

Transformation of the Cyanobacterium *Leptolyngbya boryana* by Electroporation

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[Abstract] *Leptolyngbya boryana* (*L. boryana*) (formerly *Plectonema boryanum*) is a versatile, filamentous cyanobacterium that has the ability to fix nitrogen under microoxic conditions and to grow heterotrophically with glucose in the dark, providing an excellent system to investigate photosynthesis, nitrogen fixation, and their regulatory mechanisms. While *L. boryana* is not naturally transformable different from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, it can be transformed by electroporation. Here we describe the transformation of *L. boryana* by electroporation to isolate mutants in which a targeted gene is disrupted.

Materials and Reagents

1. Bottle top filter system (0.22 µm) (Corning, catalog number: 430624)
2. Microcentrifuge tubes (1.5 ml) (Ina-optika corporation, BIO-BIK, catalog number: ST-0150F) or its equivalent, sterilized by autoclave (121 °C, 20 min)
3. Micropipettes tips (121 °C, 20 min)
4. Sterile syringe filter (Millex-GV Syringe Filter Unit, 0.22 µm), used for filter sterilization of solutions of antibiotics and glucose (Thermo Fisher Scientific, Millipore, catalog number: SLGV033RV) or its equivalent
5. Petri dish [sterile Petri dishes (90 mm x 15 mm)] (ASONE Corporation, catalog number: 1-7484-01-30) or its equivalent
6. Pulse cuvettes (Gene Pulser cuvette, 0.1 cm) (Bio-Rad Laboratories, AbD Serotec®, catalog number: 1652089)
7. Hybond N+ filter (disc 82 mm diameter) (GE Healthcare, Amersham, catalog number: RPN82B), sterilized by autoclave (121 °C, 20 min).
8. *Leptolyngbya boryana* (wild type or *dg5*) (grown on a BG-11 agar plate supplemented with 20 mM HEPES-KOH, pH 7.5 and 30 mM glucose)

*Note: The dg5 strain was isolated from the wild type as a natural mutant that grows much faster heterotrophically in the dark than wild type (Fujita et al., 1996). Recently we identified that the mutation responsible for the dg5 phenotype is one adenine insertion causing a frameshift in the *cytM* gene encoding cytochrome *c_M* (Hiraide et al., 2015).*

9. Sterilized water (150 ml)

Note: Water is purified by RiOs Essential Water Purification System (Merck Millipore Corporation) or WEX system (Yamato). Distilled water can be also used. Autoclave (121 °C, 20 min) and chill on ice before use.

10. NaNO₃ (Nacalai tesque, catalog number: 31617-35)
11. 1 M K₂HPO₄ solution (Wako Pure Chemical Industries, Siyaku, catalog number: 164-04295)
12. 7.5% (w/v) MgSO₄·7H₂O (Nacalai tesque, catalog number: 21003-75)
13. 3.6% (w/v) CaCl₂·2H₂O (Nacalai tesque, catalog number: 06731-05)
14. 2.0% (w/v) Na₂CO₃ (Nacalai tesque, catalog number: 31311-25)
15. Citric acid (Wako Pure Chemical Industries, Siyaku, catalog number: 038-05521)
16. Ammonium iron (III) citrate, brown (Wako Pure Chemical Industries, Siyaku, catalog number: 092-00802)
17. Ethylenediamine-N, N, N', N'-tetraacetic acid, disodium salt, dihydrate (EDTA-Na₂) (Dojindo, catalog number: 345-01865)
18. H₃BO₄ (Wako Pure Chemical Industries, Siyaku, catalog number: 021-02195)
19. MnCl₂·4H₂O (Nacalai tesque, catalog number: 21211-45)
20. ZnSO₄·7H₂O (Wako Pure Chemical Industries, Siyaku, catalog number: 268-00405)
21. Na₂MoO₄·2H₂O (Wako Pure Chemical Industries, Siyaku, catalog number: 197-02485)
22. CuSO₄·5H₂O (Wako Pure Chemical Industries, Siyaku, catalog number: 033-04415)
23. Co(NO₃)₂·6H₂O (Wako Pure Chemical Industries, Siyaku, catalog number: 031-03752)
24. 1 M Glucose (filter sterilized) (Wako Pure Chemical Industries, Siyaku, catalog number: 049-31165)
25. 50 mg/ml kanamycin sulfate (filter sterilized) (Wako Pure Chemical Industries, Siyaku, catalog number: 111-00344)
26. 50 mg/ml chloramphenicol (dissolve in ethanol) (Nacalai tesque, catalog number: 08027-72)
27. 10 mg/ml streptomycin sulfate (filter sterilized) (Meiji Seika Pharma, catalog number: 4987222665643)
28. 50 mg/ml erythromycin (filter sterilized) (Wako Pure Chemical Industries, Siyaku, catalog number: 054-05101)
29. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Nacalai tesque, catalog number: 17514-15)
30. Plasmid preparation kit (GenElute HP Plasmid Midiprep Kit) (Sigma-Aldrich, catalog number: NA0200)
31. Linearized plasmid solution (> 2 µg µl⁻¹)
Note: The concentration of plasmid is determined by a spectrophotometer before the enzyme digestion.
32. Bacto Agar [1.5% (w/v)] (BD, catalog number: 214010)
33. Ferric citrate solution (see Recipes)
34. Trace metal A₅+Co solution (1 L) (see Recipes)

35. 2x BG-11 solution (1 L) (see Recipes)
36. BG-11 agar plates (supplemented with 20 mM HEPES-KOH, pH 7.5, with or without appropriate antibiotics, Table 1) (see Recipes)

Equipment

1. Forceps
2. Micropipettes (P-1000, P-200 and P-20, or their equivalents) (Gilson)
3. Clean bench
4. Autoclave (TOMY DIGITAL BIOLOGY CO., model: SX-500 or its equivalent)
5. Gene pulser Xcell (Bio-Rad Laboratories, AbD Serotec®, catalog numbers: 1652666, 1652668 and 1652669)
6. Spectrophotometer (Shimadzu, model: UV-1700 or GE Healthcare, model: NanoVue Plus or its equivalent)
7. Aspirator (with a vacuum pump)
8. Growth cabinet (with appropriate fluorescence light bulbs)

Procedure

1. Plasmid for targeted mutagenesis is constructed (Figure 1). Plasmids carry a chimeric DNA fragment consisting of the upstream sequence of the target gene, drug resistance cartridge (Table 1), and downstream sequence of the target gene (Figure 1B). The upstream and downstream sequences should be more than 1 kb each, for stable homologous recombination. If the upstream and downstream fragments are obtained by PCR, their nucleotide sequences in the plasmid should be confirmed by Sanger sequencing to avoid introduction of unintended mutation(s) outside of the target gene via homologous recombination. The vectors are normally pUC18/19 derivatives.

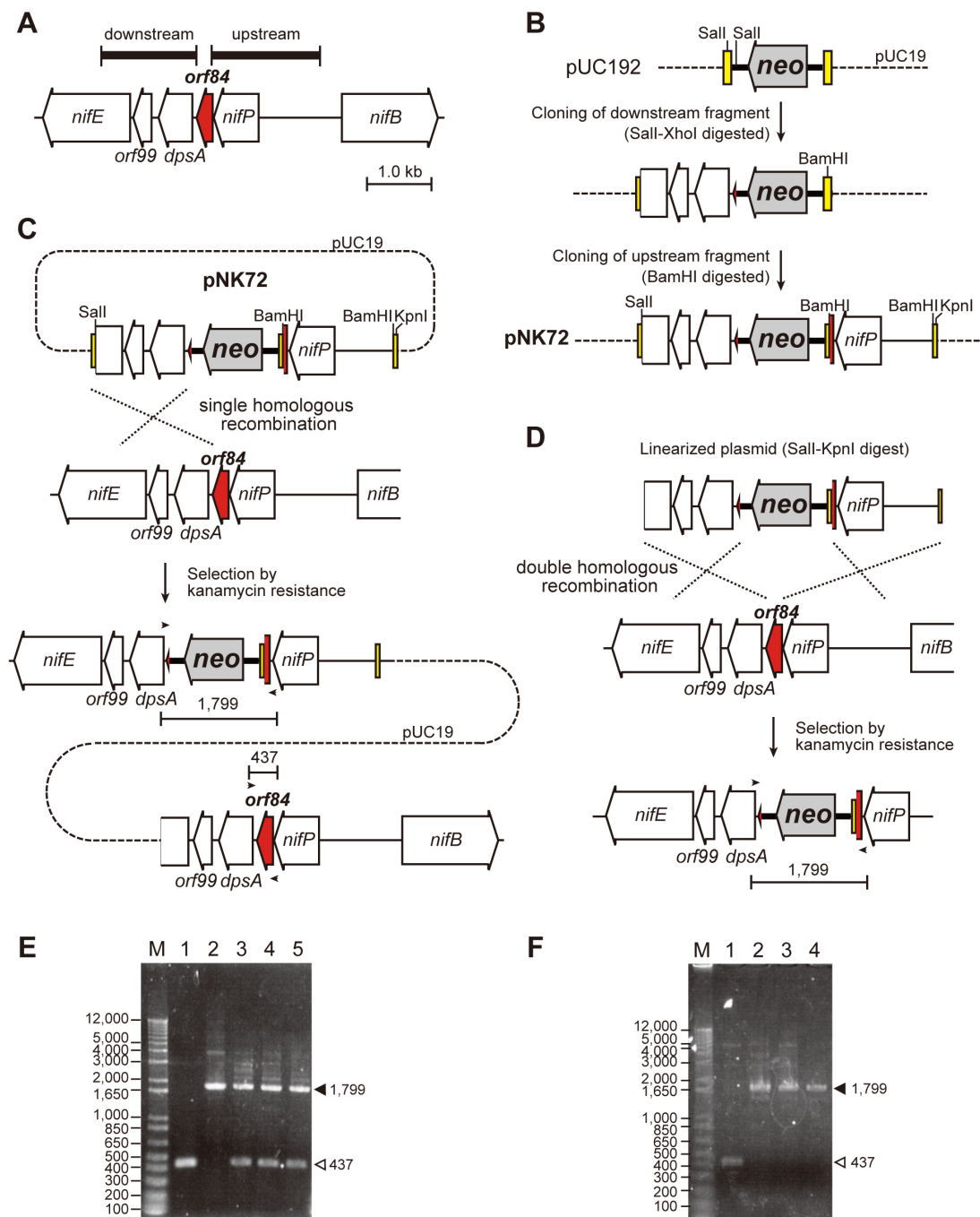


Figure 1. An example of plasmid construction, homologous recombination and PCR analysis of mutants in *L. boryana*. A. Gene arrangement of the target gene (the *orf84* gene, LBWT_23230, shown in red) in *L. boryana* (Tsujiimoto *et al.*, 2014). The 1.7-kb upstream and 1.5-kb downstream fragments (thick horizontal bars) of the *orf84* gene are amplified by PCR to construct plasmid. B. Construction of the plasmid pNK72 to isolate an *orf84*-disrupted mutant (Δ *orf84*). The two amplified fragments were cloned into a vector. In this case, pUC192 was used as the vector, which was constructed by the insertion of the *neo* gene (kanamycin resistance gene from pMC19, Fujita *et al.*, 1992) into the BamHI site of pUC19 (dashed line with its multi-cloning sites shown by yellow boxes). The 1.5-kb

downstream fragment (digested with Sall-XhoI) was cloned into the Sall sites of pUC192, and subsequently the 1.7-kb upstream fragment (digested with BamHI) was cloned into the BamHI site, yielding the plasmid pNK72. C. Gene arrangement of a single recombinant, in which the plasmid pNK72 is incorporated into the chromosome by a single homologous recombination event. Both wild-type and knock-out copies are present in the chromosome. In this case the single recombination event occurs between the 1.5-kb downstream fragment of the plasmid and the corresponding chromosomal part. Small horizontal arrows indicate primers for PCR shown in panel E. D. Gene arrangement of the $\Delta orf84$ mutant, in which the *orf84* gene was replaced with the *neo* gene by a double recombination event. The plasmid was previously digested with KpnI and Sall for electroporation to avoid to generate single recombinants. Small horizontal arrows indicate primers for PCR shown in panel F. E. PCR analysis of the single recombinants of *orf84*. Two DNA fragments were detected. The longer (a filled triangle, 1,799 bp) and shorter (an open triangle, 437 bp) fragments are corresponding to those of knock-out and wild-type copies, respectively, in the three transformants (lanes 3-5). The longer fragment was detected in PCR with the plasmid pNK72 as the template (lane 2). The shorter fragment (the wild-type copy) was detected in the wild-type cells (lane 1). F. PCR analysis of $\Delta orf84$. Only single DNA fragment corresponding to the knock-out copy of *orf84* (a filled triangle, 1,799 bp) was detected in the two transformants (lanes 3 and 4). The shorter and longer fragments (lanes 1 and 2) were the same as E. (unpublished results, Kotani and Fujita).

2. The plasmid prepared by a plasmid preparation kit was linearized by restriction enzyme(s) to minimize the probability that the plasmid would integrate into the genome via a single recombination event resulting in a merodiploid that harbors both knock-out and wild-type copies in the genome (Figure 1C). The digestion site(s) can be selected from anywhere in the vector part. It should be careful not to digest the parts of homologous recombination and the drug resistance marker. The digested plasmid was precipitated by ethanol and dissolved in sterilized water to be $>2 \mu\text{g } \mu\text{l}^{-1}$.
3. *L. boryana* is cultivated on an agar plate of BG-11 containing 30 mM glucose under low light conditions ($<10 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 30°C for 2-7 days (Note 1).
4. All procedures below should be performed under aseptic conditions. Cells on the agar plate are suspended in sterilized water (ca. 2 ml per one agar plate) and the suspension is transferred into a bottle top filter (Figure 2A-C. Notes 2 and 3). In this stage, Hybond N+ filters should be placed onto new BG-11 agar plates for cell recovery after electroporation (step 11).
5. The cells are collected on the surface of the bottle top filter by aspiration (Figure 2D).

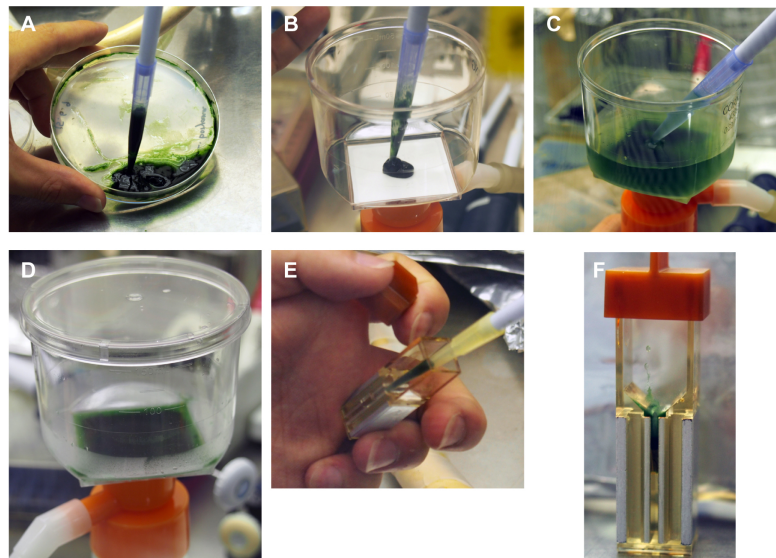


Figure 2. Preparation of *L. boryana* cells for pulse application

6. Approximately 50 ml of sterilized water, chilled on ice, is added to the filter to suspend the cells. The cells are again collected on the filter by aspiration. This washing procedure is repeated three times (Figure 3C. Notes 4-5). Centrifugation ($1,200 \times g$, 10 min) is another option for washing cells with water. In this case, note that *L. boryana* cells form very loose pellets even by high-speed centrifugation (for example, $44,000 \times g$, 30 min) due to long trichomes.
7. The cells on the filter are suspended in a small aliquot (500 μ l) of sterilized water (Figure 2D) and the suspension is transferred into a microcentrifuge tube (1.5 ml) to store on ice until pulse application (Note 3).
8. A small aliquot (50 μ l) of the suspension used for pulse application is dispensed into another microcentrifuge tube (Note 3) and the concentrated plasmid solution (typically 10 μ l of $2 \mu\text{g } \mu\text{l}^{-1}$) is added to the tube and mixed well.
9. The mixture is transferred into a cuvette with a gap width of 0.1 cm and chilled on ice (Figure 2E-F, Note 3).
10. A single exponential decay pulse (setting: voltage, 1.41 kV; capacitance, 50 μF ; and resistance, 250 ohms) is applied. The time constant will be approximately 10 ms. The time constant and the actual voltage should be recorded on your note (Notes 5-6).
11. An aliquot (350 μ l) of BG-11 liquid medium is added to the cuvette and the cell suspension is recovered with the blue tip of a P-1000 type micropipette dispenser (Figure 3A) to spread onto a Hybond N+ filter overlaid on a BG-11 agar plate without antibiotics (Figure 3B-C).



Figure 3. Recovery of pulse-applied cells from a cuvette and inoculation on a non-selective plate

12. The agar plate is incubated at 30 °C under low light conditions ($<10 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 2 days. During this period the antibiotic resistance gene is expressed to confer the cell's resistance to the specific antibiotic.
13. The Hybond N+ filter is transferred onto a new BG-11 agar plate containing appropriate antibiotics (final concentrations are shown in Table 1). The agar plate is incubated at 30 °C under high light conditions (ca. $100 \mu\text{mol m}^{-2} \text{s}^{-1}$).
14. Colonies showing resistance against the antibiotics appear after approximately 10 to 14 days (Figure 4). You can pick them up with toothpicks or tips (for a P-200-type micropipette) that should be previously autoclaved (121 °C, 20 min).
15. Some colonies are single recombinants and some are double recombinants. PCR analysis should be carried out to identify double recombinants (Figure 1E-F; Notes 7 -8).

Table 1. List of antibiotics that can be used for transformation of *L. boryana*

Antibiotics	Final concentration ($\mu\text{g ml}^{-1}$)	Resistance gene	Source of the resistance gene	References
kanamycin	15	<i>neo</i>	pKC7	Rao and Rogers, 1979
chloramphenicol	25	<i>cat</i>	pPBH201	Walton <i>et al.</i> , 1993
streptomycin ¹	10	<i>aadA</i>	pJRD215	Davison <i>et al.</i> , 1987
erythromycin	10	<i>ermC</i>	pRL425	Elhai and Wolk, 1988

¹This antibiotic has not yet been used for targeted mutagenesis. However, we confirmed that transformants harboring pJRD215 (with the streptomycin resistance gene) showed resistance to streptomycin (unpublished results).

Representative data

Representative results in targeted gene disruption are shown in Table 2. In most cases almost all transformants appeared on selective plates were double recombinants. A

procedure of repeated inoculations of transformants to segregate cells in which all wild-type copies are completely replaced with the mutant copies is required in *Synechocystis* sp. PCC 6803, but this process is not needed in *L. boryana* (Note 8).

Table 2. Examples of electroporation of *L. boryana*

Target gene(s)	Strain ¹	DNA (μg) ²	Selection marker	Single ³	Double ³	Reference
<i>nifHD</i>	WT	1	kanamycin	0	1	Fujita <i>et al.</i> , 1992
<i>chlL</i>	WT	6	kanamycin	3	2	Fujita <i>et al.</i> , 1992
<i>chlB</i>	<i>dg5</i>	4	kanamycin	1	1	Fujita <i>et al.</i> , 1996
<i>por</i>	<i>dg5</i>	3	kanamycin	0	1	Fujita <i>et al.</i> , 1998
<i>cnfR</i>	<i>dg5</i>	25	kanamycin	0	5	Tsujimoto <i>et al.</i> , 2014
<i>chlR</i>	<i>dg5</i>	8	kanamycin	0	1	Tsujimoto <i>et al.</i> , 2014
<i>nifDK</i>	<i>dg5</i>	25	kanamycin	0	6	Tsujimoto <i>et al.</i> , 2014
<i>orf84</i>	<i>dg5</i>	27	kanamycin	3	2	Kotani and Fujita, unpublished

¹WT, wild type

²Total DNA amount in the cell suspension for pulse application

³Number of colonies. Single or double recombinant was confirmed by Southern blot analysis or PCR.



Figure 4. Typical appearance of transformants on a selective plate. Two tiny green colonies (red arrows) appeared 14 days after pulse application.

Notes

1. Liquid culture of BG-11 (containing 20 mM HEPES-KOH, pH 7.5 and 30 mM glucose) can be used (Fujita *et al.*, 1992; Fujita *et al.*, 1996, Fujita *et al.*, 1998; Kimata-Ariga *et al.*, 2000).
2. To suspend cells on an agar plate, we use a sterile bent glass rod that is used for spreading *Escherichia coli* cells in transformation.

3. Concentrated cell suspension may be too viscous to dispense by a normal micropipette tip. Thus, we prepare special tips (both of P-1000 and P-200 tips) by cutting the ends of normal tips with scissors (to be a diameter of approximately 2 mm) and used them for handling dense cell suspensions. These tips are autoclaved (121 °C, 20 min) before use.
4. In the original procedure (Fujita *et al.*, 1992), 1 mM HEPES-KOH (pH 7.5) and 10% (w/v) glycerol were sequentially used for washing cells. We recently found that using water instead gives essentially the same results as described here.
5. Insufficient washing of cells and/or impurity of plasmid preparation may cause arc discharge in pulse application, resulting in transformation failure.
6. Original settings were; voltage, 1.25 kV; capacitance 25 μ F; and resistance, 600 ohms (Fujita *et al.*, 1992). Using another electroporator (BTX Electro Cell Manipulator 600), the settings are; voltage, 1.45 kV; capacitance 50 μ F; and resistance, 256 ohms.
7. While only single recombinants may be isolated in one experiment, double recombinants can be eventually isolated in further trials (Figure 1). However, if double recombinants are never isolated even after many times trials of transformation, it is probable that the target gene is essential for growth under the conditions.
8. Differentiation from a single recombinant to a double recombinant has never been observed even though many times of subculturing.
9. For transformation with a shuttle vector, derivatives of pPBH201 (Kimata-Arigo *et al.*, 2000; Yamamoto *et al.*, 2009; Yamamoto *et al.*, 2011), a relatively low concentration of plasmid ($<0.1 \mu\text{g } \mu\text{l}^{-1}$) is enough to isolate transformants.

Recipes

1. Ferric citrate solution
 - 60 mg citric acid
 - 60 mg ammonium iron (III) citrate, brown
 - 10 mg EDTA- Na_2
2. Trace metal A₅+Co solution (1 L)
 - 2.86 g H_3BO_4
 - 1.81 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
 - 0.22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
 - 0.39 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
 - 79 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 - 49 mg $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$
3. 2x BG-11 solution (1 L)
 - 3.0 g NaNO_3 dissolved in 932 ml distilled water
 - 460 μl of 1 M K_2HPO_4 solution
 - 2.0 ml of 7.5% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

2.0 ml of 3.6% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

2.0 ml of 2.0% (w/v) Na_2CO_3

20.0 ml of Ferric citrate solution

Serially add 2.0 ml of Trace metal A₅+Co

40 ml of 1 M HEPES-KOH, pH 7.5

Store at 4 °C

Note: BG-11 liquid medium (Rippka et al., 1979) supplemented with 20 mM HEPES-KOH (pH 7.5). For BG-11 liquid medium, the equal amounts of 2x BG-11 and distilled water are mixed, and autoclaved (121 °C, 20 min).

4. BG-11 agar plates

The equal amounts of 2x BG-11 and distilled water containing:

Bacto Agar [3.0% (w/v)] is separately autoclaved (121 °C, 20 min). Allow the media to cool about 50 °C (Be careful not to be solidified at this stage). The solutions of 2x BG-11 and agar are mixed in a clean bench. If needed, appropriate antibiotic solution and glucose solution are added. Then, pour about 30 ml of medium into each Petri dish. When the BG-11 agar medium solidifies, invert the agar plates and store at 4 °C.

Acknowledgments

This protocol was adapted from the previously published studies, Hiraide *et al.* (2015), Tsujimoto *et al.* (2014) and Fujita *et al.* (1992). The original protocol was described in Fujita *et al.* (1992), and we modified it as described here. We thank Yasuhiro Takahashi, Tomohiro Matsumura, Toshiharu Hase, and Hiroshi Matsubara for initial works on establishment of this transformation system. We thank Douglas K. Walton, Carl E. Bauer and Peter Wolk for donating plasmids pPBH201, pJRD215 and pRL425, respectively. We thank Chie Tomatsu for providing technical help. This work was supported by the Japan Society for the Promotion of Science (JSPS) (Grants-in-Aid for Scientific Research Nos. 05740481, 06740601, 07740617, 08836006, 11740445, 23370020, 23000007, 26660084, 15H04387 and 15H01397), Precursory Research for Embryonic Science and Technology (PRESTO) and the Advanced Low Carbon Technology Research and Development Program (ALCA).

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