

Full Length Research Paper

# Screening, identification and degrading gene assignment of a chrysene-degrading strain

Tang Yu-bin<sup>1,2\*</sup>, Yang Xu<sup>1</sup>, Chen Fang-yan<sup>1</sup>, Jiang Rui-ling<sup>1</sup> and Wang Xin-gang<sup>1</sup>

<sup>1</sup>School of Biotechnology and Environmental Engineering, Jiangsu University of Science and Technology, Sibaidu, Zhenjiang Jiangsu 212018, China.

<sup>2</sup>Zhenjiang Haida Co. Limited of Science and Technology, Zhenjiang Jiangsu 212111, China.

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A predominant chrysene-degrading strain named CT was isolated from the activated sludge of Zhenjiang coking plant. The strain was initially identified as *Paracoccus aminovorans* by the results of morphological observation, physio-biochemical test and 16S rDNA gene sequence analysis. Under the conditions of initial chrysene concentration of 40 mg/l, inoculation amount of 10% (V/V) at pH 7.0 and temperature of 35°C, the degradation efficiency of chrysene by the strain CT reached 85.2% within 8 days. Alkaline lysis was applied to the extract plasmids from strain CT to confirm the location of chrysene-degrading genes. A plasmid, greater than 15 kb, was detected. The transformants obtained the ability to degrade chrysene when the plasmid of strain CT was transformed to competent cell of *Escherichia coli* DH10B, and could remove 43% of chrysene in the solutions with concentration of 30 mg/l within 8 days. But the mutation lost the ability to degrade chrysene when its plasmid was eliminated by sodium dodecyl sulfonate (SDS) and high temperature. This indicated that the plasmid of strain CT carried chrysene-degrading genes.

**Key words:** Chrysene, degrading strain, *Paracoccus*, degrading gene, plasmid.

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds, which contain two or more benzene rings, mainly originated from incomplete combustion and thermal schizolysis of organic compounds. It is a kind of ubiquitous pollutants in the environment (Zhang et al., 2006). Some of them possess carcinogenic, mutagenic and teratogenic properties, and are linked to some health problems (Menzie et al., 1992). Chrysene is a typical high molecular weight (HMW) PAH. It has been monitored as priority pollutants by the US Environmental Protection Agency (EPA) (Nadal et al., 2004) and also been put on the blacklist of environmental pollutants by the government of China (Kuang and Xu, 2009). To control

PAHs, pollution has aroused great attention among the public in most countries. Due to its several advantages, such as efficient, low-cost and non-secondary pollution, microbial transformation and degradation has become the principal way to remove PAHs from the environment successfully. Considerable research has been focused on PAHs degradation. So far, some genera of the PAH-degrading bacteria have been isolated from specific environments. Most of them belong to the genera *Sphingomonas* (Willison, 2004), *Alcaligenes* (Møller and Ingvorsen, 1993), *Rhodococcus* (Walter et al., 1991), *Pseudomonas* (Mueller et al., 1990), *Mycobacterium* (Boldrin et al., 1993), *Bacillus* (Hunter et al., 2005), *Flavobacterium* (Abd-Elsalam et al., 2006), *Aeromonas* (Sàágua et al., 2002), *Beijerinckia* (Kiyohara et al., 1982a), *Corynebacterium* (El-Gendy et al., 2010), *Cyanobacteria* (Gamila et al., 2003), *Micrococcus* (Gerbeth et al., 2004), *Nocardia* (Zeinali et al., 2007), *Vibrio* (Hedlund and Staley, 2001) and *White-rot fungi* (Pickard, et al., 1999). With more microorganisms being screened, scientists have researched further into the degrading genes and paths of PAHs degradation. Studies

\*Corresponding author. E-mail: [ybbill@163.com](mailto:ybbill@163.com) Tel: 08651185637630. Fax: 08651185635850.

**Abbreviations:** SDS, Sodium dodecyl sulfonate; PCR, polymerase chain reaction; MR, methyl red; VP, voges-proskauer; SEM, scanning electron microscope; OD, optical density; NCBI, National Center of Biotechnology Information.

have suggested that some degradation gene of PAHs are located in the chromosome (Saito et al., 2000), while some are situated in the plasmid (Menn et al., 1993; Kiyohara et al., 1982b) and others are coded by chromosome and plasmid together (Kiyohara et al., 1994). A commonly used method to determine whether a certain phenotype of microorganism cell is controlled by plasmid DNA is first to eliminate plasmid of the strain, then to investigate whether or not the related hereditary character of mutant strain is altered. Plasmids are extra-chromosomal double-stranded, circular DNA molecules in cells. Under normal conditions, they are not essential for microbes' survival, but they play an important role under extraordinary circumstances, such as nutritional deficiency and the presence of toxic substances. Plasmids commonly exist stably in cell. So, some physical or chemical methods are usually employed to remove them. The aims of the present work were: (1) the isolation and screening of a chrysene-degrading strain from activated sludge of China Zhenjiang coking plant, (2) characterization of this strain by morphological observation, physio-biochemical test and 16S rDNA gene sequence analysis and (3) the study of the growth characteristics and degradation ability of the strain in order to determine the degradation gene of the bacterial strain. This may lay the foundation for further research and utilization of the chrysene-degrading plasmid in the bioremediation of PAH-polluted environment.

Of course, there were some objectives needed to be done in this field: more research is necessary to study the dynamic analysis of the strains mixtures and understand the mechanism of degradation at the molecular level, the control of gene expression and whether the gene transfer or not still needs further study. In addition, the exact relationship between the genetic fragments on the plasmid and the degradation process and the difference of plasmids among mutant strains should further be studied. What is more is that, we should strengthen the use of microbial molecular biology and technology, such as DNA shuffling, plasmid-assisted molecular breeding to improve plasmid characteristics, especially using biological engineering means to introduce a variety of degrading plasmid into the same microorganisms, in order to obtain the super engineering bacterium with new metabolic pathway, high efficiency and broad-spectrum degradation ability. This will provides a new way to solve environmental pollution.

## MATERIALS AND METHODS

### Materials and chemicals

The strain source for domestication was from the activated sludge of Zhenjiang coking plant, China. Chrysene was of HPLC grade, purchased from Fluka Co.; acetone, cyclohexane and other reagents and mineral salts used were all of analytical grade from local reagent providers.

The following media were used in this experiment: (1) selective

liquid medium was composed of ( $L^{-1}$ ): 1 g  $KH_2PO_4$ ; 1.3 g  $K_2HPO_4 \cdot 3H_2O$ ; 0.2 g  $MgSO_4$ ; 1 g  $NH_4NO_3$ ; 0.02 g  $CaCl_2$ ; 0.05 g  $FeCl_3$ ; 5 mg  $ZnSO_4 \cdot 7H_2O$ ; 5 mg  $MnSO_4 \cdot H_2O$ ; 1 mg  $Na_2MoO_4 \cdot 2H_2O$ ; 0.5 mg  $CuSO_4 \cdot 5H_2O$ ; 1 L  $H_2O$  (Zhang and Hiroshi, 2003). The pH of the mineral salt solution was adjusted to 7.0 and then the solution was sterilized in a high-pressure sterilizer for 20 min at 121°C. The acetone solution of chrysene after being filtered by syringe filter with membrane of 0.22  $\mu m$  diameter was added to the sterilized mineral salt solution mentioned earlier. The solutions could be used after the acetone had been completely volatilized. The composition of the selective solid medium was the same as the selective liquid medium but with 2% agar; (2) The composition of Luria-Bertani (LB) liquid culture included ( $L^{-1}$ ): 14 g yeast extract; 2 g peptone; 10.0 g NaCl; 1 L  $H_2O$  which was autoclaved for 20 min. LB solid plate contained ( $L^{-1}$  LB) 20 g agar.

### Isolation and purification of chrysene-degrading bacteria

A certain amount (about 30% v/v) of sterile distilled water was added to the activated sludge sample and aerated for 24 h. After precipitating the sludge particles for 10 to 15 min, 10 ml supernatant was transferred into 100 ml sterile selective liquid medium that contained chrysene (40 mg/l) as a sole carbon and energy source. The culture was incubated on a shaker at 120 rpm and 30°C. After been acclimated for 6 to 7 days, 10 ml of the culture solution was delivered aseptically to fresh selective liquid medium. After seven generations, 0.1 ml of the medium was spread on the LB plate.

The plate was then put into the incubator at 30°C for 5 to 6 days. When forming clear colonies, single-colony was chosen to make a further purification. The degradability of the purified strains was verified once again. The strains which grew fastest were selected as the candidate of predominant chrysene-degrading strains. Suspension of the strains was prepared and stored at 4°C for subsequent use.

### Identification of chrysene-degrading bacteria

In order to identify the isolated strain, morphological observation, physio-biochemical test and 16S rDNA gene sequence analysis were carried out.

### Morphological observation

Bacteria were inoculated on the LB solid plate and incubated at 30°C. Two days later, the morphological data of the colony were obtained by visual study. Furthermore, the strain was spread to ground slide. Then the shape and size of the cell was observed by scanning electron microscope (SEM) (model number: JSM-6480; Jeol Ltd., Japan) after vacuum coating.

### Physio-biochemical test

Biochemical and physiological characteristics were examined by previously described methods (Dong and Cai, 2001). Genomic DNA extraction, polymerase chain reaction (PCR) amplification and sequencing of 16S rDNA gene were performed: PCR amplification was performed with Veriti™ 96-Well Thermal Cycler from Applied Biosystems, using the F-primers (5'-AGAGTTTGATCMTGGC-3') and R-primers (5'-AAAGGAGGTGATCCA-3') (Shuang et al., 2006). The genomic DNA of the bacteria was extracted as template. The PCR mixture included 2  $\mu l$  10 $\times$ buffer, 2  $\mu l$  mixed dNTP, 1  $\mu l$  8f primer, 1  $\mu l$  1542r primer, 1  $\mu l$  DNA template, 0.3  $\mu l$  Taq DNA polymerase and sterile distilled water to a final volume of 20  $\mu l$ . The amplification

conditions contained an initial denaturation (95°C for 5 min), 30 cycles (94°C for 30 s, 55°C for 1 min, 72°C for 1.5 min) and a final extension (72°C for 9 min). The PCR products were separated by electrophoresis in a 1% (w/v) agarose gel and ethidium bromide staining. The target bands were cut off on UV transilluminator then recovered and purified using DNA gel purification kit (Sangon Biotechnology Co., Ltd., Shanghai). The purified DNA fragment was linked with the vector pMD18-T by T4 ligase at 16°C to stay overnight and then get the recombinant vectors into competence cells *Escherichia coli* DH10B. The PCR screening method and enzyme digestion were carried out to identify positive recombinant clone. The positive clone was picked and prepared and the plasmid was purified in which 16S rDNA gene was inserted and then sequenced (sequence by Sangon Biotechnology Co., Ltd.). Sequencing assembly was performed by DNASTar. After cutting and editing, sequence alignment and phylogenetic analyses were applied by MAGE (version 4.0).

### Analysis of chrysene

Chrysene was extracted from the culture medium by cyclohexane as extraction solvent. Chrysene content was analyzed by Shimadzu Corporation GC-2014-type gas chromatography (using flame ionization detector). Capillary chromatographic column (SE-54 type, 30 m long, 0.32 mm internal diameter and 0.25 µm film thickness) was used in the process. Then 1 µl of each sample was injected into the analytic system. A split injection was used at a split ratio of 5:1. The column temperature, inlet temperature of sample injection and detector temperature was set at 280, 280 and 300°C, respectively. Ultrapure N<sub>2</sub> was used as the carrier gas.

### Determination of microbes' growth curve and degradation of chrysene by the strains

To investigate the growth and biodegrading ability of the strain CT, the bacterial suspension (cell concentration: approximately 10<sup>7</sup> cfu/ml) were inoculated with sterile selective liquid medium (volume percentage is 10%) with an initial concentration of chrysene of 40 mg/l and at pH 7.0. All flasks with the medium were incubated on a rotary shaker (35°C, 120 rpm) in the dark. The growth of the bacteria was measured every day by determining the optical density (OD) of the culture solution via visible spectrophotometer (722-type, Tuopu Instrument Co. Ltd, Tianjin) at wavelength 600 nm and the residual concentration of chrysene was determined by GC.

### Extraction and elimination of the plasmid of the strain

Plasmid was extracted by the alkaline lysis method as described previously (Sambrook and Russell, 2000). To obtain mutant strain without plasmid, elimination of plasmid of the strain was carried out as follows: (1) activation of the strain: a single colony of the strain CT was picked from the selective solid plate, inoculated in a 3 ml of liquid LB medium and left overnight at 35°C and 250 rpm; (2) operation of elimination of strain CT: about 100 µl pre-culture was introduced into 3 ml of the LB liquid medium containing SDS with a different concentration, then was incubated for 36 h at 35°C after been treated for 1 h at 43°C; (3) initial screening of the mutant strain: sufficient amount of germ-carrying liquid was sucked from the test tubes in which bacteria have grown but turbidity of the medium was at the lowest, was spread on the LB plate and then incubated at 35°C; (4) identification of mutant strain: colonies on plate were picked at random and the plasmid was extracted to verify the result of the plasmid elimination experiment. The corresponding mutant strain from which the plasmid have been

eliminated (named ct) could then be found.

### Transformation of the plasmid of the strain

The plasmid extracted from strain CT was transferred into competence cells *E. coli* DH10B by transformation. The plasmid DNA was then prepared and purified so as to pick out the positive recombinant clone (named DHCT).

### Localization of degradation gene

In order to investigate the relationship between the plasmid and biodegradation ability, the degradation rate of chrysene by DHCT, ct, *E. coli* DH10B and CT respectively, were estimated. Strain DHCT, ct, *E. coli* DH10B and CT were inoculated with 100 ml of sterile selective liquid medium with an initial concentration of chrysene of 30 mg/l and pH of 7.0, respectively and then incubated for 8 days at 35°C and 120 rpm. The residual content of chrysene was detected as mentioned earlier.

## RESULTS

### Growth of strain CT and degradation of chrysene

The growth of strain CT and degradation of chrysene are shown in Figure 1. The results given in Figure 1 indicated that the growth of strain CT and degradation of chrysene were synchronous and presented mirror image relationship. In the beginning of the cultivation, the growth of strain CT was in the lag phase, cells grew slowly and the chrysene removal rate was inefficient. After a transitory lag period of about 2 days, the cells turned into logarithmic phase. In this stage, that is, from the second day to the seventh day, the strain used chrysene as the sole carbon and energy source for breeding and growth significantly. Chrysene in the medium was metabolized gradually to lead to the drop of chrysene concentration. Cells turned into stationary phase after this stage and the OD at 600 nm of the culture reached a maximum of 1.6. At this moment (namely the eighth day), the concentration of chrysene in the media decreased from 40 to 5.92 mg/l, the biodegradation rate reached 85.2%.

The degradation rate of chrysene (%) = (residual content of chrysene in the sample of control group - residual content of chrysene in the sample of the experimental group) / residual content of chrysene in the sample of control group × 100%.

The isolated strains had high growth and degradation rate in the culture system. This indicated that, CT was a predominant chrysene-degrading strain with high activity.

### Morphological characterization of strain CT

Strain CT, a predominant chrysene-degrading strain, was obtained by domestication, enrichment and screening from activated sludge. The colonies of the bacteria appeared rounded and milky on LB medium. They were

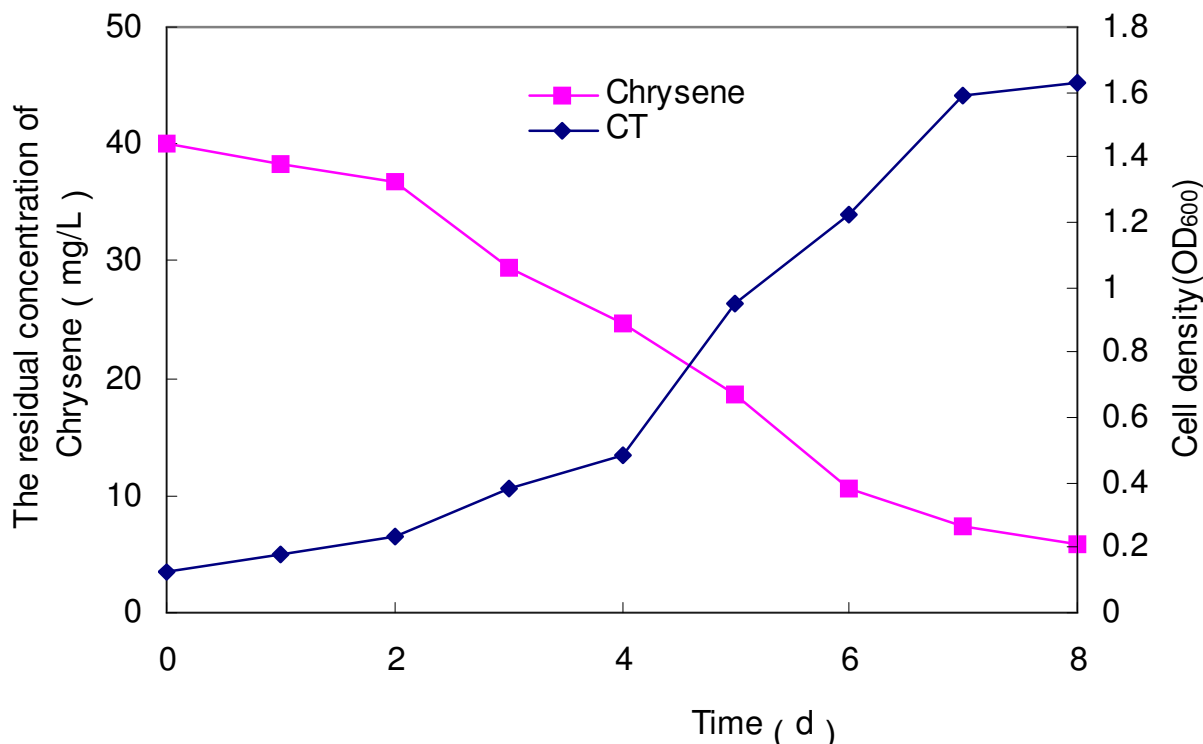


Figure 1. The growth curve of strain CT and degradation of chrysene.

applanate, hygric, glossy and opaque, ganoid, regular in edge, aequalis in texture, ropy and easy to pick up. Besides, water-soluble fluorescent pigment was not produced. In Figure 2, the cells of strain CT were coccoid or short rod-shaped, measuring 0.5 to 0.9  $\mu\text{m}$  in diameter and 0.9 to 1.2  $\mu\text{m}$  in length. They occurred singly and had no spore.

#### Physiological and biochemical characteristics of strain CT

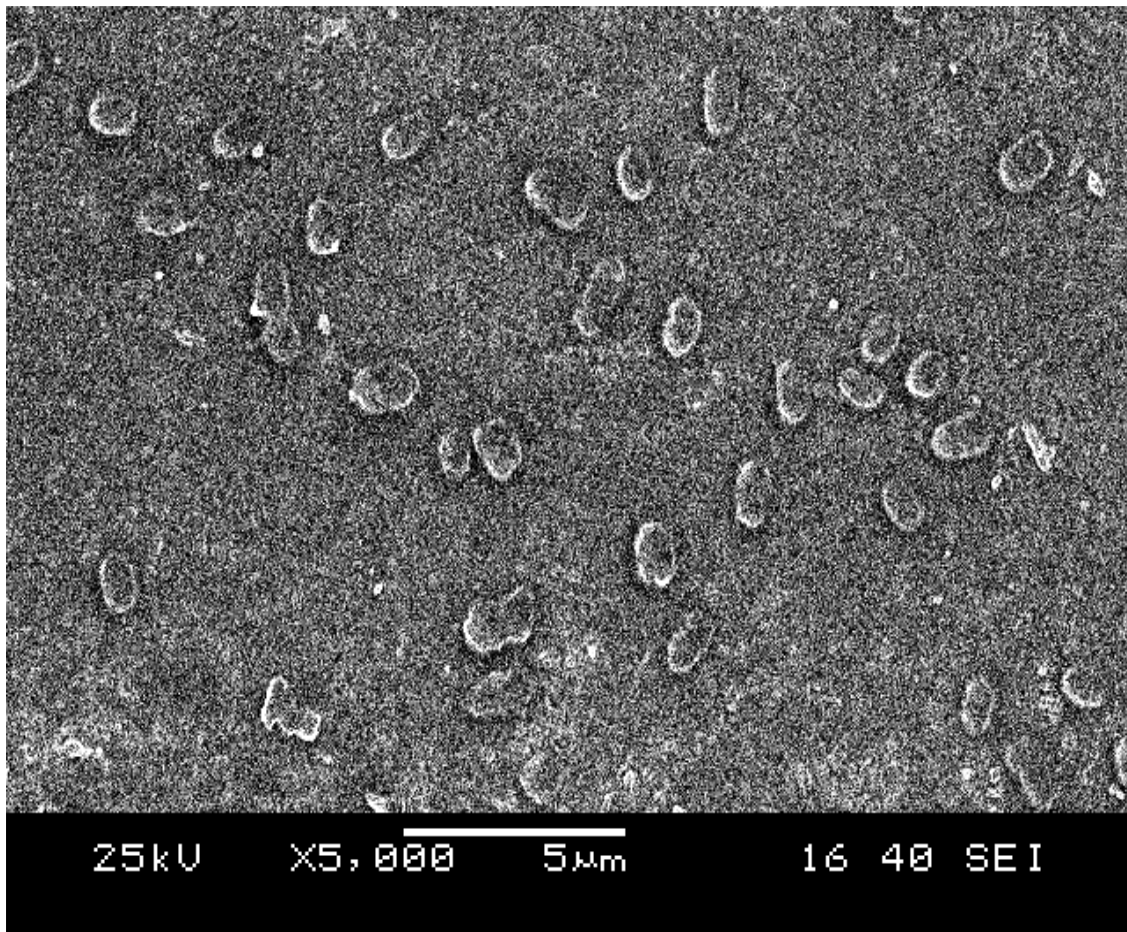
The strains CT were aerobic bacteria, gram-negative and nonmotile. The optimum temperature scope for their growth was 30 to 35°C. Strains CT could oxidize glucose but could not oxidize lactose. It could assimilate citrate and reduce nitrate. Hydrogen sulfide and indole were not produced. Methyl red (MR) and voges-proskauer (VP) test were negative. It was amylase negative and catalase positive. No urease and phenylalanine deaminase activity was detected. Gelatin was not hydrolyzed.

#### PCR amplification, clone of the 16S rDNA gene, sequence alignment and phylogenetic analyses

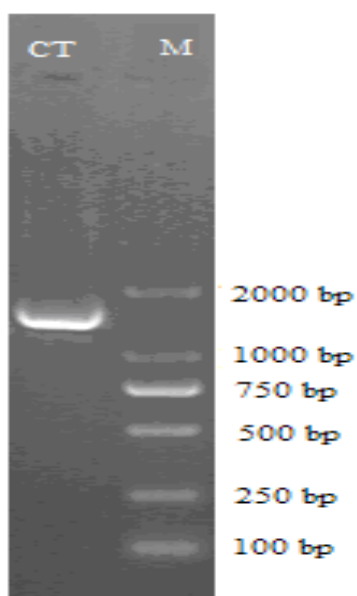
PCR amplification of the 16S rDNA gene was performed using genomic DNA of strain CT as template. The PCR products, about 1500 bp, were separated by

electrophoresis in a 1% agarose gel (Figure 3). The length of the DNA fragment was in accordance with primer designing region size. The sequence (1457 bp) was submitted to the GenBank of international nucleotide sequence databank. After examination and verification, the accession number was assigned as HQ 005404.

The 16S rDNA gene sequence of strain CT were analyzed and compared with other sequences published in GenBank database through blast on the National Center of Biotechnology Information (NCBI) website. The blast result showed that all the strains displayed on the listbox belong to genus *Paracoccus* and the isolate was most similar to *Paracoccus aminovorans* (homology 98%). The 16S rDNA gene sequences of the strains which had high homology and foregone classification as reference strains together with the isolate were used for phylogenetic analyses. Multiple alignments were applied using the MEGA software. Phylogenetic trees were constructed using the remaining multisequencing after deleting the extrude base of 5'end and 3'end so that their lengths matched that of the shortest sequence, based on the neighbor joining method (Figure 4). Phylogenetic analyses showed that this isolated strain was located at same branch with *Paracoccus aminovorans* and they were most closely related as to the genetic relationship. This was in harmony with the result of blast. So CT was initially identified as *P. aminovorans* based on 16S rDNA sequence analysis together with the results of morphological observation and physio-biochemical test



**Figure 2.** Electron micrograph of strain CT showing the morphologic characteristic. Magnification:  $\times 5,000$ .



**Figure 3.** Electrophoresis of clone PCR products of the strain CT.

(Urakami et al., 1990).

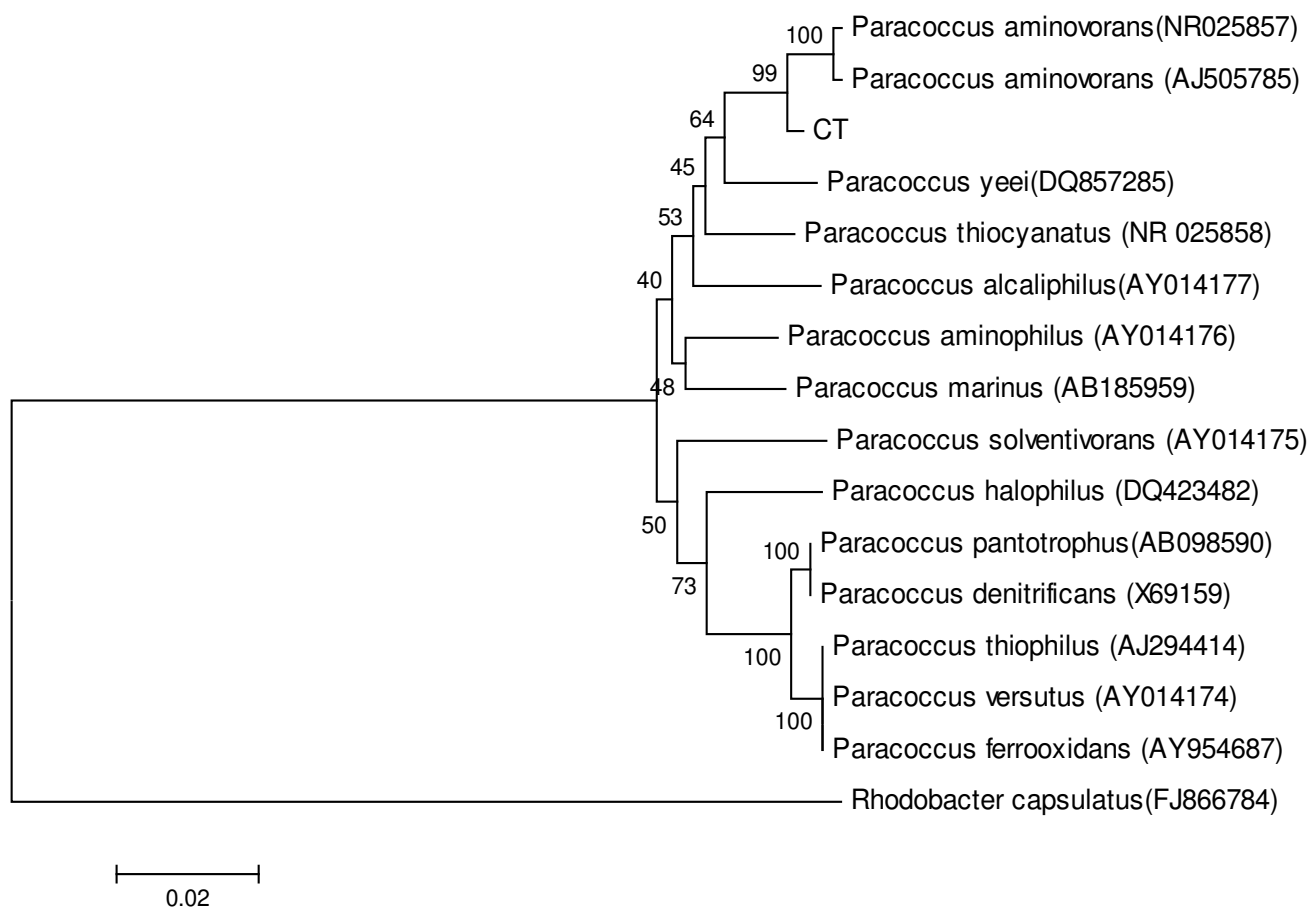
#### Extraction and elimination of plasmid of the strain

Electrophoresis in 0.7% agarose gel was carried out to check plasmid extraction result. Electrophoresis pattern (Figure 5) showed that plasmid with better quality could be obtained by alkaline lysis method. It was found that the plasmid was more than 15 kb. Comparing with marker standard zone, it belonged to big plasmid.

#### Transformation of plasmid of the strain

In order to verify whether plasmids of the strain CT had been transformed into competence cells *E. coli* DH10B, the extraction of plasmids from transformants DHCT was carried out. The electrophoresis pattern for transformation of plasmid of the strain is shown in Figure 6.

As shown in Figure 7, transformants DHCT contained



**Figure 4.** Unrooted phylogenetic tree based on comparison of 16S rDNA gene sequences demonstrating the evolutionary relationships between strains CT and relating species. The GenBank accession number for each microbes used in the analysis is shown in parentheses after the species name. Bootstrap values (%) are indicated at the nodes.

plasmids as big as the strain CT. This indicated that, the plasmids had been successfully transferred into the competence cells *E. coli* DH10B.

#### Location of degradation gene

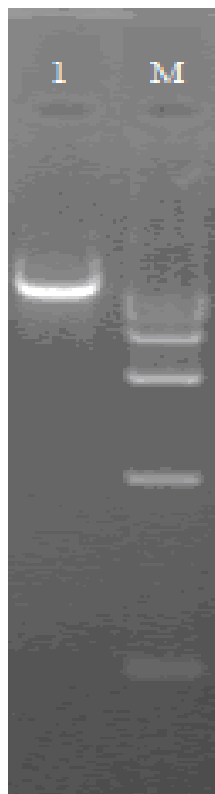
In order to elucidate the relationship between the plasmid and chrysene-biodegradability of strain CT, chrysene-biodegradability of the isolated CT (wild strain), transformant DHCT (transformed to harbor a plasmid), mutant strain ct (CT's plasmid missed) and competence cells *E. coli* DH10B were studied.

Degradation rate of chrysene by strain CT and transformants DHCT was 87 and 43%, respectively, while mutant strain ct and *E. coli* DH10B in the control group did not have the ability to degrade chrysene. This indicated that the transformant was endowed with the degradation ability of chrysene at the same time of obtaining the plasmid. So, we can conclude that the plasmid of strain CT carried chrysene-degrading genes.

#### DISCUSSION

A *Paracoccus* strain, capable of degrading chrysene, was isolated and screened in this study. Compared with related report, the isolated strains had strong degradation ability with a brief period of adjustment in the culture system (Hadibarata, 2009; Dhote et al., 2009). Several reports have suggested that genus *Paracoccus* has versatile degrading capacity. Besides HMW PAHs (containing 3 or more aromatic rings), genus *Paracoccus* could degrade pyridine (Qiao and Wang, 2010), dimethoate (Li et al., 2010), theophylline (Xu et al., 2002), monocrotophos (Jia et al., 2006), trimethylamine (Kim et al., 2001) and so on. It can be seen clearly that *Paracoccus* bacteria may play an important role in degrading toxic environmental pollutants.

Bacterial plasmids is important in aspect like construction of genetic engineering bacterium, microbial genetics and strain improvement, the biological treatment of environmental pollution; so the research on plasmid characteristics is of great significance. In this research,



**Figure 5.** Agarose gel electrophoretogram of plasmid DNA purified from strain CT. M, Marker DL15000; 1, the plasmid DNA of strain CT. The plasmid of strain CT was drastically eliminated after treating strain CT by 60 mg/l of SDS and 43°C for 1 h (lane 3). The result of the experiment for elimination of plasmid of the strain CT is shown as Figure 6.



**Figure 6.** Agarose gel electrophoretogram of the plasmid DNA purified from strain CT treated with SDS and high temperature. (1) Plasmid DNA of strain CT; (2) residual plasmid DNA of strain CT treated with SDS and high temperature (40°C, 0.5 h; SDS: 45 mg/l); (3) plasmid DNA of strain CT was removed successfully (43°C, 1 h; SDS: 60 mg/l).

alkaline lysis was applied to the extract plasmids from strain CT. The method for plasmid extraction, to some extent, depends on the magnitude of plasmid. Alkaline lysis is a mild method. It can avoid damaging the plasmid DNA because of fierce lysis. So, it is appropriate for the extraction of big plasmid. It is also worth noting that there existed a plasmid in a naphthalene-degrading strain we obtained from the sludge of Zhenjiang coking plant at the same time, which was similar in size to the plasmid of strain CT. The naphthalene-degrading strain does not belong to the genus *Paracoccus*. We conjectured that the horizontal transfer of the plasmid between the cells of strain CT and the naphthalene-degrading strain was carried out in the selective conditions (PAHs existed). Furthermore, this work utilized the method of SDS with high temperature to eliminate the plasmid of strain CT. To eliminate plasmid is an important method of judging whether a certain phenotype of the cell is controlled by the plasmid DNA. Most of the plasmids are stable in cell. So, to obtain a strain missed plasmids, it is necessary to

use an appropriate physical or chemical method to interfere the duplication or reduce stability of the plasmids, thus, the loss frequency of plasmids can be promoted (Lou et al., 2002). This work also confirmed that the plasmid of the isolated strains was a degrading plasmid. In 1972, the SAL plasmid, which confers salicylate metabolism, was first found by the American Scholar Chakrabarty (Chakrabarty, 1972). More than 30 kinds of degrading plasmid were discovered from natural isolated strains, which can degrade camphor (Rheinwald et al., 1973), octane (Chakrabarty et al., 1973), naphthalene (Connors and Barnsley, 1982), toluene (Chakrabarty et al., 1978), xylol (Friello et al., 1976), polychlorinated biphenyl (Chaudhry and Chapalamadugu, 1991) and so on. Previous reports demonstrated that plasmid detection rate of the bacteria in the polluted environment are much higher than the bacteria in clean environment because the former can formed widespread plasmid to adapt to the surrounding environment by adjusting their structure and physiological condition. In



**Figure 7.** Agarose gel electrophoretogram of plasmid DNA purified from transformants DHCT. (1) Plasmid DNA of strain CT; (2) plasmid extraction of *E. coli* DH10B (negative control); (3) plasmid DNA of transformants DHCT.

this way, related genes normally exist in plasmid. Perhaps, the plasmid of CT is formed just like this; But if continually, pressure is dispensed to the selection of the strain, related degradation genes of the strain is likely to be gotten from evolution through gene recombination and mutation or straightforward, integrate the degradation genetic fragments on the plasmid into their chromosomes to avoid poisoning itself caused by the loss of the plasmid and the corresponding capability.

## Conclusions

A predominant chrysene-degrading strain was isolated and screened from the sludge of Zhenjiang coking plant. It utilized chrysene as sole carbon source to grow and multiply under the conditions of this work. The strain was initially identified as *P. aminovorans* by morphological observation, physio-biochemical test and 16S rDNA sequence analysis. Under the conditions of initial concentration of 40 mg/l chrysene, 10% (v/v) inoculation amount, pH 7.0 and temperature 35°C, the degradation

efficiency of chrysene by the strain CT reached 85.2% within 8 days.

It was also described that this organism contained a stable plasmid of more than 15 kb. The transformant obtained the ability to degrade chrysene when the plasmid of strain CT was transformed to the competent cell of *E. coli* DH10B. This indicated that the plasmid of strain CT participate in coding enzyme system related with the biodegradation of chrysene.

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