Broad host range plasmid-based gene transfer system in the cyanobacterium *Gloeobacter violaceus* which lacks thylakoids*

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Abstract *Gloeobacter violaceus*, a cyanobacterium lack of thylakoids, is refractory to genetic manipulations because its cells are enveloped by a thick gelatinous sheath and in colonial form. In this study, a large number of single cells were obtained by repeated pumping with a syringe with the gelatinous sheath removed. And an exogenous broad host range plasmid pKT210 was conjugatively transferred into *G. violaceus*. Analyses with dot-blot hybridization and restriction mapping showed that the exogenous plasmid pKT210 had been introduced into *G. violaceus* and stably maintained with no alteration in its structure. pKT210 extracted from *G. violaceus* exconjugants could be transformed into the *mcr*− *mrr*− *E. coli* strain DH10B but not the *mcr*+ *mrr*+ strain DH5α, which suggests that a methylase system may be present in *G. violaceus*.

Keywords: *Gloeobacter violaceus*, conjugation, pKT210.

Gloeobacter violaceus, a cyanobacterial photoautotroph with a unique cellular structure, was first isolated and purified by Rippka et al. in 1974[1]. Its cells are rod-shaped, with typical cyanobacterial pigments and phycoerythrin, appearing in the characteristic violet color, and 3 ~ 4 fold lower in chlorophyll content. In natural state, the cells are enveloped by a gelatinous sheath and associated in colonial form. *G. violaceus* stands for a minor phylogenetic branch of cyanobacteria. The most significant feature of its cellular structure is the absence of thylakoid membrane that is usually found in other cyanobacteria or chloroplasts in plants, while phycobilisome and photosynthetic apparatus are all located on the cytoplasmic membrane, wherein photosynthesis is performed[2]. For this point, the cyanobacterium is of special value in the study of morphogenesis of thylakoid membrane, an important biological problem. The Kazusa DNA Research Institute in Japan will announce its whole genome sequence in the near future (http://www.kazusa.or.jp/cyano/cyano.html). However, there has been no gene transfer system reported in this cyanobacterium, which seriously impedes the genetic approaches with this special cell model.

So far, gene transfer methods including natural transformation[3], electroporation[4] and conjugation[5] have been widely used in cyanobacteria. Among these methods, conjugation is a process of DNA transfer mediated by a conjugative plasmid and cell contact. It is currently applicable to many cyanobacteria, mainly in filamentous species, and successful applications in unicellular ones have also been documented[6,7]. The gelatinous sheath of *G. violaceus* forms a natural barrier to the entry of exogenous genes into the cells. For this reason, a stable gene transfer system was established in this study, in which the gelatinous sheath was mechanically removed and an exogenous plasmid pKT210[8] was conjugatively transferred into *G. violaceus*. With its replicon derived from the broad host range plasmid RSF1010, this plasmid can be mobilized by the conjugative plasmid RP4 into many gram-negative bateria, including some cyanobacteria.

1 Materials and methods

1.1 Plasmids and strains

All of the plasmids used in this study including pKT210, pRL443, pDS4101 and pRL623 were from Professor Wolk of the DOE Plant Research Laboratory at Michigan State University. *Gloeobacter violaceus* ATCC 29082 (PCC 7421) was from the American Type Culture Collection and free of contaminating bacteria by mechanical treatments and streaking on plates.

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1.2 Culture conditions

The wild type cyanobacterium was cultured in BG11 medium at 28 ~ 30 ℃ in the light (10 ~ 20 $\mu E \cdot m^{-2} \cdot s^{-1}$) without shaking. Selection of exconjugants was performed on solidified BG11 medium containing 5 μg/mL streptomycin. When grown in liquid medium, 2 μg/mL streptomycin and 50 μg/mL kanamycin were supplemented. *E. coli* strains were cultured in LB medium at 37 ℃. The concentration of streptomycin and chloramphenicol used for selection of *E. coli* transformants were 20 μg/mL and 25 μg/mL respectively.

1.3 Pretreatment of *G. violaceus*

G. violaceus at logarithmic phase of growth was collected and subject to pumping with a needle-less syringe under axenic conditions until 80% of the colonies were dispersed into single cells. The cyanobacterial cells were sampled and examined microscopically for the gelatinous sheath after staining with alcian blue[9], a stain for polysaccharide layer.

1.4 Conjugation

The basic procedure was as described by Elhai et al.[10] with slight modifications. Fifty milliliters of cyanobacterial cells were collected and pretreated to remove the gelatinous sheath, washed twice with BG11 and resuspended in 2.5 mL of BG11. At this point, the cell concentration was ca. 3.2×10^8/mL. *E. coli* containing relevant plasmids and *G. violaceus* were mixed and spread on cellulose ester filters overlaid on BG11 plates. After incubation in light for 36 h, the filters were transferred onto the plates containing 5 μg/mL streptomycin for selection of exconjugants.

1.5 DNA manipulations

DNA extraction from *G. violaceus* was performed following the procedure described by Xu et al.[11] except the culture conditions. Extraction, transformation and digestion of the *E. coli* plasmid were performed as standard molecular biology methods.

1.6 Dot blot hybridization

Dig-labeled plasmid pKT210 was used as the probe. The Dig-labeling and detection kit was purchased from Roche of Germany. Labeling, hybridization and immunological detection were according to the manufacturer's protocols[12].

2 Results

2.1 Sensitivity of *G. violaceus* to antibiotics

Because there is no report on the sensitivity of *G. violaceus* to often used antibiotics, before trying to introduce an exogenous plasmid into it, we first tested its growth on plates containing 5 different antibiotics respectively (Table 1). After incubation in light for 30 days, it was found that *G. violaceus* is resistant to 50 μg/mL kanamycin (Km) and 100 μg/mL chloramphenicol (Cm) but sensitive to 2.5 μg/mL streptomycin (Sm), 15 μg/mL ampicillin (Ap) and 1.0 μg/mL erythromycin (Em). In the following research, 5 μg/mL Sm was used to select exconjugants and 50 μg/mL Km was used to remove the remaining *E. coli* donor cells. Because cyanobacterial cells are more sensitive in liquid medium, 2 μg/mL Sm was used instead, when exconjugants were grown in liquid medium.

Table 1. Sensitivity of *G. violaceus* to different antibiotics

Antibiotics	Concentration (μg/mL)					
	0.5	1.0	2.5	5.0	15.0	50.0
Km	+	+	+	+	+	+
Sm	+	+	−	−	−	−
Em	+	−	−	−	−	−
Ap	+	+	+	+	−	−
Cm	1.0	5.0	10.0	20.0	40.0	100.0
	+	+	+	+	+	+

"+" stands for growth at the specified concentration, "−" stands for no growth.

2.2 Conjugative transfer of pKT210 into *G. violaceus*

For no small plasmid detected in *G. violaceus*, the plasmid pKT210 with a broad host range was tested for conjugative transfer. pKT210, carrying a Sm resistance marker and the RSF1010 replicative region, can be transferred between cells under the drive of RP4 or its derivatives (such as pRL443). The Gloeobacter cells are enveloped with a multi-layered gelatinous sheath that prevents cell-to-cell contact between *E. coli* and the cyanobacterium. A method that reduces the gelatinous sheath by increasing EDTA in medium[11] was tested, but showed little effect on this cyanobacterium, nor did sonication treatment. Repeated pumping with a syringe, however, turned more than 80% colonies into single cells, which lacked gelatinous sheath as shown under a microscope and were fit for conjugation (Fig. 1). The mating ex-

periments were divided into 3 groups: (1) *E. coli* HB101 (pRL443 + pRL623) + *E. coli* DH10B (pKT210) + *G. violaceus*; (2) *E. coli* HB101 (pRL443) + *E. coli* HB101 (pDS4101) + *E. coli* DH10B (pKT210) + *G. violaceus*; (3) controls (i) *E. coli* HB101 (pRL443 + pRL623) + *G. violaceus*, (ii) *E. coli* HB101 (pRL443) + *G. violaceus*, (iii) *E. coli HB*101 (pDS4101) + *G. violaceus*, (iv) *E. coli* DH10B (pKT210) + *G. violaceus*. Of these plasmids, pRL623 and pDS4101 are non-essential to the conjugative transfer of pKT210. Use of them was to test whether the methylase genes[10] of pRL623 could increase the conjugation efficiency. The cyanobacterial cells mixed with *E. coli* according to the above combinations were cultured on BG11 plates with 5 μg/mL streptomycin for 30 ~ 40 days. It was found that there were no or very few colonies formed in the controls, while many exconjugants formed in groups (1) and (2) (Table 2). The two groups showed a certain difference in conjugation efficiency, which were 1.67×10^{-5} and 4.63×10^{-6} exconjugants/recipient cell respectively.

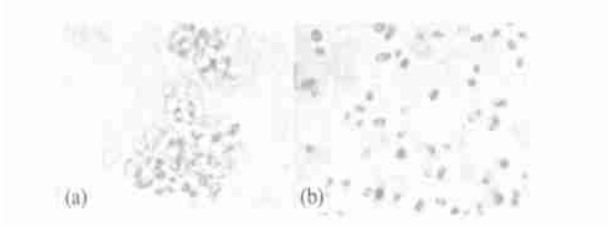

Fig. 1. Microphotographs showing the effect of mechanical treatment on *G. violaceus*. (a) Cells before treatment; (b) cells treated by pumping with a syringe.

Table 2. Conjugative transfer of pKT210 into *G. violaceus*

Groups		Exconjugants/plate	Efficiency (exconjugants/recipient cell)
(1)		267	1.67×10^{-5}
(2)		74	4.63×10^{-6}
(3)	i	0	0
	ii	0	0
	iii	0	0
	iv	0	0

All data are the means of two independent experiments. Totally 20 plates for each of groups (1) and (2), 4 plates for each subgroup of (3).

2.3 Extraction of pKT210 from *G. violaceus* and transformation back into *E. coli*

To verify that pKT210 had been introduced into *G. violaceus*, we inoculated some exconjugants into Sm-containing liquid BG11 medium. Under the same conditions, wild type *G. violaceus* became bleached and dead after 20 ~ 30 days, while the monoclonal exconjugants were able to grow. When the exconjugant cells were transferred to the medium containing Km and Sm to continue growing and selected for the co-existence of *E. coli* donor cells, the culture was eventually free of the donor cells as shown by the tests on LB plates after washing with BG11 medium. The crude plasmid DNA was then extracted from the exconjugant cells of *G. violaceus* and used to transform *E. coli* DH5α and DH10B respectively. We found that the former produced no transformants and the latter showed a high efficiency of 2.2×10^{3} transformants/μgDNA. Transformation of *E. coli* DH5α and DH10B with DNA from wild type *G. violaceus* resulted in no transformants. As the control, pKT210 from *E. coli* DH5α was used to transform DH5α and DH10B, resulting in 4.8×10^{5} and 4.3×10^{5} transformants/μg DNA respectively, which are virtually of no difference to each other.

2.4 Restriction analysis and dot blot hybridization

The original plasmid and the plasmid pKT210 recovered from *G. violaceus* by transformation back into *E. coli* were analyzed by restriction with *Nco*I or *Pst*I + *EcoR*I. They demonstrated an identical pattern in restriction mapping, suggesting that pKT210 in *G. violaceus* was not changed in its structure (Fig. 2). The total DNA of wild type *G. violaceus* and an exconjugant were probed with Dig-labeled pKT210 in dot-blot hybridization. The former showed a negative signal, while the latter was positive (Fig. 3). Southern blot hybridization also con-

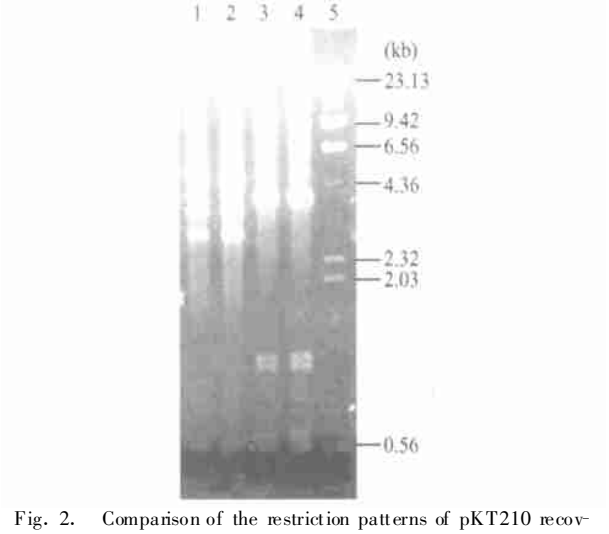

Fig. 2. Comparison of the restriction patterns of pKT210 recovered from *G. violaceus* and the original pKT210. Lane 1, pKT210 recovered from *G. violaceus* by transformation back into *E. coli* cut with *Pst*I + *EcoR*V; lane 2, the orginal pKT210 cut with *Pst*I + *EcoR*V; lane 3, the recovered pKT210 cut with *Nco*I; lane 4, the original pKT210 cut with *Nco*I; lane 5, DNA maker.

firmed that pKT210 had been transferred into *G. violaceus* (data not shown).

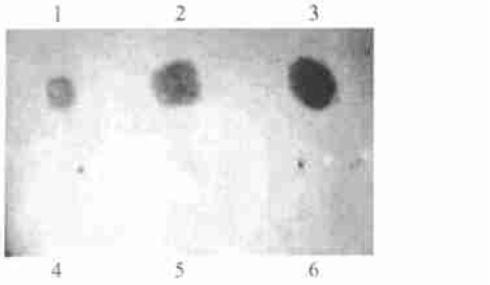

Fig. 3. Dot blot hybridization using pKT210 as the probe. 1 ~ 3, 50 ng, 100 ng and 200 ng of total DNA from *G. violaceus* exconjugants respectively; 4 ~ 6, 50 ng, 100 ng and 200 ng of total DNA from wild type *G. violaceus* respectively.

3 Discussion

Establishment of a gene transfer system in *G. violaceus* was confronted with difficulties in several aspects: (1) it is of gelatinous sheath, which forms the physical barrier to the entry of exogenous DNA[13]; (2) the cells are mainly in colonial form and of poor plating efficiency; (3) it grows slowly with the mean doubling time of ca. 73 h. To remove the gelatinous sheath of *G. violaceus*, we tried raising EDTA concentration in medium, or sonication and other methods, but all failed to solve this problem. In the end, using repeated pumping with a syringe we broke ca. 80% of the colonies into single cells and removed their gelatinous sheath. Consequently, the plating efficiency of the cyanobacterial cells was improved, and the cell-to-cell contact between *E. coli* and cyanobacteria became feasible. It should be noted that the gelatinous sheath of this cyanobacterium is notably regenerative; such treated cells should be applied to conjugative transfer immediately. In this study, the plasmid pKT210 with a broad host range was successfully introduced into *G. violaceus*, exconjugants were produced and the recovered pKT210 was shown to be unchanged in structure. These results indicate that the RSF1010 replicon works in *G. violaceus* also. This is the first report of a gene transfer system in *G. violaceus*, which opens a window for genetic manipulations with this special model.

Currently, genetically amenable cyanobacteria are only of a limited number, basically a handful of model species from several genus. Conjugation was first applied to filamentous cyanobacteria, later on to some unicellular species. In cyanobacterial cells, there are often restriction enzymes that may reduce the conjugation efficiency or completely block the formation of exconjugants. The conjugation efficiency of groups (1) and (2) of our research showed a certain difference, which was supported by t-test ($p < 0.01$). However, the difference was only in a range of 3 ~ 4 fold, which may not necessarily imply the presence of restriction enzymes or the protective effect of methylases encoded by pRL623.

Kreps et al. found that plasmid DNA extracted from cyanobacteria, being methylated, could not be transformed into $mcrA^+$ host strain *E. coli* HB101[6]. In some *E. coli* strains, there are Mcr and Mrr restriction systems that specifically cut methylated DNA. The McrA restriction enzyme recognizes DNA with the sequence 5′-C^{me}CGG-3′, McrBC recognizes DNA containing 5′-R^{me} C-3′[14, 15], while Mrr restricts DNA containing N6-methyl-adenine[16, 17]. The presence of these restriction systems prevents *E. coli* DH5α (mcr^+ mrr^+) from being transformed by certain methylated plasmid DNA. In *E. coli* DH10B, however, the *mcr* and *mrr* genes are deficient in the genome, so there is no such restriction barrier. The fact that the plasmid DNA from *G. violaceus* could only be transformed into DH10B but not into DH5α probably implies the presence of a methylase system in this cyanobacterium.

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