BIOMEDICAL AND ENVIRONMENTAL SCIENCES **20**, 113-118 (2007)

Directed Molecular Evolution of Nitrite Oxido-reductase by DNA-shuffling[1]

JUN-WEN LI[2], JIN-LAI ZHENG, XIN-WEI WANG, MIN JIN, AND FU-HUAN CHAO

Institute of Environment and Health, 1 Dali Road, Tianjin 300050, China

Objective To develop directly molecular evolution of nitrite oxido-reductase using DNA-shuffling technique because nitrobacteria grow extremely slow and are unable to nitrify effectively inorganic nitrogen in wastewater treatment. **Methods** The *norB* gene coding the nitrite oxido-reductase in nitrobacteria was cloned and sequenced. Then, directed molecular evolution of nitrite oxido-reductase was developed by DNA-shuffling of 15 *norB* genes from different nitrobacteria. **Results** After DNA-shuffling with sexual PCR and staggered extension process PCR, the sequence was different from its parental DNA fragments and the homology ranged from 98% to 99%. The maximum nitrification rate of the modified bacterium of X16 by DNA-shuffling was up to 42.9 mg/L·d, which was almost 10 times higher than that of its parental bacteria. Furthermore, the modified bacterium had the same characteristics of its parental bacteria of *E. coli* and could grow rapidly in normal cultures. **Conclusion** DNA-shuffling was successfully used to engineer *E. coli*, which had *norB* gene and could degrade inorganic nitrogen effectively.

Key words: Directed evolution; DNA-shuffling; Nitrite oxido-reductase; *norB* gene; Nitrobacteria

INTRODUCTION

High content of inorganic nitrogen such as ammonia and nitrite not only is poisonous to organisms, but also causes eutrophication in the water. Treatment of wastewater containing nitrogenous pollutants can be physical, chemical, or biological. The biological treatment has the greatest prospect for development[1]. In a biological treatment, nitrobacteria play a crucial role by transforming ammonia into nitrate and then into nitrogen. Nitrifying bacteria mainly comprise two physiological groups: *nitrosomonas* and nitrobacteria. The former oxidize ammonia nitrogen into nitrite nitrogen and the latter oxidize nitrite nitrogen into nitrate nitrogen[2-5]. The protein responsible for nitrification in nitrobacteria is nitrite oxido-reductase (nor) coded by *norB* gene[6-7]. Nitrobacteria rely on oxido-inorganic nitrogen as a sole energy source, thus they grow extremely slow[1]. In fact, the most common isolation and purification methods, such as the most probable number, limited dilution and selective plate, are unable to isolate and select nitrobacteria[8-9]. This will affect the study and application of nitrobacteria in wastewater treatment.

DNA-shuffling is a directed evolution technique of molecule *in vitro* that can greatly improve or enhance some features of target gene products[10]. It has been widely applied in the recombination of all sorts of proteins such as gene medicine, enzymes for industrial purposes and proteins for scientific research[11-13]. However, there are no reports to date on recombination of nitrobacteria gene using DNA-shuffling technique on pollutant degradation bacteria except for recombination of atrazine and arsenate degradation genes[14].

The present study cloned, sequenced and reconstructed *norB* gene of the nitrobacteria, using DNA-shuffling. Engineering bacteria with *norB* gene were modified to improve culture characteristics of nitrobacteria and enhance their ability to transform nitrite nitrogen.

MATERIALS AND METHODS

Bacterial Strains and Expression Vectors

Nitrobacteria X01-X15 were isolated from soil or wastewater samples, and identified as *Nitrobacter*, *Nitrococcus*, and *Nitrospina* based on the *Bergey's*

[1]This study was supported by the National High Technology Research and Development Program of China (863 Program) (No. 2001AA214191).

[2]Correspondence should be addressed to Jun-Wen LI, No. 1, Dali Road, Tianjin 300050, Department of Environment and Health, Institute of Environment and Health. Tel: 86-22-84655345. Fax: 86-22-23328809. E-mail: junwen9999@hotmail.com

Competing interests: None declared.

0895-3988/2007
CN 11-2816/Q

Manual of Determinative Bacteriology[15]. The expression vectors pGEM-T, pGEM-4Z, and *E. coli* JM109, DE3 were obtained from Takara Biotechnology (Dalian) Co., Ltd.

Determination of Nitrification Rate

The single strain on plate was directly inoculated in nitrobacterial inorganic salt basal medium and cultured at 30℃ with shaking air exposure. The concentration of nitrite nitrogen was measured colorimetrically. Nitrification rate was calculated according to the following formula[16]:

Nitrification rate (mg/L·d) = (nitrite nitrogen concentration of the controlled group – nitrite nitrogen concentration in experiment group)/time (d).

NorB Gene Amplification and PCR Product Purification

Primer design The primer was designed according to *norB* gene sequence by Oligo 6.0 software. *EcoR* Ⅰ and *Hind*Ⅲ sites (black in the primers) were incorporated in the primers used for PCR, respectively.

F：5′ggt**aagctt**atggacatccgagctcaagtt

R：5′gca**gaattc**ttactagaagcctatggtcctct

DNA extraction The separated strain was cultured on solid culture medium plate for one week and collected. DNA was extracted according to the manual of the DNA extraction kit from Takara Biotechnology (Dalian) Co., Ltd. After electrophoresis, the target DNA fragment were selected and sliced for recovery and purification.

NorB gene amplification The 50 μL PCR reaction mixture contained 2 μL of 100 μmol/L of each dNTP, 1 μL of 50 pmol of each primers, 5 μL of 10×buffer (contain 25 mmol/L $MgCl_2$), 2U Taq polymerase, and 4 μL of about 1 mol/L of template DNA. The polymerase chain reaction (PCR) temperature program was 94℃ for 3 min followed by 94℃ for 1 min, 52℃ for 1 min and 72℃ for 3 min for 35 cycles, finally 72℃ for 12 min. After amplification, the PCR products were analyzed by electrophoresis on 0.8% agrose gels stained with ethidium bromide.

Recovery and purification of PCR products Recovery and purification were conducted with the silver beads DNA gel recovery kit produced by Shanghai Sengon Biological Engineering Technology Service Ltd.

Ligation and Transformation

DNA (5 μL), T_4 DNA ligate enzyme (1 μL), vector (1 μL) and 10×buffer (2 μL), and water were added till the overall volume reaching 20 μL, ligated at 16℃ over night. Competent cells were prepared and transformed according to Molecule Cloning Experiment Guidance.

DNA Shuffling

Sexual PCR *NorB* gene (1542 bp) was obtained by PCR and purified from 1% low-melting-point agarose gel using the Wizard PCR prep DNA purification system (Promega). The purified PCR products (2-3 μg) was digested with 0.15 units of DNase I (Promega) for 10 min at room temperature in 100 μL of 50 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L $MgCl_2$. The reaction was stopped by addition of 1 mmol/L EDTA and 0.1% SDS and incubation at 65℃ for 10 min. Fragments of 200-300 base pairs were purified from 2% low-melting-point agarose gel. The purified fragments were resuspended in a primerless 25-μL PCR (0.2 mmol/L each dNTP, 2.2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mol/L Tris-HCl (pH 8.8), 0.1% Triton X-100, and 2 units of Taq (Promega)) at 20-30 ng/μL. A PCR program of 94℃ for 2 min and 40 cycles of 94℃ for 40 s, 53℃ for 40 s, and 72℃ for 40 s was followed in a Perkin-Elmer DNA thermal cycler (2400). The product of this reaction was diluted 40-fold in a 50-μL PCR (0.2 mmol/L each dNTP, 2.2 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 0.1% Triton X-100, and 5 units of Taq with the F/R primers at 0.2 pmol/μL, followed by a PCR program of 94℃ for 3 min and 25 cycles of 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 2 min. The correctly sized band was purified and digested with *EcoR* Ⅰ and *Hind*Ⅲ enzymes before being cloned into the pGEM-4Z phagemid vector. The *in vitro* recombined *norB* library was transformed into *E. coli* DE3 cells, plated on LB plates containing 100 μg/mL ampicillin and 2% glucose, and grown overnight at 30℃.

Staggered extension process PCR Primer was introduced to amplify the purified *norB* gene. The PCR temperature program was set at 95℃ for 10 min followed by 94℃ for 1 min, 56℃ for 5 s and 72℃ for 5 s for 55 cycles, finally 72℃ for 10 min. Another PCR was performed with template of the first PCR product. Gel electrophoresis was conducted to check if recombination succeeded, and then treated as described above.

Plasmid Extraction, Gene Sequencing, and Homology Analysis

Plasmid was extracted as above and sequenced by Takara Biotechnology (Dalian) Co., Ltd. The sequences were forwarded to the official website of US National Health Institute *via* Internet and blasted in GenBank for homology analysis.

NorB Gene Stimulation Expression

Host bacteria with target gene were inoculated in medium containing relevant antibiotics, saturated and cultured at 37℃ over night. Saturated culture (100 μL) was then inoculated in antibiotic medium and cultured for 4 h at 37℃. IPTG was added to stimulate target gene expression. Samples were taken after 0.5, 1.0, 2.0, 3.0, and 4.0 h and centrifuged rapidly at room temperature. Bacteria were collected with 100 μL loading buffer added, split in 100℃ water, and stored at 4℃ for future use.

Gel Electrophoresis

Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were denatured by incubation with denaturing buffer (2% SDS and 0.64 M mercaptoethanol) at different temperature. Low-MW protein markers (Takara) were used as standards. Once bromphenol blue reached the bottom of isolation gel in electrophoresis, power was shut down. Glass plates were removed from electrohoresis device and gel was taken into carbinol-acetic acid condense solution containing Coomassie brilliant blue for immersion of several hours, and then decolored with carbinol-acetic acid condense solution for several hours and then the results were observed.

Enzyme Assays

The activity of nitrite oxido-reductase was determined by measuring the nitrification rate as described above.

Nucleotide Sequence Accession Number

The sequence of the *norB* gene is available in GenBank under accession no. Af001268. The sequence of the *norB* gene of *N*. hamburgensis *norB* gene for nitrite oxidoreductase (beta subunit) is available in GenBank under accession no. X66067.1.

RESULTS

Cloning and Sequencing of NorB Gene

The *norB* gene amplified with designed primer was 1542 bp. The positive PCR product of *norB* gene could be obtained in every strain of X01-X15, as shown in Figs.1a and 1b. The homology between all 15 nitrobacterial and Nitrobacterium hamburgensis *norB* genes in Genbank was from 96% to 98%.

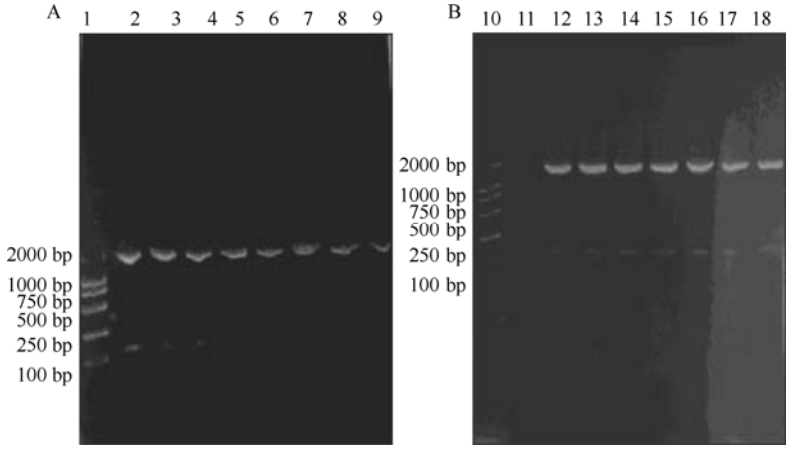

FIG. 1a. Electrophoretogram of *norB* gene of Nitrobacteria by PCR. (1: Marker DL2000, 2-9: X01-X08 Nitrobacteria *norB* gene); 1b. Electrophoretogram of *norB* gene of Nitrobacteria by PCR (10: Marker DL2000, 11: negative control, 12-18: X09-X15 Nitrobacteria *norB* gene).

Recombination of NorB Gene

NorB genes of all X01-X15 strains were mixed equally and recombined with sexual PCR and staggered extension process PCR, respectively. It was proved that the recombination product was 1542 bp after DNA-shuffling with either method, matching the whole length of *norB* genes of X01-X15 strains amplified with conventional PCR.

Cloning, Transformation, and Selection of Positive Transformant

Enzyme-digested and purified target DNA was ligated to plasmid vector pGEM-T and transformed to *E. coli* JM109 strain competent cells. Positive transformants were selected after blue-white assay selection. The recombination bacterium by DNA-shuffling was named as X16.

Sequencing and Homology Analysis

Positive transformants were sequenced with T7 sequencing primer and sequences were analyzed. The homology of X01-X16 target genes was analyzed by Genedoc software in terms of nucleic acid and protein sequence level (Table 1).

TABLE 1

Homology of X01-X16 Target Genes After 55 Rounds of Directed Evolution

	X01	X02	X03	X04	X05	X06	X07	X08	X09	X10	X11	X12	X13	X14	X15	X16
X01	-	97	98	96	98	98	98	98	96	98	98	98	98	98	98	97
X02	-	-	97	96	97	97	97	97	98	97	97	97	97	97	97	97
X03	-	-	-	96	98	98	99	98	96	99	98	99	98	99	98	98
X04	-	-	-	-	96	96	96	96	97	96	96	96	96	96	96	96
X05	-	-	-	-	-	99	99	99	96	99	99	98	98	99	98	97
X06	-	-	-	-	-	-	99	98	96	99	99	98	98	99	98	98
X07	-	-	-	-	-	-	-	99	96	99	99	99	99	99	99	98
X08	-	-	-	-	-	-	-	-	96	99	99	98	98	99	98	97
X09	-	-	-	-	-	-	-	-	-	96	96	96	96	96	96	96
X10	-	-	-	-	-	-	-	-	-	-	99	99	99	99	99	98
X11	-	-	-	-	-	-	-	-	-	-	-	99	98	99	98	97
X12	-	-	-	-	-	-	-	-	-	-	-	-	98	99	98	97
X13	-	-	-	-	-	-	-	-	-	-	-	-	-	99	99	97
X14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99	98
X15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	97
X16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The results showed that *norB* gene of recombination bacterium X16 was not exactly the same as its parent's gene sequences, and contained different mutation alkali that came from one or several X01-X15 strains. The homology was from 96% to 99% (Fig. 2).

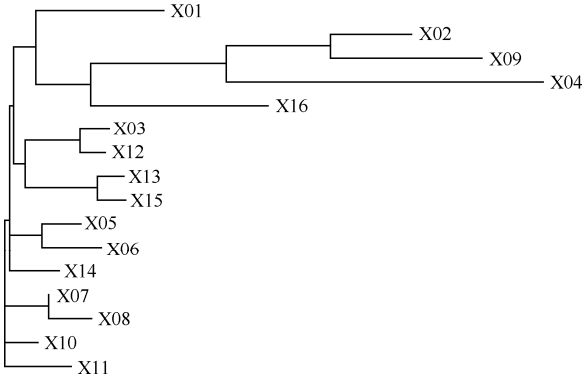

FIG. 2. Phylogenetic trees for nitrobacteria based on *norB* gene sequence homology.

SDS-PAGE Electrophoresis Assay

The X16 strain stimulated by IPTG for 4 hours was forwarded to SDS-PAGE electrophoresis. As shown in Fig. 3, X16 strain produced a 57-KD band identical with expected-size of protein encoded by *norB* gene, while two controls had no band. The isoelectropoint of this product was pH 6.4.

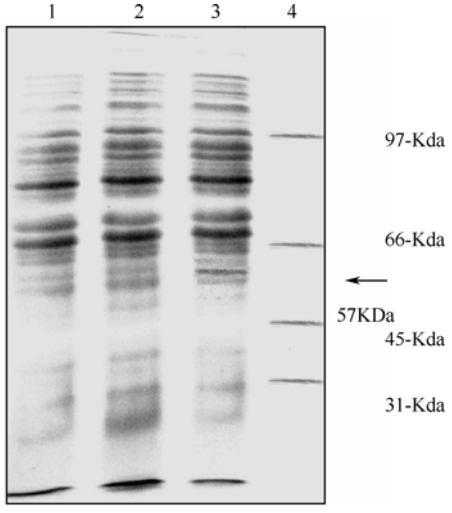

FIG. 3. SDS-PAGE of the expression product of *norB* gene. (1-3: *E. coli* DE3, *E. coli* DE3 with pGEM-4Z, *E. coli* DE3 with pGEM-4Z+*norB*, 4: Protein Marker).

Nitrite Nitrogen Transformation Efficiency

The nitrification rates of 15 bacteria strains selected in this study ranged from 4.4 mg/L·d to

5.0 mg/L·d. The nitrification rate of *E. coli* DE3 used as a host bacterium was 0.0 mg/L·d. The maximum nitrification rate of the X16 strain modified by DNA-Shuffling was up to 42.9 mg/L·d, which was about 10 times higher than that of its parental nitrobacteria of X01-X15 (Table 2).

TABLE 2

Nitrite Nitrogen Transformation Efficiency

Bacteria	Nitrification Rate (mg/L·d)
X01	4.6
X02	4.6
X03	4.6
X04	4.8
X05	4.5
X06	4.7
X07	4.4
X08	4.4
X09	4.6
X10	4.5
X11	4.6
X12	5.0
X13	4.6
X14	4.6
X15	4.5
X16	42.9
E. coli DE3	0.0

DISCUSSION

Water pollution is serious worldwide. China also faces rather grave environment pollution after rapid economic development for nearly 20 years. According to the annual Report on the State of the Environment in China[17], major lakes and rivers countrywide have been seriously polluted, and the primary pollutants are ammonia and organic matters. Many lakes and rivers have been suffering from eutrophication for a long time. Conventional physical and chemical methods for ammonia nitrogen treatment so far have worked poorly. The current study on nitrogen pollution treatment focused on the biological treatment using nitrobacteria and other microorganisms.

As being energy autotrophic, nitrobacteria can not rely on organic substances and their growth is usually restricted with existence of organic substances[1]. Nitrobacteria grow very slow and take 24 hours to complete a division cycle. In solid culture process, they take about one month to form colony. Besides, the strongly aerobic nitrobacteria are unable to grow in acid conditions and are highly sensitive to the characteristics of wastewater, for example pH and temperature[1]. Such characteristics make it too difficult to separate them from environments and apply to denitrogenation in practices.

DNA-shuffling has become a directed evolution technology for protein molecule since the end of last century, which introduces mutations *via* two experimental solutions: sexual PCR and staggered extension process PCR[18-19]. Sexual PCR linking mutually complementary DNA fragments is mutual templates or primers with non-primer PCR and recombines them into a new nucleic acid molecules, thus introducing mutation. Staggered extension process PCR introduces mutation *via* exchanging templates after brief annealing and extension in the process. Both methods can recombine genes but staggered extension process PCR is easier to operate and incurs a higher frequency of introduced mutation. For a higher frequency of mutation in sexual PCR, the working time of DNA enzyme must be prolonged with fragment cut smaller. However, a smaller fragment is hard to be recovered and tends to incur mistakes in non-primer PCR. Comparatively, staggered extension process PCR does not have such shortcomings and only needs to shorten annealing and extension time optimally for a higher mutation frequency. On this, the mutation frequency of staggered extension process PCR is related to temperature change rate of PCR. Sensitive PCR device changes faster in temperature and mutation is frequently higher and *vice versa*.

This study applied DNA-shuffling in modifying of *norB* gene from 15 nitrobacterial strains isolated from environments in order to obtain a higher effective transforming nitrite bacterium. Both sexual PCR and staggered extension process PCR could achieve satisfying results. The recombination product is a new gene formed by splicing different fragments from different *norB* genes of 15 strains. Sequencing of the isolated clone revealed 10-15 point mutations, while these mutations did not produce amino acid substitutions. Such mutational mechanisms have already been observed by DNA-shuffling[20]. Although the amino acid sequences kept identical with those of its parental bacteria, the maximum nitrification rate of the modified bacterium by DNA-Shuffling was much higher than that of its parental nitrobacteria.

Furthermore, the modified bacterium using DNA-shuffling had the similar characteristics of its host bacterium-*E. coli* as the heterotrophic bacterium, which not only reproduced very fast (colony growth could be seen within 16-18 hours in solid culture) and was cultured easily (common broth culture generates

massive bacterium), but also had a high adaptive ability to environment.

This study has laid a foundation for further research on physiological, biochemical, and genetic characteristics of nitrobacteria and recombination of highly effective nitrobacteria for denitrogenation in wastewater treatment field.

REFERENCES

1. Sharma B, Ahlert R C (1977). Nitrification and nitrogen removal. *Water Res* **11**, 897-925.
2. Bock E, Koops H P, Ahlers B, *et al.* (1992). Oxidation of inorganic nitrogen compounds as energy source. In The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, ed. A Balows, H G Tru, M Dworkin, *et al.* Springer, New York. EPA Draft.
3. Rittmann B E, Langeland W E (1985). Simultaneous denitrification with nitrification in single-channel oxidation ditches. *J Water Pollut Control Fed* **57**, 300-308.
4. Robertson L A, Kuenen J G (1984). Aerobic denitrification: a controversy revived. *Arch Microbiol* **139**, 351-354.
5. Robertson L A, Kuenen J G (1992). Nitrogen removal from water and waste. In Microbial Control of Pollution, ed. J C Fry, G M Gadd, R A Herbert, *et al*. Watson-Craik. Cambridge University Press, Cambridge.
6. Klotz M G, Norton J M (1995). Sequence of an ammonia monooxygenase subunit A-encoding gene from *Nitrosospira* sp. NpAV. *Gene* **163**, 159-160.
7. Maron P A, Coeur C, Pink C, *et al.* (2003). Use of polyclonal antibodies to detect and quantify the NOR protein of nitrite oxidizers in complex environments. *J Microbiol Methods* **53**, 87-95.
8. Mendum T A, Sockett R E, Hirsch P R (1999). Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the beta subdivision of the class *proteobacteria* in arable soils to nitrogen fertilizer. *Appl Environ Microbiol* **65**, 4155-4162.
9. Schramm A, Beer D, Heuvel J C, *et al.* (1999). Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira* spp. along a macroscale gradient in a nitrifying bioreactor: quantification by *in situ* hybridization and the use of microsensors. *Appl Environ Microbiol* **65**, 3690-3696.
10. Stemmer W P (1994). Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370**, 389-391.
11. Harayama S (1998). Artificial evolution by DNA shuffling. *Trends Biotechnol* **16**, 76-82.
12. Zhao H, Giver L, Shao Z, *et al*. (1998). Molecular evolution by staggered extension process (StEP) *in vitro* recombination. *Nat Biotechnol* **16**, 258-260.
13. Chang C C, Chen T T, Cox B W, *et al*. (1999). Evolution of a cytokine using DNA family shuffling. *Nat Biotechnol* **17**, 793-801.
14. Wackett L P (1998). Directed evolution of new enzymes and pathways for environmental biocatalysis. *Ann N Y Acad Sci* **864**,142-52.
15. Bergey Holt, J G, Krieg N R, Sneath P H A (1994). Bergey's Manual of determinative Bacteriology. Lippincott Williams & Wilkins.
16. APHA, AWWA, WEF (1998). Standard methods for the examination of water and wastewater. 20th ed. APHA, Wahington D. C.
17. State Environmental Protection Administration of China (SEPAC) (2005). Report on the Environmental Conditions in China.
18. Yano T, Kagamiyama H (2001). Directed evolution of ampicillin-resistant activity from a functionally unrelated DNA fragment: A laboratory model of molecular evolution. *Proc Natl Acad Sci USA* **98**, 903-907.
19. Salamone P R, Kavakli I H, Slattery C J, *et al.* (2002). Directed molecular evolution of ADP-glucose pyrophosphorylase. *Proc Natl Acad Sci USA* **99**, 1070-1075.
20. Xirodimas D P, Lane D P (1999). Molecular evolution of the thermosensitive PAb1620 epitope of human p53 by DNA shuffling. *J Biol Chem* **274**, 28042-28049.

(*Received January* 2, 2006 *Accepted August* 15, 2006)