Establishment of the reporter system for a thylakoid-lacking cyanobacterium, *Gloeobacter violaceus* PCC 7421

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**A B S T R A C T**

*Gloeobacter violaceus* PCC 7421 is considered, by molecular phylogenetic analyses, to be an early-branching cyanobacterium within the cyanobacterial clade. *G. violaceus* is the only known oxygenic photosynthetic organism that lacks thylakoid membranes. There is only one report on the development of a transformation system for *G. violaceus* [H. Guo, X. Xu, Prog. Nat. Sci. 14 (2004) 31–35] and further studies using the system have not been reported. In the present study, we succeeded in introducing an expression vector (pKUT1121) derived from a broad-host-range plasmid, RSF1010, into *G. violaceus* by conjugation. The frequency of transformation of our system is significantly higher than that described in the previous report. In addition, luciferase heterologously expressed in *G. violaceus* functioned as a reporter. The established system will promote the molecular genetic studies on *G. violaceus*.

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1. Introduction

Cyanobacteria are considered to be the first oxygenic photosynthetic organisms that emerged about 2.7 billion years ago. Most of the genes that are responsible for photosynthesis are widely conserved from cyanobacteria to eukaryotic photosynthetic organisms, this conservation is a convincing evidence of the endosymbiotic acquisition of eukaryotic chloroplast from a cyanobacterium. This high conservation has prevented us from understanding the evolution of photosynthetic mechanisms from the primordial one. Therefore, cyanobacteria diverged from early stage of the cyanobacterial evolution may be helpful in studying the evolution of photosynthetic mechanisms, because such cyanobacteria are expected to retain a part of primordial properties that had been lost during the evolution of other major cyanobacteria. However, “primordial cyanobacteria” that retain a part of primordial properties rarely exist nowadays.

*Gloeobacter violaceus* PCC 7421 (hereafter referred to as *G. violaceus*) is a unicellular cyanobacterium, and is considered to be an early-branching cyanobacterium within the cyanobacterial clade, by the molecular phylogenetic analyses [1–4]. Almost all oxygenic photosynthetic organisms form the internal membranes called thylakoid membranes, which are the site for the light reaction of photosynthesis. *G. violaceus* is the only known oxygenic photosynthetic organism that lacks the thylakoid membranes [5]. This unique property has been found only in this organism. Accordingly, both the photosystems and the respiratory chain in *G. violaceus* are localized at the cytoplasmic membrane. This indicates that photosynthetic activity per cell in *G. violaceus* is much lower than those in other cyanobacteria and eukaryotic photosynthetic organisms. For these unique characteristics, the complete genome of *G. violaceus* was sequenced in 2003 [6]. The genome sequence revealed that a part of the genes that are responsible for photosynthesis was not found in *G. violaceus*, whereas those genes are highly conserved among other oxygenic photosynthetic organisms [6]. Therefore, in recent years, protein complexes that are responsible for photosynthesis (e.g. photosystem I and phycobilisome) in *G. violaceus* were biochemically analyzed based on the genome information [7–11]. These recent results partly solved unique features previously reported [12–14]. Recently, it was reported that both the photosynthetic and respiratory complexes were concentrated at the respective domains, which may have specialized functions, in the cytoplasmic membrane of *G. violaceus* [15]. Moreover, the comparison of state transitions between *G. violaceus* and *Synechocystis* sp. PCC 6803 showed the commonalities and differences [16]. *G. violaceus* exhibited state transitions and non-photochemical fluorescence quenching like *Synechocystis* sp. PCC 6803 [16]. In *G. violaceus*, the structure of phycobilisome was quite different from other cyanobacterial phycobilisomes [12]. Nevertheless, orange carotenoid protein that binds to phycobilisome was also correlated with blue-light-induced heat dissipation in *G. violaceus*, like *Synechocystis* sp. PCC 6803 [16]. These results suggest that *G. violaceus* is an ideal organism for investigating the evolution of photosynthetic system by comparison of other

**Abbreviations:** CBP, Coomassie Brilliant Blue; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Sm, streptomycin

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cyanobacteria.

Molecular genetics, such as the production and analysis of mutants, is a preferable method to analyze the function of individual genes in *G. violaceus*. Unfortunately, molecular genetic analysis cannot be applied to *G. violaceus* because of the lack of a highly-reproducible transformation system for this organism. Only one report on the development of a transformation system for *G. violaceus* has been published to date [17]. However, there is no subsequent paper that describes the functional expression of the foreign genes in *G. violaceus* using the system. In the present study, we re-examined the transformation system reported previously, and developed a highly-reproducible transformation system for *G. violaceus*. We succeeded in introducing an expression vector derived from a broad-host-range plasmid into *G. violaceus* by conjugal gene transfer. Using this system, we introduced a luciferase gene into *G. violaceus*, and the resultant transformant exhibited significant luciferase activity.

2. Materials and methods

2.1. Culture of *G. violaceus*

*G. violaceus* was grown photoautotrophically in BG11 medium [18] under the continuous white light (10 μmol photons m$^{-2}$ s$^{-1}$) at 25 °C, and air was supplied via an air filter (Milllex-FG, Millipore, Massachusetts, USA). For transformants, 10 μg ml$^{-1}$ streptomycin (Sm) was added to the medium. BG11 agar medium containing 1 mM TES–NaOH (pH 8.2) was used for solid culture.

2.2. Construction of plasmids and transformation of *G. violaceus*

We used a plasmid vector pKUT1121 [19], which was constructed from a broad-host-range plasmid RSF1010 [20], to establish a transformation system for *G. violaceus*. The coding region of firefly luciferase gene (*luc*) was amplified by polymerase chain reaction (PCR) using pGL3-Basic vector (Promega, Wisconsin, USA) as a template. The PCR product containing *luc* gene with additional restriction sites for Ndel and Xhol at the 5′- and 3′-ends, respectively, was amplified using the following primers: 5′-GGCGCATATGGAGACGCCAAAAACAT-3′, 5′-GGCGGAAAGATCGCCGTGAATCTGAGAAA-3′. After the PCR product was subcloned into pZErO-2 (Invitrogen, California, USA), the sequence of cloned *luc* gene was confirmed by sequencing. The *luc* gene was excised from the plasmid by Ndel and Xhol treatment, and subcloned into pKUT1121 to yield pKUT-luc.

Transformation was performed by diparental mating basically according to the method of Elhai and Wolk [21]. First, a conjugative helper plasmid, pRK2013 [22], was introduced into *Escherichia coli* XL1-Blue MRF‡ (Agilent Technologies, California, USA). Subsequently, the expression vector (pKUT1121 or pKUT-luc) was introduced into XL1-Blue MRF‡ (pRK2013). Equal amounts of resultant transformant cells and *G. violaceus* cells were mixed, and then aliquots of the mixture were spotted onto nitrocellulose membrane on a BG11 agar medium. Following a 48 h incubation under the light of 5 μmol photons m$^{-2}$ s$^{-1}$, the membrane was transferred onto BG11 agar medium containing 5 μg ml$^{-1}$ Sm. Streptomycin-resistant colonies appeared after several months, and each colony was finally cultured in BG11 liquid medium containing 10 μg ml$^{-1}$ Sm. Total DNA was prepared from *G. violaceus* cells using hexadecyl-trimethyl-ammonium bromide [23]. The presence of marker gene in the total DNA was checked by PCR.

2.3. SDS–PAGE and Western blotting

Total protein of *G. violaceus* cells was prepared by the following procedure. *G. violaceus* was resuspended with a buffer (20 mM MES–NaOH (pH 6.5), 1 mM MgCl$_2$, 0.5 mM CaCl$_2$, 1 mM NaCl, 0.6 M betaine). The suspended cells were disrupted by repeated agitation with glass beads (φ = 0.1 mm) at 4 °C. After the debris was removed by centrifugation (2000 × g, 5 min, 4 °C), Triton X-100 was added to the supernatant at the final concentration of 1% to solubilize the membrane. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [24] using 12% (w/v) of polyacrylamide gel. Total proteins (10 μg) were loaded on each lane of a gel, and the gel was stained with Coomassie Brilliant Blue (CBB) after electrophoresis. For Western blotting, separated proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (CBB) after electrophoresis. For Western blotting, separated proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (CBB) after electrophoresis. For Western blotting, separated proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (CBB) after electrophoresis. For Western blotting, separated proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (CBB) after electrophoresis.

2.4. Luciferase assay

The concentration of *G. violaceus* cells was adjusted to 1.0 × 10$^7$ cells ml$^{-1}$ with BG11 medium. After the cells were adapted to darkness for 5 min, background luminescence was measured with a luminometer (GloMax$^\text{TM}$ 20/20n Luminometer, Promega). Then, luciferin (Beetle Luciferin, Promega) was added to the cells at the final concentration of 100 μM, and the luminescence derived from luciferase reaction was measured.

3. Results

3.1. Antibiotic susceptibility of *G. violaceus*

First, we tried to culture *G. violaceus* at 28 °C under the light of 20 μmol photons m$^{-2}$ s$^{-1}$ according to Guo and Xu [17], however, cells were not able to survive. Therefore, we applied our routine culture conditions to further study. We examined the antibiotic susceptibility of *G. violaceus* for the use of antibiotic resistance genes as marker genes of transformant. We tested gentamicin, hygromycin, spectinomycin and zeocin in addition to antibiotics used in Guo and Xu [17]. Table 1 summarizes the result of antibiotic susceptibility test of wild type *G. violaceus*. Three antibiotics showed same susceptibility as described in Guo and Xu [17], however, the others showed different susceptibility (for details, see Section 4). Three of nine antibiotics, erythromycin, Sm and spectinomycin exhibited antibiotic activity against *G. violaceus* within the range of 1–50 μg ml$^{-1}$. For these three antibiotics, we also checked the antibiotic activity against *G. violaceus* on the agar medium. *G. violaceus* cells adjusted to the concentration of 1.0 × 10$^3$ to 1.0 × 10$^9$ cells ml$^{-1}$ were spotted onto nitrocellulose membrane on BG11 agar medium including each antibiotic. As a result, Sm was the most effective for killing cells at lower concentration (5 μg ml$^{-1}$). Therefore, we chose Sm resistance gene (*aadA*) as a marker gene for the screening of transformant.

3.2. Development of transformation system for *G. violaceus*

Because *G. violaceus* was sensitive to Sm (Table 1), we tried to introduce a broad-host-range plasmid derived expression vector, pKUT1121 [19] that possesses Sm resistance gene cassette, by conjugal gene transfer. After the treatment of exconjugants with Sm, Sm resistant colonies appeared (Fig. 1A). In contrast, no colony was formed in the spot of negative control (Fig. 1B). The frequency of transformation of *G. violaceus* was approximately 1.2 × 10$^{-4}$ per recipient cell for pKUT1121. Total DNA prepared from the Sm resistant strain and wild type were used as template of PCR (Fig. 2) to confirm successful introduction of the plasmid. As a marker gene,
Table 1
Antibiotic susceptibility of G. violaceus.

| Antibiotics | Concentration (μg ml⁻¹) |
|-------------|-------------------------|
|             | 1          | 5          | 15         | 50         |
| Ap          | R          | R          | R          | R          |
| Cm          | R          | R          | R          | S          |
| Em          | S          | S          | S          | S          |
| Gm          | R          | R          | R          | S          |
| Hyg         | R          | R          | R          | R          |
| Km          | R          | R          | R          | R          |
| Sm          | R          | S          | S          | S          |
| Sp          | R          | S          | S          | S          |
| Zeo         | R          | R          | R          | R          |

Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Hyg, hygromycin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Zeo, zeocin. R, resistant; S, sensitive.

aadA was amplified by PCR, and a PCR product that exhibited the similar migration to that of positive control (Fig. 2A, lane 2) was observed in the Sm resistant strain by electrophoresis (Fig. 2A, lane 3). In contrast, no amplification was found in wild type (Fig. 2A, lane 1). Furthermore, we transformed two strains of E. coli (XL1-Blue MRF and DH5α) with the total DNA. For each E. coli strain, a lot of Sm resistant colonies appeared after the transformation with the total DNA prepared from Sm resistance strain. On the contrary, no colony formed after the transformation with the total DNA from wild type. Then, plasmids prepared from the E. coli transformants were digested with restriction enzymes. In agarose gel electrophoresis, restriction patterns of the prepared plasmids were identical to that of the original pKUT1121 (data not shown). These results demonstrated that the Sm resistant strain harbors pKUT1121 as a plasmid. Therefore, we concluded that the transformation system for G. violaceus was established. We named the G. violaceus transformant pKUT1121 strain, and used this strain as a control for further experiments.

3.3. Introduction of a luc gene into G. violaceus as a reporter gene

Because we succeeded in developing the transformation system for G. violaceus by conjugation, we evaluated the use of a luc gene as a reporter gene in G. violaceus cells. The luc gene was subcloned into pKUT1121, and the resultant vector (pKUT1121-luc) was introduced into G. violaceus. By conjugation, Sm resistant colonies were obtained at the frequency of transformation of approximately 3.6 × 10⁻⁵ per recipient cell. Total DNA was prepared from this Sm resistant strain (pKUT1121-luc strain), and used as a template of PCR. Two marker genes, aadA and luc were successfully amplified by PCR from total DNA of pKUT1121-luc strain (Fig. 2, lanes 5), whereas luc gene was not amplified from the total DNA of both wild type and pKUT1121 strain (Fig. 2B, lanes 1 and 3). Transformation of E. coli with the total DNA prepared from pKUT1121-luc strain confirmed that pKUT1121-luc was maintained as a plasmid in pKUT1121-luc strain.

3.4. Activity of luciferase expressed in the pKUT1121-luc strain

We examined whether the luc gene was functionally expressed in the pKUT1121-luc strain, by Western blotting and luciferase assay. Total proteins prepared from cells were analyzed by SDS–PAGE and following Western blotting. Although no specific band was found among wild type, pKUT1121 strain and pKUT1121-luc strain by CB staining of the gel (Fig. 3A), luciferase was immunologically detected only in the pKUT1121-luc strain (Fig. 3B). For wild type and transformants, we measured luciferase activity in vivo (Table 2). The background luminescence was measured after the dark adaptation of the cells, and the values were quite low in all samples. After the addition of luciferin to the cells, approximately 1000 times higher luminescence than background was observed in pKUT1121-luc strain, whereas virtually no difference was found in wild type and pKUT1121 strain (Table 2).
The luciferase assay revealed that there is no significant background activity in *G. violaceus* (Table 2). Therefore, the luc gene can be available for promoter assay using *G. violaceus* as a host. Although we could not succeed in accumulating luciferase at a high level (Fig. 3), this low-level expression will be enough for the metabolic engineering of *G. violaceus*. The genes for enzymes that are responsible for chlorophyll, carotenoid and lipid biosynthesis are candidates for alteration, because those molecules affect photosynthetic activity.

In the present study, we established the highly-reproducible transformation system on *G. violaceus*, and demonstrated the introduction and functional expression of the luc gene in *G. violaceus*. Using our system, other molecular genetic techniques such as transposon tagging and gene targeting will be developed in the future, and the analyses of *G. violaceus* will progress by the novel techniques.

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References

[1] Nelissen B., Van de Peer Y., Wilmotte A., De Wacher R. (1995) An early origin of plastids within the cyanobacterial divergence is suggested by evolutionary trees based on complete 16S rRNA sequences. Mol. Biol. Evol. 12, 1166–1173.
[2] Swingley W.D., Blankenship R.E., Raymond J. (2008) Integrating Markov clustering and molecular phylogenetics to reconstruct the cyanobacterial species tree from conserved protein families. Mol. Biol. Evol. 25, 643–654.
[3] Falcón L.I., Magallón S., Castillo A. (2010) Dating the cyanobacterial ancestor of the chloroplast. ISME J. 4, 777–783.
[4] Gujra R.S., Mathews D.W. (2010) Signature proteins for the major clades of cyanobacteria. BMC Evol. Biol. 10, 24.
[5] Ripka R., Waterbury J., Cohen-Bazire G. (1974) A cyanobacterium which lacks thylakoids. Arch. Microbiol. 100, 419–436.
[6] Nakamura Y., Kaneko T., Sato S., Mimuro M., Miyashita H., Tsuji T. et al. (2003) Complete genome structure of Gloeobacter violaceus PCC 7421, a cyanobacterium that lacks thylakoids. DNA Res. 10, 137–145.
[7] Innate H., Tsuji T., Sato M., Miyashita H., Kameko T., Tabata S. et al. (2004) Unique constitution of photosystem I with a novel subunit in the cyanobacterium *Gloeobacter violaceus* PCC 7421. FEBS Lett. 578, 275–279.
[8] Koyama K., Tsuji T., Akiimoto S., Mikioka Y., Miyashita H., Mimuro M. (2006) New linker proteins in phycobilisomes isolated from the cyanobacterium *Gloeobacter violaceus* PCC 7421. FEBS Lett. 580, 3457–3461.
[9] Sisera C.L., Brown C.M., Cherage O., Vass I., Campbell D.A. (2008) The psaA gene family responds differentially to light and UVB stress in *Gloeobacter violaceus* PCC 7421, a deeply divergent cyanobacterium. Biochim. Biophys. Acta. 1777, 130–139.
[10] Dreher C., Hielscher R., Prodohl A., Hellwig P., Schneider D. (2010) Characterization of two cytochrome b6 proteins from the cyanobacterium *Gloeobacter violaceus* PCC 7421. Photosynt. Res. 106, 247–261.
[11] Mendez-Hernandez G., Perez-Gomez B., Krogmann D.W., Gutierrez-Corlos E.B., Gomez-Lojero C. (2010) Interactions of linker proteins with the phycobiliproteins in the phycobilisome substructures of *Gloeobacter violaceus*. Photosynth. Res. 72, 307–319.
[12] Guglielmi G., Cohen-Bazire G., Bryant D.A. (1981) The structure of *Gloeobacter violaceus* and its phycobilisomes. Arch. Microbiol. 129, 181–189.
[13] Koening. S., Schmidt M. (1995) *Gloeobacter violaceus* – investigation of an unusual photosynthetic bacterium. Abundance of the long wavelength emission of the photosystem I in all 7 K fluorescence spectra. Physiol. Plant. 94, 621–628.
[14] Mangels D., Krupj. B., Söggew N., Römer M., Boekema E.J., Koening F. (2002) *Photobacterium* I from the unusual cyanobacterium *Gloeobacter violaceus*. Photosynth. Res. 72, 307–319.
[15] Rexroth S., Mullineaux C.W., Ellinger J., Sendtke E., Römer M., Koening F. (2011) The plasma membrane of the cyanobacterium *Gloeobacter violaceus* contains segregated bioenergetic domains. Plant Cell. 23, 2379–2390.
[16] Renat C., Schreiher U., Sendtke E., Stadnichuk N., Kreyath S., Römer M. et al. (2012) Unique properties vs. common themes: the atypical cyanobacterium *Gloeobacter violaceus* PCC 7421 is capable of state transitions and blue-light-induced fluorescence quenching. Plant Cell Physiol. 53, 528–542.
[17] Guo H., Xu X. (2004) Broad host range plasmid-based gene transfer system in the cyanobacterium *Gloeobacter violaceus* which lacks thylakoids. Prog. Nat. Sci. 14, 31–35.
[18] Allen M.M. (1968) Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4, 1–4.

Table 2

| Samples         | Background (RLU) | +Luciferin (RLU) |
|-----------------|------------------|-----------------|
| Wild type       | 118 ± 16         | 106 ± 32        |
| pKUT1121 strain | 83 ± 5           | 87 ± 9          |
| pKUT-luc strain | 87 ± 11          | 110437 ± 10431  |

The values represent the averages and standard deviations of triplicate measurements.

* RLU, relative luminescence units.

4. Discussion

Development of the transformation system for *G. violaceus* was already reported by Guo and Xu [17]. They demonstrated the introduction of a RSFI1010 derived plasmid into *G. violaceus* by conjugation. However, we could not reproduce even their culture condition (28–30 °C, 10–20 μmol photons m⁻² s⁻¹). Because *G. violaceus* was reported to be a cyanobacterium that can grow only under the dim light [5], we presumed that *G. violaceus* used by Guo and Xu [17] was adapted to slightly stronger light condition during long cultivation. Since the higher-light adapted *G. violaceus* was not obtained in our experiment, we examined proper conditions suitable for the transformation of *G. violaceus* using our routine culture. Sensitivity of *G. violaceus* to erythromycin and Sm (Table 1) was similar to that described in Guo and Xu [17]. However, sensitivity to ampicillin and chloramphenicol was different from the former result, which demonstrated that ampicillin was effective at the concentration of 15 μg ml⁻¹ and chloramphenicol was ineffective up to 100 μg ml⁻¹ [17]. We newly evaluated the four antibiotics (gentamicin, hygromycin, spectinomycin and zeocin) for *G. violaceus*. Among them, only spectinomycin was effective.

We used expression vectors derived from a broad-host-range plasmid RSFI1010, because *G. violaceus* have no endogenous plasmid [6]. Guo and Xu [17] reported that no transformant was obtained by the transformation of *E. coli* DH5α with DNA prepared from plasmid-introduced *G. violaceus* whereas transformants appeared with high efficiency in the case of a methylation-restriction mutant, DH10B as a host strain. They concluded that the difference in the transformation efficiency reflect the presence of DNA methylation in *G. violaceus*, which caused the restriction by *E. coli*. On the other hand, our results using DH5α and a methylation-restriction mutant, XL1-Blue MRF demonstrated that there is no difference in the transformation efficiency of two strains. Guo and Xu [17] used a vector, pKT210 [25], which is different from our vector, pKTU1121 that was recently constructed [19]. Both vectors are derived from a broad-host-range plasmid RSFI1010, however, pKTU1121 is 3.3 kb smaller than pKT210. Therefore, the transformation efficiency of pKTU1121 might be higher than that of pKT210 for *E. coli*. However, we assumed that the difference is not related to DNA methylation in *G. violaceus* because DH10B was transformed with high efficiency using pKT210 [17]. Hence, this inconsistency also indicated that the properties of *G. violaceus* reported in 2004 was quite different from our strain.

In the transformation of bacteria, restriction-modification system in the cell affects the frequency of transformation. In the case of *Anabaena* sp. PCC 7120, type II DNA restriction-modification system is major barrier against transformation [21]. This problem was overcome by the coexpression of DNA methylases that were associated with type II restriction enzymes in the *E. coli* [21]. Guo and Xu [17] reported that the frequency of transformation of *G. violaceus* raised from 4.63 × 10⁻⁶ to 1.67 × 10⁻² by the coexpression of three DNA methylases that were effective for the transformation of *Anabaena* [26]. In our result, the frequency of transformation of pKTU1121 strain was 1.2 × 10⁻⁴ without coexpression of *Anabaena* DNA methylases. The frequency of transformation of our system is significantly higher than that described in the previous report. Moreover, no genes for type II restriction-modification system was found in the genome of *G. violaceus* [6]. These results indicate that the barrier of the restriction system for transformation is not high in *G. violaceus* unlike *Anabaena*.
[19] Tsuchiya T., Mizoguchi T., Akimoto S., Tomo T., Tamaki H., Mimuro M. (2012) Metabolic engineering of the Chl d-dominated cyanobacterium Acaryochloris marina: production of a novel Chl species by the introduction of the chlorophyllide a oxygenase gene. Plant Cell Physiol. 53, 518–527.

[20] Scholz P., Haring V., Wittmann-Liebold B., Ashman K., Bagdasarian M., Scherzinger E. (1989) Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene. 75, 271–288.

[21] Elhai J., Wolk C.P. (1988) Conjugal transfer of DNA to cyanobacteria. Methods Enzymol. 167, 747–754.

[22] Figurski D.H., Helinski D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA. 76, 1648–1652.

[23] Wilson K. (1997) Preparation of genomic DNA from bacteria. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Current Protocols in Molecular Biology. Hoboken, NJ: John Wiley & Sons, pp. 2.4.1–2.4.5.

[24] Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680–685.

[25] Bagdasarian M., Lurz R., Ruckert B., Franklin F.C.H., Bagdasarian M.M., Frey J. et al. (1981) Specific-purpose plasmid cloning vectors. II. Broad host range. high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. Gene. 16, 237–247.

[26] Elhai J., Vepritskiy A., Muro-Pastor A.M., Flores E., Wolk C.P. (1997) Reduction of conjugal transfer efficiency by three restriction activities of Anabaena sp. strain PCC 7120. J. Bacteriol. 179, 1998–2005.