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Expression of organophosphorus-degradation gene (*opd*) in aggregating and non-aggregating filamentous nitrogen-fixing cyanobacteria*

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Abstract Genetic engineering in filamentous N_2-fixing cyanobacteria usually involves *Anabaena* sp. PCC 7120 and several other non-aggregating species. Mass culture and harvest of such species are more energy consuming relative to aggregating species. To establish a gene transfer system for aggregating species, we tested many species of *Anabaena* and *Nostoc*, and identified *Nostoc muscorum* FACHB244 as a species that can be genetically manipulated using the conjugative gene transfer system. To promote biodegradation of organophosphorus pollutants in aquatic environments, we introduced a plasmid containing the organophosphorus-degradation gene (*opd*) into *Anabaena* sp. PCC 7120 and *Nostoc muscorum* FACHB244 by conjugation. The *opd* gene was driven by a strong promoter, P_{psbA}. From both species, we obtained transgenic strains having organophosphorus-degradation activities. At 25°C, the whole-cell activities of the transgenic *Anabaena* and *Nostoc* strains were 0.163±0.001 and 0.289±0.042 unit/μg Chl *a*, respectively. However, most colonies resulting from the gene transfer showed no activity. PCR and DNA sequencing revealed deletions or rearrangements in the plasmid in some of the colonies. Expression of the green fluorescent protein gene from the same promoter in *Anabaena* sp. PCC 7120 showed similar results. These results suggest that there is the potential to promote the degradation of organophosphorus pollutants with transgenic cyanobacteria and that selection of high-expression transgenic colonies is important for genetic engineering of *Anabaena* and *Nostoc* species. For the first time, we established a gene transfer and expression system in an aggregating filamentous N_2-fixing cyanobacterium. The genetic manipulation system of *Nostoc muscorum* FACHB244 could be utilized in the elimination of pollutants and large-scale production of valuable proteins or metabolites.

Keyword: cyanobacteria; aggregating species; conjugative gene transfer; organophosphorus-degradation

1 INTRODUCTION

A phosphotriesterase in bacterium *Pseudomonas diminuta* MG (McDaniel et al., 1988) or *Flavobacterium* sp. ATCC27551 (Murbry et al., 1986; Murbry et al., 1989) can effectively hydrolyze a group of organophosphate insecticides, and the encoding gene *opd* (organophosphorus degradation) is located on a large plasmid of both two bacterial strains (Harper et al., 1988). The enzyme shows activity over a wide range of temperature and can hydrolyze diverse substrates (Munnecke, 1976); therefore, the phosphotriesterase could be applied in a variety of environments to eliminate organophosphate pollutants.

Based on a conjugative gene transfer system (Elhai et al., 1988), a few filamentous N_2-fixing cyanobacteria have been used as hosts to express foreign genes. Genetically engineered cyanobacteria have been suggested for mosquito control or pollutant elimination. For example, *Anabaena* sp. PCC 7120 (hereafter referred to as *Anabaena* 7120) expressing crystal protein genes from *Bacillus sphaericus* or *Bacillus thuringiensis* showed high toxicities against mosquito larvae, providing a tool

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for persistent mosquito control (Xu et al., 1993; Wu et al., 1997; Manasherob et al., 2003). The same cyanobacterium expressing *linA* from *Pseudomonas paucimobilis* or *fcbABC* from *Arthrobacter globiformis*, showed enhanced dechlorination of hexachlorocyclohexane and 4-chlorobenzoate (Kuritz et al., 1995). Thus, transgenic cyanobacteria might be applied to promote the degradation of chlorinated pollutants in aquatic environments. Transgenic *Anabaena* was also suggested for the elimination of heavy metals from polluted water (Shao et al., 2002). However, all the above-cited studies used *Anabaena* sp. 7120 or *Nostoc ellipsosporum*, which are both non-aggregating species. To date, only aggregating *Anabaena* or *Nostoc* species have demonstrated large-scale production with plastic-covered shallow ponds (Shen et al., 1993). In particular, harvesting of non-aggregating species is consumes much more energy than that of aggregating species. In this study, we first introduced *opd* into *Anabaena* 7120 and obtained recombinant cells with high Opd activity. Then, we tested many aggregating species of *Anabaena* and *Nostoc* and identified *Nostoc muscorum* FACHB244 (hereafter referred to as *Nostoc* 244) as a strain that can be genetically manipulated using the conjugative gene transfer system (Elhai et al., 1988). Introduction of *opd* into *Nostoc* 244 also resulted in recombinant cells with Opd activity.

2 MATERIAL AND METHOD

2.1 Strains, culture conditions and conjugative gene transfer

Flavobacterium sp. ATCC27551 was purchased from ATCC (American Typical Culture Collection, USA), *Anabaena* sp. PCC 7120 and *Nostoc muscorum* FACHB 244 were from FACHB (Freshwater Algae Culture Collection at Institute of Hydrobiology, China). *Flavobacterium* sp. was cultured at 30°C in nutrient broth medium (McDaniel et al., 1988); *Anabaena* 7120 and *Nostoc* 244 were grown in BG11 medium (Stanier et al., 1971) at 30°C in the light of 30 μE m^{-2} s^{-1}. For recombinant strains, a final concentration of 20 μg ml^{-1} neomycin was supplemented in the medium.

Conjugative gene transfer from *Escherichia coli* to *Anabaena* or *Nostoc* species was conducted as previously described (Elhai et al., 1988; Elhai et al., 1997). For *Nostoc* 244, the aggregates were sequentially dispersed into separate filaments using a 5-ml and a 1-ml glass tissue homogenizer. A suspension of filaments was collected and used in the conjugation with *E. coli* HB101 (pRL443+pRL623+ pHB303), in which pRL443 was a conjugative plasmid, pRL623 was a helper plasmid, and pHB303 was the cargo plasmid.

2.2 DNA manipulations

Molecular manipulations were performed according to standard protocols. Enzymes were used according to the instructions provided by the manufacturers. The sticky ends of DNA fragments were blunted using T4 DNA polymerase (Promega, USA). Restriction enzymes and T4 DNA ligase were purchased from Takara (Dalian, China).

PCR mixtures consisted of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 μmol/L primers, 50 μmol/L dNTPs, 50 ng of template DNA, and 2.5 U Taq DNA Polymerase (MBI Fermentas). PCR reactions comprised initiation at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; and a final extension of 6min at 72°C. PCR products were purified by recovery from agarose gels using a glass milk kit (MBI Fermentas).

The *opd* gene from *Flavobacterium* sp. ATCC27551 (NCBI GenBank accession no. M22863) was cloned by PCR using primers opd-1 (5'-caggatccgaggtggcagcatgcaaacgagaagg-3') and opd-3 (5'-tggaattcatgacgcccgcaaggtc-3'). A BamHI restriction site and a ribosome-binding site were incorporated into the opd-1 primer, while an EcoRI site was incorporated into opd-3. The purified PCR product was digested with BamHI and EcoRI and cloned between BamHI and EcoRI sites in pUC19, resulting in pHB300. The cloned PCR product was confirmed by sequencing. *opd* was excised from pHB300 with EcoRI and BamHI and placed downstream of P_{psbA} in pRL439 (Elhai, 1993), resulting in pHB302. The P_{psbA}-*opd* fragment was excised with PstI and EcoRI from pHB302, blunted with T4 DNA polymerase, and cloned into SalI-digested and T4-DNA polymerase-blunted pDC8 (Xu et al., 1993), resulting in pHB303 (Fig.1).

The green fluorescent protein gene, *gfp*, was excised from pRL2378 with SmaI and EcoRI, and inserted into pRL439 downstream of P_{psbA} between SmaI and EcoRI, resulting in pHB1115. Plasmid pRL2378 contained the *gfp* gene used for construction of pRL2391a and pRL2392a (Xu et al., 2001). P_{psbA}-*gfp* was excised from pHB1115 with PstI, blunted with T4 DNA polymerase, redigested with EcoRI, and cloned into pDC8, which was cut with BamHI, blunted with T4 DNA polymerase, and redigested with EcoRI, resulting in pHB1153.

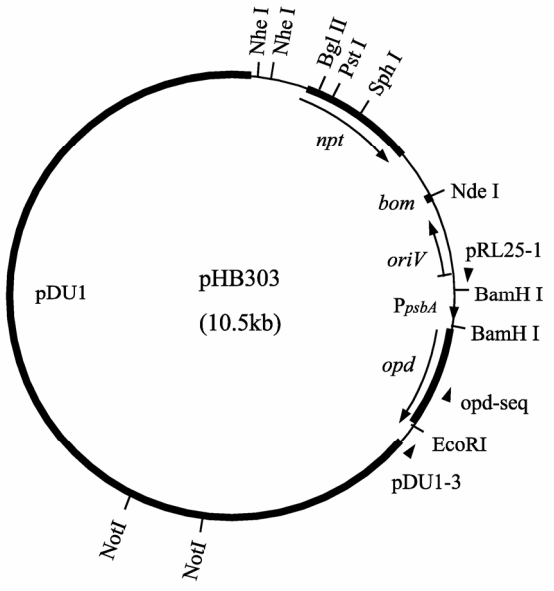

Fig.1 Structure of pHB303

Arrows denoted with pRL25-1, opd-seq and pDU1-3 stand for relative positions of sequencing primers

2.3 Organophosphorus degradation (Opd) activity assay

E. coli DH5α and *Anabaena* 7120 were collected by centrifugation; *Nostoc* 244 was dispersed with a tissue homogenizer to generate a suspension of filaments. The collected cells were washed twice with 10 mmol/L Tris-HCl+1 mmol/L EDTA (pH 8.0) and re-suspended in 10 mmol/L Tris-HCl (pH 8.0). For assays using cell extracts, cyanobacterial cells were disrupted by sonication (10 rounds of 30 s) on ice and centrifuged at 13 000 *g* for 10 min to remove intact cells and cell debris. Opd activity was assayed in 10 mmol/L Tris-HCl (pH 8.0) containing 130 μmol/L paraoxon; the reaction buffer was pre-adjusted to the indicated temperatures in a water bath. One hundred microliters of cell suspension or extracts were added to 1.5 ml of reaction buffer. After incubation at indicated temperature for 20 min, the reactions were stopped by centrifugation at 13 000 *g* for 30 sec, and the produced *p*-nitrophenol in the supernatant was quantified according to the absorbance at 400 nm. *E. coli* or cyanobacterial cells without the *opd* gene were used as negative controls. The Opd activity was calculated as unit (nmol *p*-nitrophenol produced/min)/OD_{600} (for *E. coli*) or unit/μg Chl *a* (for cyanobacteria). Chl *a* was extracted with methanol and measured as previously described (Lichtenthaler, 1987).

2.4 Microscopy

Fluorescence and bright-field images were captured through an Olympus BX41 microscope with a JVC3 charge-coupled device (CCD) color video camera (TK-C1381) (Victor Company of Japan, Limited), assisted with the software 10 moons-2 000/PRO (V4.5). Images for GFP expression were visualized using a Sapphire GFP filter set (Chroma Technology Corp. [Brattle-boro, Vt.] Exciter D395/440, dichroic 425DCLP, and emitter D510/540).

3 RESULT AND DISCUSSION

3.1 Expression of *opd* in *Anabaena* 7120

We cloned *opd* from *Flavobacterium* sp. using PCR and positioned the *opd* gene under the control of P_{psbA} in a pDU1-based shuttle vector pDC8 (Xu et al., 1993), resulting in the plasmid pHB303 (Fig.1). The cloned *opd* gene was confirmed by sequencing. P_{psbA} is the promoter of *psbA* in spinach chloroplasts, which is also very active in *Escherichia coli* and cyanobacteria (Elhai, 1993). pDU1 is a plasmid initially found in a strain of *Nostoc muscorum*. Hybrid plasmids carrying pDU1 can replicate in *Anabaena* and *Nostoc* species. *E. coli* DH5α cells harboring pHB303 can rapidly degrade paraoxon. At 25°C, the recombinant *E. coli* showed organophosphorus degradation (Opd) activity of 18.0±0.3 unit/OD_{600}.

Plasmid pHB303 was then transferred by conjugation into *Anabaena* 7120. Twenty exconjugants were cultured and assayed for Opd activity. Using paraoxon as the substrate, one of the exconjugants showed activity of 0.163±0.001 unit/μg Chl *a* at 25°C (Opd^+), while the rest showed no activity (Opd^-). Different batches of conjugative transfer experiments produced similar results: only a small proportion of exconjugants were active in organophosphorus degradation. The Opd activity was also assayed with cell extracts. Cell extracts were 10 fold more active than intact cells, attaining 1.84±0.04 unit/μg Chl *a* at 25°C. Opd activity was also detected with intact cells or extracts at 4°C and 40°C, and showed increasing activity with the temperature from 4°C to 40°C (Fig.2). Opd activity was also detectable in the medium of old cultures. However, after washing and resuspending with fresh medium, the Opd activity was not found in the medium within 4 hours (data not shown). The Opd enzyme should be located within cells, but might be very slowly released into the medium due to spontaneous cell lysis. These results indicate that *opd* can be expressed in *Anabaena* 7120 from P_{psbA} and might provide sufficient Opd activity in recombinant cyanobacteria for practical uses.

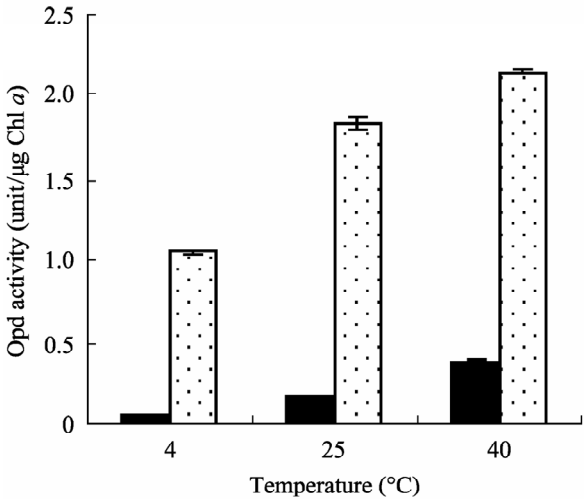

Fig.2 Activity of an Opd$^+$ strain of *Anabeana* 7120 (pHB303)
Dotted bars, cell extracts; solid bars, intact cells

3.2 Expression of *opd* in *Nostoc* 244

To identify an aggregating N_2-fixing species that can be genetically manipulated, we tested over 20 strains of aggregating *Anabaena* and *Nostoc* from the Freshwater Algal Culture Collection at Institute of Hydrobiology (FACHB), and identified *Nostoc* 244 as such a strain. To perform conjugation with an aggregating species, we manually homogenized the cyanobacterial clumps to prepare a suspension of filaments, and used the filaments in conjugation experiments with *E. coli*. With the conjugative plasmid pRL443 and the helper plasmid pRL623 (Elhai et al., 1997), pHB303 was successfully transferred from *E. coli* to *Nostoc* 244. Under our conditions, each plate produced 150–400 exconjugants. The exconjugants initially formed single colonies and later released filaments that spread out. At the stage that single colonies were separate from each other, 20 exconjugants were picked up and cultured. Again, only one colony was able to degrade paraoxon. At 25°C, intact Opd$^+$ cells showed activity of 0.289±0.042 unit/μg Chl *a*. Different batches of conjugation experiments also showed that only a small proportion of the exconjugants possessed the Opd activity. The Opd$^+$ strains retained their aggregating abilities, and the filament clumps floating in the culture could be collected without centrifugation. It has been reported that *Synechococcus* sp. PCC 7942 expressing *opd* possessed a very high whole-cell activity (Chungjatupornchai et al., 2008). In our study, the genetically engineered strain of aggregating N_2-fixing cyanobacterium showed high whole-cell Opd activity. The Opd$^+$ *Nostoc* strain could potentially be produced on a large scale in shallow ponds.

3.3 Analyses of P_{psbA}-*opd* in Opd$^-$ strains of *Anabaena* 7120 (pHB303) and *Nostoc* 244 (pHB303)

To find out why most exconjugants showed no Opd activity, we examined some of them by PCR and sequencing. PCR was performed using primers pRL25-1 (5′-ggtctgacgctcagtggaac-3′) and pDU1-3 (5′-gactacctcgttaggcagcaa-3′) (Fig.1) to detect the P_{psbA}-*opd* region. Fig.3a shows the PCR products of eight Opd$^-$ exconjugants (lanes 1-8) and one Opd$^+$ exconjugant (lane 9) of *Anabaena* 7120. Of the eight Opd$^-$ exconjugants, one showed an altered band (lane 7) compared with the Opd$^+$ control (lane 10), four showed no or very faint bands (lanes 1, 2, 4 and 6), and three showed a band of the same size (lanes 3, 5 and 8) as the control. Sequencing of the latter three PCR products with primers pRL25-1, pDU1-3 and opd-seq (5′-ctcaagccttcggactcaa-3′) (Fig.1) showed that one had a 14-bp deletion between P_{psbA} and *opd*, while the other two had no mutation in this region. We also analyzed the exconjugants of *Nostoc* 244 (Fig.3b). Of seven Opd$^-$ exconjugants (lanes 1-3 and 5-8), two showed altered PCR bands (lanes 7 and 8), while the remaining five showed no difference from the control. Sequencing of the P_{psbA}-*opd* region showed no mutations. Therefore, the Opd$^-$ strains can be classified into two types: I) those with mutations and II) those without mutations, which, in this study, can be either deletions or rearrangements within the plasmid.

3.4 The expression of P_{psbA}-*gfp* in *Anabaena* 7120

Expression of certain exogenous genes from a strong promoter in cyanobacteria can exert physiological stress on the host cells, and mutations that inactivate or silence the gene's expression should alleviate the stress. The relatively high frequency of mutation upon expressing P_{psbA}-*opd* in *Anabaena* and *Nostoc* species might reflect the stress caused by overexpression of *opd* in cyanobacetria. For those Opd$^-$ exconjugants with no mutation, there could be other genetic or non-genetic alterations in the cyanobacteria that silenced the *opd* activity.

We also tested the expression of green fluorescent protein gene (*gfp*) from P_{psbA} in *Anabaena* 7120. Plasmid pHB1 153 harboring P_{psbA}-*gfp* was introduced into *Anabaena* 7120. Among cell lines derived from eight exconjugants, one showed a strong GFP signal in all cells, while the other seven showed mosaic or no expression of GFP in filaments (Fig.4). Therefore, the difference in expression from a strong promoter among exconjugants is not unique to the organophosphorus degradation gene.

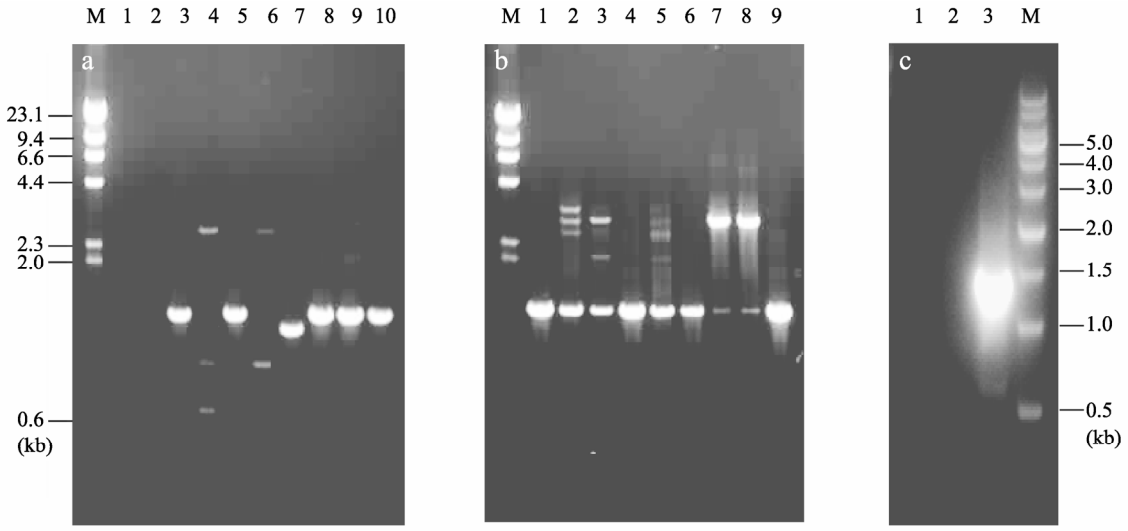

Fig.3 PCR examination of P_{psbA}-*opd* in *Anabaena* 7120 and *Nostoc* 244 transformed with pHB303

PCR primers used were pRL25-1 and pDU1-3; the positive control was the PCR product generated using pHB303 as the template; molecular weight markers (M) were fragments of lambda DNA cut with HindIII (a and b) or 0.5-kb ladders (c); Electrophoresis was performed with 0.7% agarose gel and 0.5×TAE buffer

a. *Anabaena* 7120 transformed with pHB303. Lanes 1-4 and 6-9, Opd^- strains; lane 5, an Opd^+ strain; lane 10, positive control; b. *Nostoc* 244 transformed with pHB303. Lanes 1-3 and 5-8, Opd^- strains; lane 4, an Opd^+ strain; lane 9, the positive control; c. Lane 1, *Anabaena* 7120; lane 2, *Nostoc* 244; lane 3, positive control

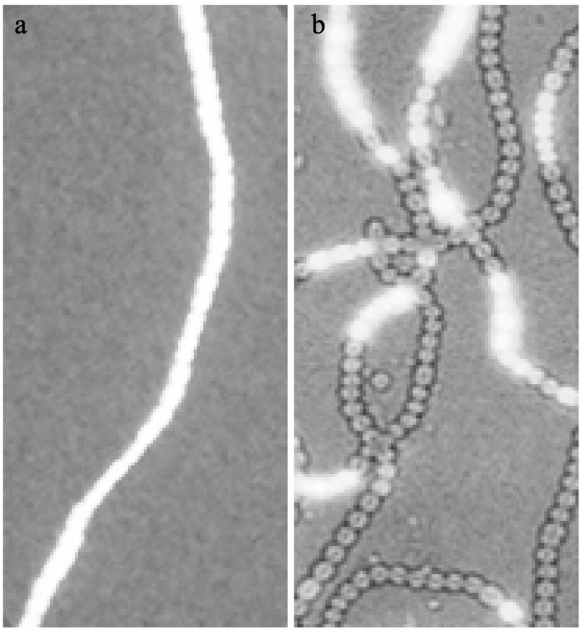

Fig.4 GFP fluorescence in two cell lines of *Anabaena* 7120 (pHB1153)

An image of GFP-based fluorescence is superimposed upon the bright-field image; a. Cell line showing GFP fluorescence in all cells; b. one of seven cell lines that showed GFP fluorescence in a small proportion of cells

Our results suggest that in genetic engineering of filamentous N_2-fixing cyanobacteria using a strong promoter, screening of many exconjugants is necessary to obtain highly expressing strains. Cyanobacteria possess multiple genome copies per cell. Recently, we re-sequenced the whole genome of *Anabaena* 7120, and found many point mutations in different copies of the genome (data not shown). It merits further investigation whether the difference of gene expression from P_{psbA} is due to different combinations of mutations in the genomes of different exconjugants.

4 CONCLUSION

In this study, we introduced P_{psbA}-*opd* into *Anabaena* 7120 and the aggregating strain, *Nostoc* 244. While some exconjugants showed high Opd activities, most showed no organophosphorus degradation activity. Expression of *gfp* from P_{psbA} in *Anabaena* sp. PCC 7120 showed similar results. For the first time, a gene transfer system was established in an aggregating filamentous N_2-fixing cyanobacterium. The high Opd activity of the selected cell lines revealed the potential to eliminate organophosphate pollutants with transgenic *Anabaena* or *Nostoc* species. In particular, our results clearly indicated that a population of transgenic cells of *Anabaena* or *Nostoc* should be screened to obtain high-expression strains.

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