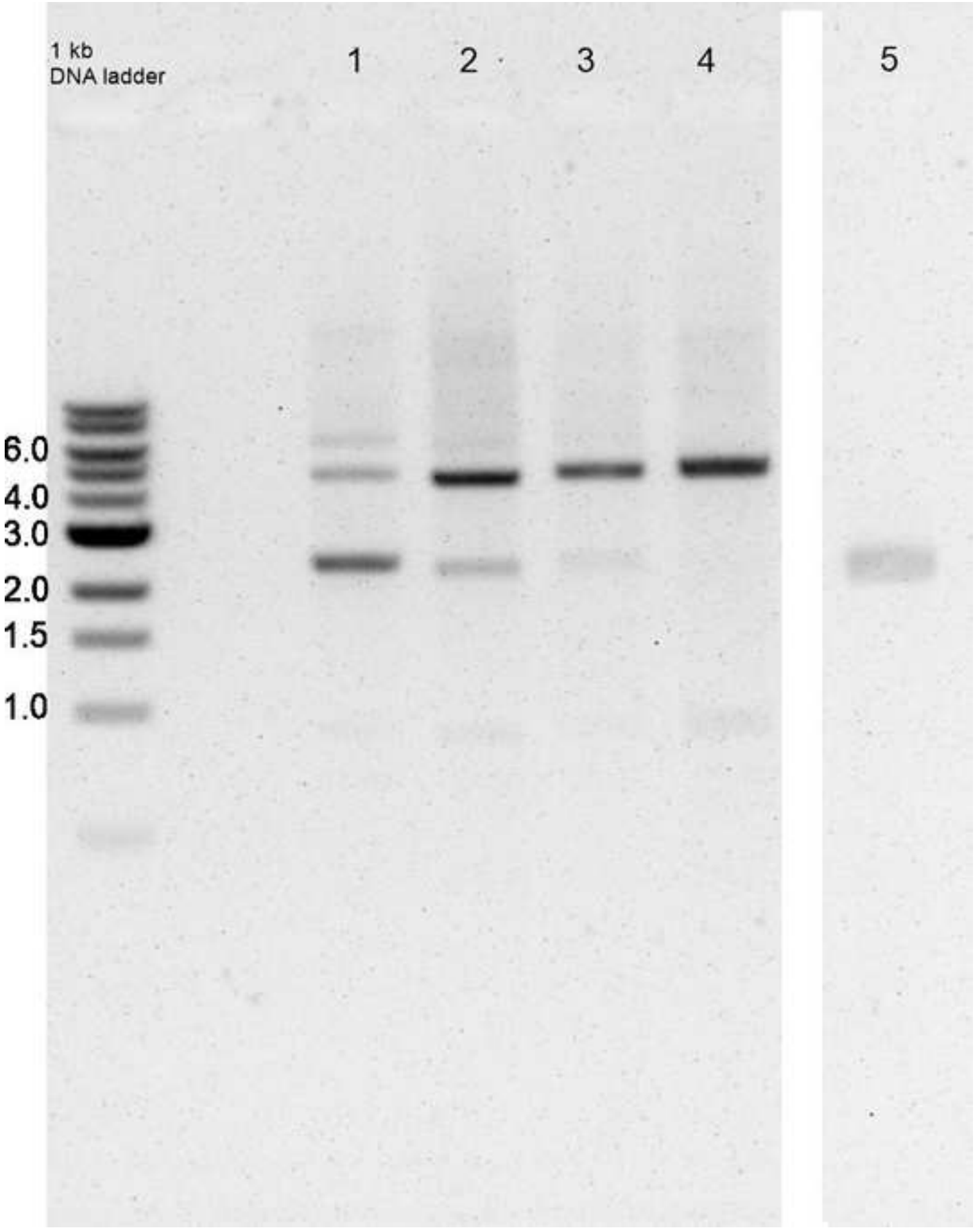

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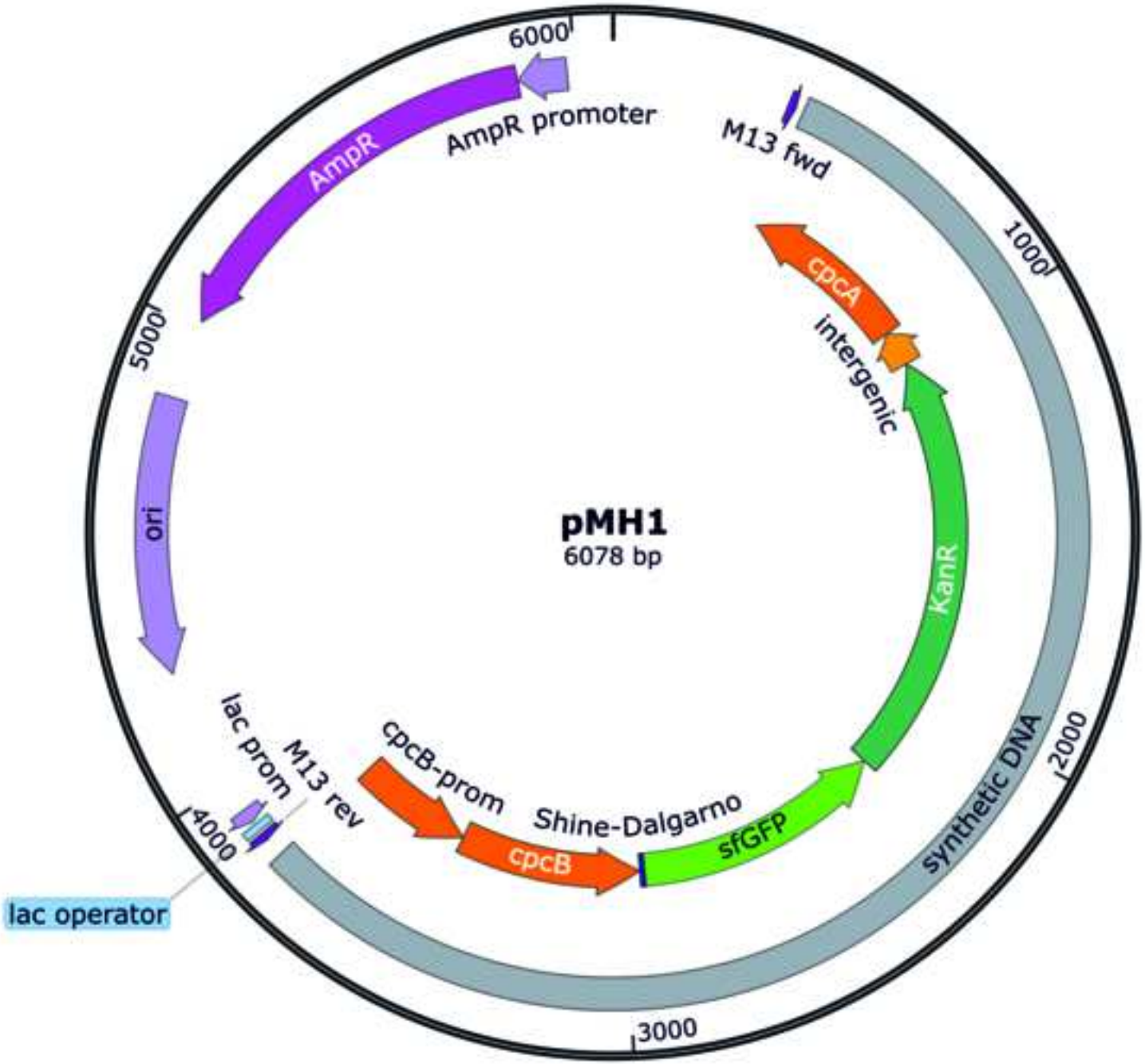

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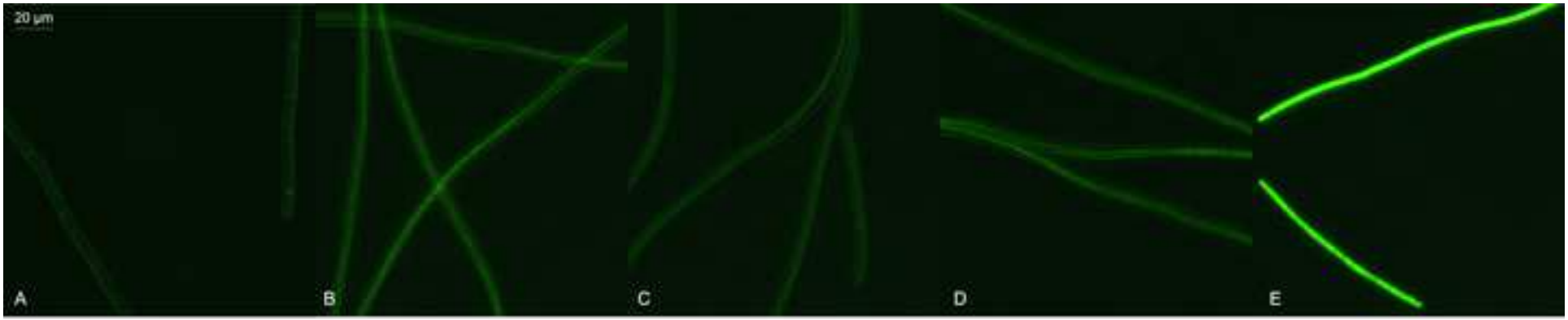

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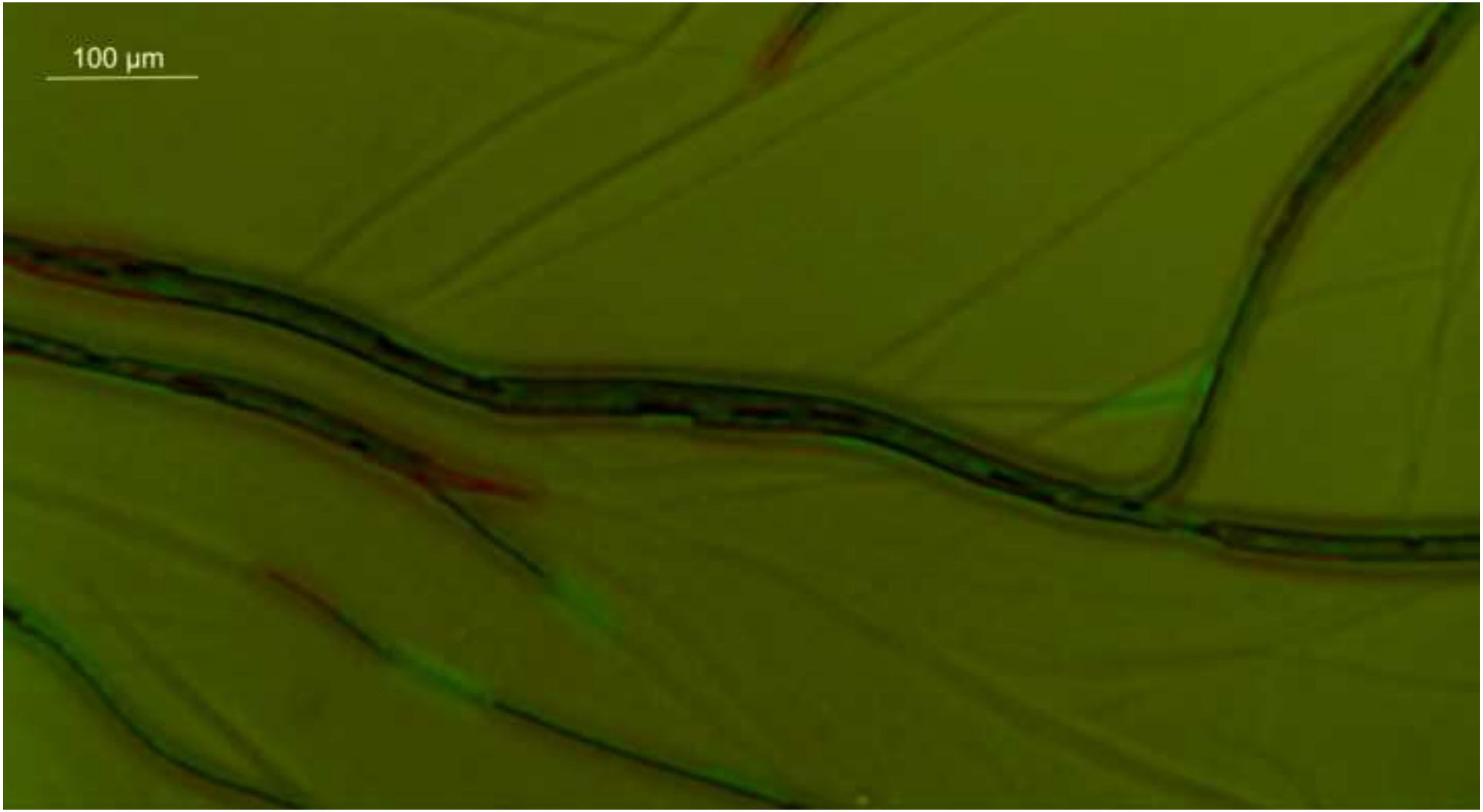

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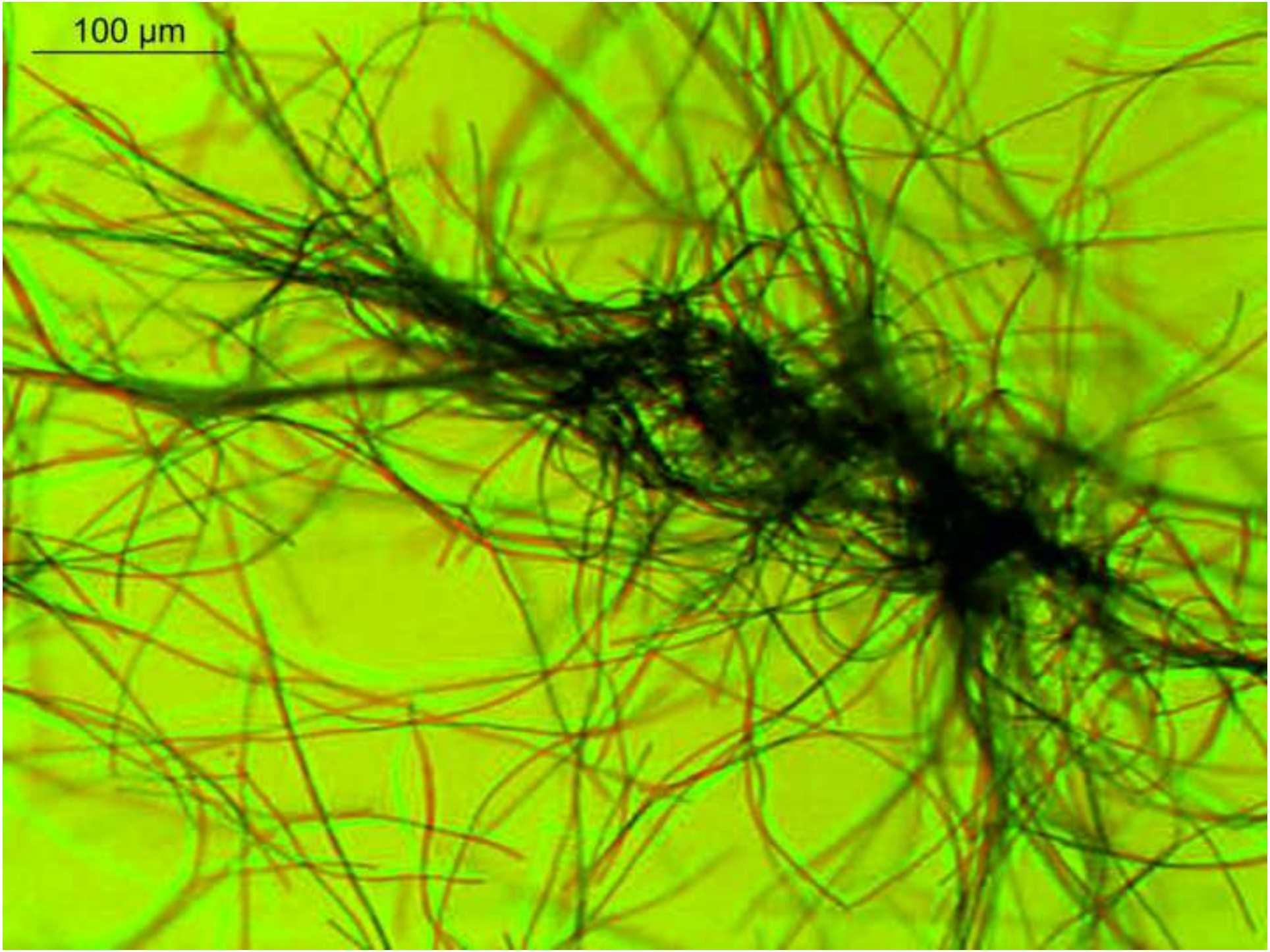

Table 1

strain	HE10JO	HE10DO	GI08AO	GI09CO	HE15M2G1
Contigs	104	174	218	102	154
total bp	4,819,017	4,788,491	4,778,775	3,669,922	4,598,395

Table 2.

	Gi09CO	HE10DO	HE10JO	HE15M2G1
Gi08AO	42	40	0	42
Gi09CO		2	42	0
HE10DO			40	2
HE10JO				42

Table 3.

Cell density OD 750 nm	1	1	3	3	5	5	7	7
Washes	1	2	1	2	1	2	1	2
Synechocystis PCC 6803	2/5	5/5	1/4	4/4	0/4	4/4	0/4	3/4
Phormidium lacuna HE10DO	4/4	4/4	4/4	4/4	3/ 4	4/4	3/3	3/3

Rebuttal letter

We have followed all comments that we found in the manuscript. The new version still contains these comments and my reply. There are now 7 separate files (besides tables and figures). One with the scripts of the minicomputer, one with cloning details, and 5 with sequences of the strains.

Is there some advice how to make videos?

With many regards

Tilman Lamparter

Supplemental file 1

Natural Transformation, Protein Expression, and Cryoconservation of the Filamentous Cyanobacterium *Phormidium lacuna*

Cloning of vectors for transformation of *P. lacuna*

For pAK1, pAK2 and pAK3, the inserts of pBK47, pBK54, and pBK55 were amplified by PCR, respectively, and the PCR products were cloned into pFN1. Plasmids and primers are given in the tables below. Cloning and PCR were performed according to standard procedures[1]. The sequence of pMH1 was generated by DNA synthesis and cloned into pUC19.

List of transformation vectors

Vector	**Purpose**	**Size [bp]**	**References**
pFN1	*P. lacuna* knock out of *chwA*, KanR resistance	6,400	[2]
pBK47	*sfGFP* expression in *Synechococcus* sp. PCC 7002, *cpc560* promoter	6,301	[3]
pBK54	*sfGFP* expression in *Synechococcus* sp. PCC 7002, A2813 promoter	6,244	[3]
pBK55	*sfGFP* expression in *Synechococcus* sp. PCC 7002, psbA2s promoter	5,949	[3]
pAK1	*sfGFP* expression in *P. lacuna*, *cpc560* promoter	7,697	this work
pAK2	*sfGFP* expression in *P. lacuna*, A2813 promoter	7,640	this work
pAK3	*sfGFP* expression in *P. lacuna* , *psbA2s* promoter	7,345	this work
PMH1	*sfGFP* expression in *P. lacuna*	6,078	this work

List of primers used for cloning of pAK1, pAK2, and pAK3 and for detection of integrated DNA in *P. lacuna*: Primer names contain "fwd" forward primers and "rev" for reverse primers. Restriction sites are indicated by the name of the restriction enzyme, followed by "site". The last column shows the vectors for which the primers were designed. Vector that are used as template are given in parentheses.

primer	**purpose**	**sequence**	**(template) and target**
AK1fwd	cloning, amplification of *psbA2s* / *sfGFP*, NheI site	ATGCGTGCTAGCCAGGTAAAC	(pBK55), pAK3
AK21fwd	cloninng, amplification of A2813 / *sfGFP*, NheI site	ATGCGTGCTAGCCCGATTTAAG	(pBK54), pAK2
AK5fwd	cloning, amplification of *cpc560* / *sfGFP* , NheI site	ATGCGTGCTAGCACCTGTAGA	(pBK47), pAK1
AK6rev	cloning, amplification of *psbA2s*, *A2813*, *CPC560* and *sfGFP,* PacI site	GTGGTGTTAATTAAGTATGCTCTTCTG CTCCTGCAG	(pBK47, pBK54, pBK55) pAK1 pAK2 pAK3
AK3fwd	cloning, amplification of pFN1 (without KanR) for integration DNA into chwA homologous sites, PacI site	GTGGTGTTAATTAAGGCTGACA TAGAGTTTGCCTCG	(pFN1) pAK1 pAK2 pAK3
AK4rev	cloning, amplification of pFN1 (without KanR) for integration DNA into chwA homologous sites, NheI site	GTGGTGGCTAGCGCCTCGCAAA CTTTGCTTTGC	pAK1 pAK2 pAK3
AK9fwd	detection, outer PCR *chwA*	ATCGACATCCCAATCCTCTGC	pAK1 pAK2 pAK3
AK10rev	detection, outer PCR *chwA*	CCTCGCACTCGTTTGGC	pAK1 pAK2 pAK3
F25fwd	detection, inner PCR *chwA*	GGTCTAGGTGAGGCAATCC	pAK1 pAK2 pAK3
F28rev	detection, inner PCR *chwA*	ACCTGATTTGTTTATATCTGAC GC	pAK1 pAK2 pAK3
MH1fwd	detection,, outer PCR *cpcB-cpcA*	GTTCGTCCATCATGGCTCAG	pMH1
MH2rev	detection, outer PCR *cpcB-cpcA*	CGACGGAATCGACATAGGAGT C	pMH1
MH3fwd	detection, inner PCR *cpcB-cpcA*	CGAGAATCACTCAGGTGAAGA G	pMH1
MH4rev	detection, inner PCR *cpcB-cpcA*	CGTAGTCGAGGTAGGAGTTGG	pMH1

F29 fwd	detection, KanR	CTATGACCATGATTACGAATTCCC	any
F30 rev	detection, KanR	AAGCCGTTTCTGTAATGAAGG	any
cpc560_pBK47_fwd (A23)	detection *cpc560* (and sfGFP)	GCTGTGGTTCCCTAGGC	any
GFP_pBK47_rev (A24)	detection (*cpc560*) and GFP	CAAGAAGGACCATGTGGTC	any

1 Sambrook, J., Russell, D. W. *Molecular Cloning. A Laboratory Manual. 3rd edition.*, Cold Spring Harbor Laboratory Press (2001).

2 Nies, F., Mielke, M., Pochert, J., Lamparter, T. Natural transformation of the filamentous cyanobacterium *Phormidium lacuna*. *PLoS One.* **15** (6), e0234440 (2020).

3 Kachel, B., Mack, M. Engineering of *Synechococcus* sp. strain PCC 7002 for the photoautotrophic production of light-sensitive riboflavin (vitamin B2). *Metabolic Engineering.* **62**, 275–286 (2020).

Sequence of pMH1 in gb format

```
LOCUS Exported 6078 bp DNA circular SYN 14-DEC-2021
DEFINITION Standard E. coli vector with a multiple cloning site (MCS) for
DNA
cloning. The MCS is reversed in pUC18.
ACCESSION .
VERSION .
KEYWORDS puc19_cpcB_sfGFP
SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
REFERENCE 1 (bases 1 to 6078)
AUTHORS .
TITLE Direct Submission
JOURNAL Exported Tuesday, Dec 14, 2021 from SnapGene Viewer 5.3.0
https://www.snapgene.com
COMMENT See also GenBank accession L09137.
FEATURES Location/Qualifiers
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/mol_type="other DNA"
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LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGAS
LIKHW"
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replication"
promoter 2144..2174
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protein_bind 2182..2198
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relieved by adding lactose or
isopropyl-beta-D-thiogalactopyranoside (IPTG)."
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/label=M13 rev
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/note="common sequencing primer, one of multiple similar
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```
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//
```

Supplemental file 2

Shell scripts (sh) for Raspberry Pi minicomputer

Microscope camera (see the **Table of Materials**) is coupled to USB port of minicomputer.

```
#program focus.sh
#!/bin/sh
NOW=0
NOW=$(date +%d%m%y%H%M)
while true; do
BEFORE=$NOW
NOW=$(date +%d%m%y%H%M)
webcam -F 10 -S 10 im1.jpg
fbi im1.jpg
done
#used to focus the filaments. Button must be pressed for new image.
```

```
#program 1minute.sh
#!/bin/sh
NOW=0
NOW=$(date +%d%m%y%H%M)
while true; do
BEFORE=$NOW
NOW=$(date +%d%m%y%H%M)
If [“$NOW” gt “$BEFORE” ]
then
fswebcam -F 30 -F 40 anyname_$NOW.jpg
fi
done
#If the time increases by 1 min, a photograph is taken and stored as a file.
```

Supplemental File 3

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Supplemental Coding Files

HE15M2G1_1.fna

Supplemental File 4

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Supplemental Coding Files

Gi08AO_1.fna

Supplemental File 5

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Supplemental Coding Files

Gi09CO_1.fna

Supplemental File 6

Click here to access/download

Supplemental Coding Files

HE10DO_1.fna

Supplemental File 7

Click here to access/download

Supplemental Coding Files

HE10JO.fna

Supplemental Video S1

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Supplemental Coding Files

renamed_489c0.mp4

Supplemental Video S2

Click here to access/download

Supplemental Coding Files

Video2_ Wt He10do 24H-1-1.mp4