Journal of Environmental Sciences Vol.15, No.5, pp.590—594, 2003 ISSN 1001—0742 CN11—2629/X

Article ID: 1001-0742(2003)05-0590-05 **CLC number**: X17 **Document code**: A

Analysis of parental strain DNA fragments existing in GEMs-Fhhh

HAO Chun-bo[1], YAN Jun[1], QU Meng-meng[1], WANG Dong[1], CHENG Shu-pei[1]*, GU Ji-dong[2], QIU Wan-fei[2], WANG Yin-yin[2]

(1. The National Key Laboratory of Pollution Control and Resource Reuse, School of Environmental Science, Nanjing University, Nanjing 210093, China. E-mail: chengsp@nju.edu.cn; 2. Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong, China)

Abstract: There were 6 target DNA fragments of the three parental strains existing in the cell of GEMs(genetically engineered microorganism strain) Fhhh measured in this research by PCR(polymerase chain reaction). The determination showed that GEMs Fhhh contained all the 6 target DNA fragments, *mnp*1, *mnp*2, *lip*1, *lip*2, *FLO*1 and 16S rDNA, and had the molecular genetic stability. Meanwhile the PCR production of each parental strain could only had its target DNA fragments and was different from each other. It may illustrate that the technique of the inter-kingdom protoplast fusion for the construction of GEMs Fhhh through the process of intercellular gene recombination could be used as a reliable bioengineering technique to create the specific functional stain for the pollution control.

Keywords: GEMs; protoplast fusion; target gene; DNA fragment; PCR; pollution control

Introduction

Using protoplast fusion technique to create new strains has made great achievements in the range of medicine, food, fermentation and agriculture, and approximately 1000 patents of them published(Suzuki, 1996; Vazquez, 1997; Zanke, 1997; Treeradakorn, 1998; Collonnier, 2001). But still now there is no patent reported in pollution control. Recently in China, the research of constructing GEMs by protoplast fusion for the wastewater treatment is in an ascending phase(Xu, 2001; Cheng, 2002).

Fhhh was a GEMs(genetically engineered microorganism strain) constructed by the inter-kingdom protoplast fusion of the three parental strains. The three parental strains were PC (*Phanerochaete chrysosporium*, eukaryote cell); SC(*Saccharomyces cerevisiae*, eukaryote cell) and the native bacterium YZ1(prokaryote cell). GEMs Fhhh could integrate the potentials of its three parental strains for wastewater treatment, which were the high degradation ability from the parental strain PC(Barr, 1994; Bogan, 1996), the high adaptability from the parental strain YZ1 and the high flocculation ability from the parental strain SC(Watari, 1994). The potentials of GEMs Fhhh for the degradation of PTA(purified terephthalic acid) wastewater and the regulation of the transcription and expression of GEMs Fhhh genes for enhancing degradation effective have been reported(Zhong, 2000; Yan, 2001; Cheng, 2002).

The 6 DNA fragments from the three parental strains existing in the cell of GEMs Fhhh were demonstrated by PCR (polymerase chain reaction) reported in this paper. Among the 6 target DNA fragments, 4 of them are *mnp*1, *mnp*2, *lip*1 and *lip*2 gene from PC with the high degradability for purification of hazardous organic pollutants(Godfrey, 1990; Naidou, 1992); the gene *FLO*1 is from SC with high flocculation(Teunissen, 1993) and 16S rDNA is a genetic indicator for all bacteria including YZ1 bacterium(Griffiths, 2000). Synthesis of the 6 pairs premiers according to the sequences of the 6 target DNA fragments, extraction of the chromosome DNA(Ch-DNA) of Fhhh and its three parental strains as the templates, the result of PCR showed that Fhhh contained the total 6 DNA fragments of its three parental strains simultaneously after isolating and purifying of over 700 generations, and each parental strain could only have its own DNA fragments.

Foundation item: The National 863 Hi-Tech Foundation of China(No.2001AA214191) and NSF of Jiangsu Province(No.BK-99033) and the China Environmental Protection Company of Hong Kong; * Corresponding author

The result indicated that the inter-kingdom protoplast fusion technique is an effective bioengineering technique for constructing GEMs by *in vivo* gene recombination. It can overcome the difficulties of genetic barriers and expand the range of gene communication and integration.

1 Materials and methods

1.1 Strain

Fhhh was a GEMs constructed by our research group. It was isolated and incubated over 700 generations. Prof. Wang provided the first parental strain PC, *Phanerochaete chrysosporium*. The second parental strain, the native bacterium YZ1, was isolated from the activated sludge of PTA wastewater treatment plant of NJYZ. And the third parental strain SC, *Saccharomyces cerevisiae*, was the production of Dongguan Sugar Mill of Guangdong Province.

1.2 Media

The liquor medium(LM) of 1000 ml contained the juice of 200 g potato, 20 g glucose and distilled water, with pH 6 - 9, and autoclaved at 121℃ under 100 kPa for 15 min. The solid medium was LM + 1.5% agar.

1.3 Equipments

Incubation shaker: G25-KLC, New Brunswick Scientific. Co, USA; gel imaging system: TSTC GIS-7000 gel imaging system, TSTC Shanghai, China; electrophoresis instrument: DY-602V, Nanda Company, NJU, China; Microscope: Nikon E600, Japan; digital camera: DXM-1200, Japan; PCR authorized thermal cycler: Mastercycler Personal. Eppendorf, Germany.

1.4 PCR

DNA marker: 26—501 bp, pUC19 DNA/*Msp*1(*Hpa* Ⅱ), MBI Fermentas, USA; 100—3000 bp, GeneRulerTM 100 bp DNA Ladder Plus, MBI Fermentas, USA.

PCR kit: Bioengineering Company, Shanghai, China.

Template: After putting 1 ml strain liquor of SC, PC, YZ1 and Fhhh in each Eppendorf tube, centrifuged at 10000 r/min for 10 min, suspended the cells with STE buffer, the cell walls of SC, PC and Fhhh were fractured with snail enzyme and those of YZ1 bacterium were fractured with lysozyme at 37℃ for 1 h. The reaction for releasing the Ch-DNA of the cells with SDS(sodium dodecyl sulfate) was conducted at 60℃, then the samples were kept on blue ice for 0.5 h, centrifuged at 10000 r/min for 10 min. CH_3CONH_4 was used to remove the protein from the supernatant, 95% of cold alcohol was used to set down Ch-DNA as the DNA template. The DNA templates of the 4 strains obtained were suspended in each 20 μl of double distilled water for PCR.

Primers: There were 6 pairs of primers synthesized for PCR of the 6 DNA fragments shown in Table 1 (Godfrey, 1990; Naidou. 1992; Teunssion, 1992; Griffiths, 2000).

Table 1 The designation and synthesis of DNA sequences for 6 pairs of primers in PCR

Targeted DNA	Primer number	DNA sequences of primers	References	Synthesis institutions
*mnp*1	Primer 1	5'-ATG GGA GTA GCG GAA GCA-3'	Godfrey, 1990	PPI, Shanghai
	Primer 2	3'-CGA GTG CGG TGA AGA GTA-5'	Godfrey, 1990	PPI, Shanghai
*mnp*2	Primer 1	5'-ATG TAG GCA GAC TGC AAG-3'	Godfrey, 1990	PPI, Shanghai
	Primer 2	3'-GAG GTG CTG CTC AAG GTA-5'	Godfrey, 1990	PPI, Shanghai
*lip*1	Primer 1	5'-ATG TCG TCG AGA AGC GTC-3'	Naidou, 1992	PPI, Shanghai
	Primer 2	3'-ACA TCG GTC TCG ACG GTA-5'	Naidou, 1992	PPI, Shanghai
*lip*2	Primer 1	5'-ATG AAG CGA CCA CGA CAG-3'	Naidou, 1992	PPI, Shanghai
	Primer 2	3'-CGA CTC TCA CCA CTC GTA-5'	Naidou, 1992	PPI, Shanghai
*FLO*1	Primer 1	5'-CGG AAT TCC TCC AAC TAC TG-3'	Teunissen, 1993	BEC, Shanghai
	Primer 2	3'-CAG AAG CGC AGG CTT AAG GC-5'	Teunissen, 1993	BEC, Shanghai
16S rDNA	Primer 1	5'-GGT TAC CTT GTT ACG ACT T-3'	Griffiths, 2000	BEC, Shanghai
	Primer 2	3'-GAC TCG GTC CTA GTT TGG AG-5'	Griffiths, 2000	BEC, Shanghai

Polymerase chain reaction: The amplification of the 6 DNA fragments needed 6 PCR. In each PCR, the DNA templates were predenaturated at 95℃ for 10 min before the formal reaction. One cycle of the PCR was conducted with denaturation of 1 min at 93℃, renaturation of 1 min at 50℃ and extension of 2 min at 72℃. The total process of the PCR for each target DNA fragment was 35 cycles, and the extension time of the 35th cycle was 10 min and stop at −20℃. The system of the PCR was 50 μl reaction volume, which included double distilled-water, dNTPs, 10 × PCR buffer, primers and DNA template (Cheng, 1995).

Electrophoresis: The PCR productions were separated by horizontal electrophoresis on 1.5% agarose gel with 0.5 μg/ml ethidium bromide(EB) in TBE buffer with 5 V/cm for 2.5 h. After that, the agar was visualized and screened with the Gel Photo System GIS-7000.

1.5 Cell morphology

Dyeing: The cells of YZ1 and mycelia of PC were dyed with crystal violet, and that of SC and Fhhh with methyl blue(Zhu, 1994).

Photo taken: All the cells were taken their photos with microscope Nikon E600 and digital camera DXM-1200, and magnified 1000 fold.

2 Results and discussion

2.1 Comparison of PCR productions between Fhhh and its three parental strains

The results of PCR of the 6 targets DNA fragments between Fhhh and its three parental strains are shown in Table 2.

The PCR productions of Fhhh had all of the 6 target DNA fragments of its three parental strains. But that of the three parental strains only contained the target DNA fragments of their own, and they were distinctly different from each other as shown in Table 2.

Fhhh and PC had two equal bands of DNA MW for *mnp*1 gene; one similar band of DNA MW for *mnp*2 gene; two close bands of DNA MW for *lip*1 gene and one close band of DNA MW for *lip*2 gene. The four DNA fragments of *mnp*1, *mnp*2, *lip*1 and *lip*2 existing in Fhhh could only be from its parental strain PC, because the other two parental strains, SC and YZ1, did not contain the four DNA fragments(Table 2).

Table 2 DNA-MW (bp) of the equal/similar bands between Fhhh and parental strains

Strain	Fhhh	PC	SC	YZ1
*mnp*1	48.54	48.54	No	No
*mnp*1	51.36	51.36	No	No
*mnp*2	114.64	113.2	No	No
*lip*1	29.53	31.03	No	No
*lip*1	46.02	44.9	No	No
*lip*2	108.81	106.21	No	No
*FLO*1	11.58	No	11.75	No
16S rDNA	32.55	No	No	28.51
16S rDNA	73.71	No	No	70.53

Notes: "No" means no equal/similar band of DNA MW for PCR productions between Fhhh & its parental strains; "MW" means molecular weight; "bp" means the numbers of base pair of DNA

Fhhh and SC had one similar band of DNA MW for *FLO*1 gene. The DNA fragments of *FLO*1 gene existing in Fhhh could only be from its parental strain SC, because the other two parental strains, PC and YZ1, did not contain the DNA fragments of *FLO*1 gene (Table 2).

Fhhh and YZ1 had one similar band of DNA MW for 16S rDNA. The DNA fragments of 16S rDNA existing in Fhhh could only be from its parental strain YZ1, because the other two fungi parental strains, PC and SC, did not contain the DNA fragments of 16S rDNA(Table 2).

2.2 Comparison of the cell morphology between Fhhh and its three parental strains

The cells of YZ1 and mycelia of PC were dyed with crystal violet, and that of SC and Fhhh with methyl blue. All the cells of Fhhh and its three parental strains magnified 1000 fold were taken photo with the microscope Nikon E600 and digital camera DXM-1200 shown in Fig.1 to Fig. 4.

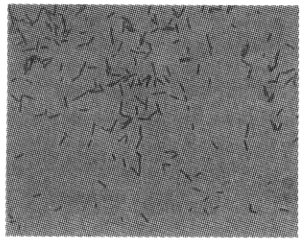

Fig.1 Cell shape of the parental strain YZ1

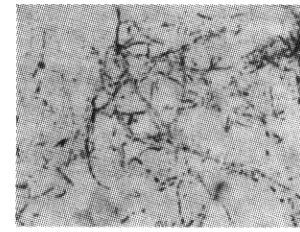

Fig.2 Mycelium of the parental strain PC

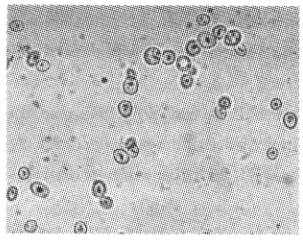

Fig.3 Cell shape of the parental strain SC

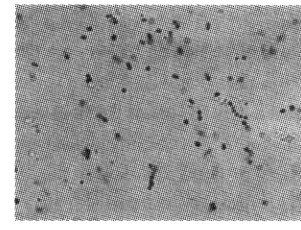

Fig.4 Cell shape of GEMs Fhhh

Fig.1—3 are the photos of the three parental strain cells. The shapes of the three parental strain cells are quite different from each other.

Table 3 Comparison of the cell sizes between Fhhh and its two parental strains

Strain	GEMs Fhhh	Parental strain SC	Parental strain YZ1
Long axle, μm	1.708 ± 0.139	4.122 ± 0.092	4.206 ± 0.244
Short axle, μm	1.357 ± 0.155	3.636 ± 0.087	0.618 ± 0.065
Volume, μm^3	1.678 ± 0.442	28.542 ± 1.379	1.273 ± 0.270
Statistic examination(10 samples for each strain)			
P value between Fhhh and SC		P value between Fhhh and YZ1	
$P = 0.023$, $P < 0.05$		$P = 5.175 \times 10^{-22}$, $P < 0.01$	

The calculation results of the cell sizes for each strain except PC are listed in Table 3.

Table 3 shows that there are obvious/extreme differences between the cell sizes of Fhhh and that of its two parental strains, SC and YZ1, by the statistic examination(Tong, 1986).

3 Conclusions

The cell size and shape of Fhhh were different from any of the parental strain. There were the 6 target DNA fragments of Fhhh's three parental strains existing in Fhhh cell. The parental strains of PC, SC and YZ1 only contained their own DNA fragments respectively. Fhhh had the molecular genetics stability after isolating and purifying over 700 generations.

The biotechnology of the inter-kingdom protoplast fusion could be used to construct GEMs through *in viro* gene recombination and integration for pollution control.

Acknowledgements: The authors appreciate Prof. Wang for PC strain and Prof. Jiang for digital photography.

References:

Barr D P, Aust S D, 1994. Mechanisms of white rot fungi used to degrade pollutants[J]. Environ Sci Technol, 28:78—89.

Bogan B W, Schoenike B, Lamar R T *et al*., 1996, Mnp mRNA and enzyme activity levels during bioremediation of PAH-contaminated soil with *Phanerochaete chrysosporium*[J]. Appl Environ Microbiol, 62: 2381—2384.

Cheng S P, 1995. A laboratory manual of environmental biotechnology[M]. Nanjing: Nanjing University Press. 61—65.

Cheng S P, Pei D P, You L *et al*., 2000. Optimal parameters for *Phanerochaete. chrysosporium* degradation of PTA wastewater[J]. Toxico Environ Chem, 76: 229—238.

Cheng S P, Chen L, Yan J *et al*., 2002. Degradation of purified terephthalic acid and expression of *mnp* gene for GEM Fhhh[J]. Acta Scientiae Circumstantiae, 22(1):1—5.

Collonnier C, Mulya K, Fock I *et al*., 2001. Source of resistance against *Ralstonia solanacearum* in fertile somatic hybrids of eggplant (*Solanum melongena* L.) with *Solanum aethiopicum*[J]. Plant Science, 160(2):301—313.

Godfrey B J, Mayfield M B, Brown J A *et al*., 1990. Characterization of a gene encoding a MnP from *Phanerochaete chrysosporium*[J]. Gene, 93: 119—124.

Griffiths R I, Whiteley A S, O'Donnell A G *et al*., 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition[J]. Appl Environ Microbiol, 12(66): 5488—5491.

Naidou P S, Reddy C A, 1992. Nucleotide sequence of a new lignin peroxidase gene GLG3 from the white-rot fungous, *Phanerochaete chrysosporium*[J]. Nucleic Acid Res, 18(23): 7173—7177.

Suzuki P, 1996. Breeding of yeast for wastewater treatment by protoplast fusion[J]. Nippon Jozo Kyokaishi, 7:521—526.

Teeradakorn S, Kishimoto M, Seki T *et al*., 1998. Process development and simulation of glucose isomerase production from birch wood xylan by a *Streptomycetes* fusant[J]. Biochemical Engineering Journal, 1(2):159—168.

Teunissen A W R H, Holub E, Van Der Hucht J *et al*., 1993. Sequence of the open reading frame of the *FLO*1 gene from *Saccharomyces cerevisiae*[J]. YEAST, 9:423—427.

Tong Y Z, 1986. Statistics in biology[M]. Changsha: The Press of Hunan Science and Technology. 176—214.

Vazquez F, Heluane H, Spencer J F T *et al*., 1997. Fusion between protoplast of *Pichia stipitis* and isolated filamentous fungi nuclei[J]. Enzyme and Microbial Technology, 21(1): 32—38.

Watari J, Takata Y, Ogawa M *et al*., 1994. Molecular cloning and analysis of the yeast flocculation gene *FLO*1[J]. Yeast, 10: 211—225.

Xu Y B, Yang R D, Liu H *et al*., 2001. Stability of the extreme enzyme in wastewater treatment[J]. Environmental Protection in Chemical Engineering, 21(1):21—24.

Yan J, Gu J D, Cheng G *et al*., 2001. Effects of metal elements on the level of *mnp* gene expression for degradation of PTA wastewater[J]. Journal of Nanjing University, 3(37): 342—347.

Zanke C, Hemleben V, 1997. A new *Solanum* satellite DNA containing species-specific sequences which can be used for identification of genome parts in somatic hybrids of potato[J]. Plant Science, 126(2):185—191.

Zhong Z H, Lu P, Cheng S P, 2000. Characters of Fhhh in degradation of PTA wastewater[J]. Journal of Nanjing University, 3:312—316.

Zhu G J, Wang Z X, 1994. Experimental manual of industrial microorganism[M]. Beijing: Light Industry Press of China. 53—84.

(Received for review June 10, 2002. Accepted July 4, 2002)