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# Characterization of Phenol Biodegradation by *Comamonas testosteroni* ZD4-1 and *Pseudomonas aeruginosa* ZD4-3

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**Objective** To investigate the characteristic and biochemical mechanism about the phenol biodegradation by bacterial strains ZD 4-1 and ZD 4-3. **Methods** Bacterial strains ZD 4-1 and ZD 4-3 were isolated by using phenol as the sole source of carbon and energy, and identified by 16S rDNA sequence analysis. The concentrations of phenol and total organic carbon (TOC) were monitored to explore the degradation mechanism. The biodegradation intermediates were scanned at 375 nm by using a uv-vis spectrophotometer. The enzyme assays were performed to detect the activities of dioxygenases. **Results** Bacterial strains ZD 4-1 and ZD 4-3 were identified as *Comamonas testosteroni* and *Pseudomonas aeruginosa* by 16S rDNA sequence analysis, respectively. The growth of the two strains was observed on a variety of aromatic hydrocarbons. The strains ZD 4-1 and ZD 4-3 metabolized phenol via *ortho*-pathways and *meta*-pathways, respectively. In addition, the results of enzyme assays showed that the biodegradation efficiency of phenol by *meta*-pathways was higher than that by *ortho*-pathways. Finally, the results of induction experiment indicated that the catechol dioxygenases, both catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O), were all inducible. **Conclusion** The strains ZD 4-1 and ZD 4-3 metabolize phenol through *ortho*-pathways and *meta*-pathway, respectively. Furthermore, the biodegradation efficiency of phenol by *meta*-pathways is higher than that by *ortho*-pathways.

**Key words:** Aromatic compounds; Phenol; Biodegradation; *Ortho*-pathways; *Meta*-pathways; C12O; C23O

## INTRODUCTION

Aromatic compounds are widely used in industrial and agricultural activities, and often released into the environment during their use. Due to their toxic properties and persistence in nature, the biodegradation of these compounds has attracted strong attention[1-3]. Phenol, a typical and characteristic aromatic compound, is regarded as a priority contaminant by China Environmental Protection Agency[4]. It occurred widely in wastes, rivers, groundwater and even in the atmosphere. Given the wide spectrum of pollution potential, the ability of bacteria to degrade phenol is of considerable interest and environmental importance.

The bacterial catabolism of phenol has been studied in some detail and the sequences have been identified[5,6]. In general, phenol is degraded via catechol which is further degraded either by cleavage between two hydroxyl groups by catechol 1,2-dioxygenase (C12O) via an *ortho*-pathway or by cleavage adjacent to the hydroxyl groups by catechol 2,3-dioxygenase (C23O) via a *meta*-pathway, respectively. However, little is known

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concerning the biodegradation efficiency and the mineralization extent of phenol by two different pathways.

We are interested in the construction of genetically engineered bacteria, which can biodegrade a wide range of aromatic hydrocarbon pollutants efficiently in hostile natural environment. As a prelude to detailed investigations of the pathways and genetic organization of aromatic hydrocarbon metabolism in these two strains, this manuscript describes initial characterization of the biochemistry and preliminary genetics of *C. testosteroni* ZD 4-1 and *P. aeruginosa* ZD 4-3.

## MATERIAL AND METHODS

### *Media and Culture Conditions*

The LB medium (pH 7.0) contained 10 g of ploypeptone, 5 g of yeast extract, and 5 g of NaCl per liter. The mineral salts medium (MS, pH 7.0) contained 0.1 g of $MgSO_4$. $7H_2O$, 0.02 g of $CaCl_2.2H_2O$, 0.68 g of $KH_2PO_4$, 1.73 g of $K_2HPO_4$, 0.03 g of $MnSO_4$. $H_2O$, 1.0 g of $NH_4NO_3$, 0.03 g of $FeSO_4.7H_2O$ per liter. In preparation of phenol mineral salts medium (PMS), a certain volume of phenol from a prepared stock solution was added to the medium directly to give the desired initial concentrations. Solid media were prepared by adding 1.8% agar.

### *Isolation and Identification of Phenol-degrading Bacteria*

Sludge samples were obtained from a pesticide-manufacturing factory in Hangzhou, China. One-gram sludge was inoculated into 125 mL of PMS medium. Phenol was provided in the medium as the sole carbon source. Cultures were incubated at 30℃ with shaking at 150 rpm in a shaker bed. Phenol concentrations in the PMS were monitored by high-performance liquid chromatography (HPLC, see below). 10% (about 10 mL) of the culture medium was transferred to fresh PMS when the concentrations of the phenol in the culture medium decreased and the cultures became turbid. After one-month enrichment, samples were spread on LB agar plates supplemented with phenol as the sole carbon source. The colonies were purified by classical purification procedure after they formed on the LB plates.

To identify the isolates, the 16S rRNA genes were amplified by PCR with primer pairs[7]. BSF8/20: 5'-AGAGT TTGAT CCTGG CTCAG-3' and BSR1541/20: 5'-AAGGA GGTGA TCCAG CCGCA-3'. PCR mixtures (50 μL final volume) contained 10×PCR buffer 5 μL, 25 mM $MgCl_2$ 4 μL, 5 mM dNTP 2 μL, each primer 1μL, template DNA 100 ng, *Taq* polymerase (10 000 U/mL) 0.5 μL, sterile distilled water 35.5 μL. The following amplification parameters were used: initial denaturation at 94℃ for 2 min, then at 94℃ for 1 min, at 58 ℃ for 1 min and at 72℃ for 2 min (29 cycles), and a final extension at 72℃ for 10 min, then stored at 4℃ for 10 min. The PCR products were purified by using a QIA purification kit (QIA Gene Corp.). A partial sequence of the PCR products was determined by Shanghai Shenyou Biotechnology Company. Related sequences were obtained from the Genbank Database (National Center for Biotechnology Information (NCBI)) by using the BLASTN program[8].

### *Phenol Degradation*

The Isolates were grown in LB medium at 30℃ for overnight. Cells were aseptically harvested, washed twice in sterile phosphate buffer (0.1M, pH 7.5), and resuspended in 10 mL

of the same buffer. The cell suspensions were transferred to 250 mL sterile flasks. Sterile PMS was added to the phenol final concentration of 500 mg/L. The concentrations of phenol and total organic carbon (TOC) were detected at a 12 h interval by HPLC (see below) and by TOC analyzer (TOC, Apollo 9000, USA), respectively. At the same time, the bacterial biomass was monitored spectrophotometrically at $OD_{600}$.

A negative control without inoculum was also included. All treatment was conducted in duplicate.

*Growth on Other Aromatic Compounds*

The ability of phenol-degrading strains to use other aromatic compounds as growth substrates was screened by auxanography[9].

*Curing Experiment*

Acridine orange was added at a final concentration of 50 mg/L to LB medium inoculated with about $10^6$ exponential-phase cells per mL. After 24 h incubation at 30℃, the culture was diluted with saline and plated out onto LB agar plates. The colonies appearing on the plates were replicated onto PMS plates to examine phenol-degrading ability.

*Detection of Intermediates of Phenol Metabolism*

The bacteria were spread on LB medium and incubated overnight. A 250 mg/L phenol solution was sprayed onto the plate. The bacteria were incubated at 30℃ for 15 min. The plates were eluted with a small amount of phosphate buffer (0.2M, pH 7.0) and centrifuged at 4℃, 10 000 rpm. The supernatant was scanned with a Shimadzu UV-1206 spectrophotometer. The yellowish compound 2-hydroxymuconic semialdehyde (HMS) gives a characteristic absorption peak at 375 nm[10].

*Preparation of Enzyme Extracts*

Cells collected were washed twice with cold 100 mM potassium phosphate buffer (pH 7.5) and resuspended in the same buffer. The cell suspension was disrupted by sonication with 99×3s bursts on ice. Unbroken cells and cell debris were removed by centrifugation at 12000×g for 10 min at 4℃.The clear supernatant obtained was used for enzyme assays. The protein concentration of cell extracts was determined by the method of Bradford[11] with bovine serum albumin as a standard.

*Enzyme Assays*

Enzyme activities were measured with UV-visible spectrophotometer (Shimadzu UV-1206) at 25℃. Reaction mixtures contained 2 mL EDTA (40 mM), 13.8 mL phosphate buffer (50 mM, pH 7.5), 0.2 mL catechol (30 mM) and 4 mL cell-free extracts. The reaction was started by adding catechol. Activities of C12O and C23O were measured spectrophotometrically by monitoring the formation of reaction products at 260 nm and 375 nm, respectively. The crude extracts used for C12O activity measurements were pre-treated for 5 min with $H_2O_2$ (0.01%), to suppress the activity of C23O[12].

For experiments with induced cells, cultures containing phenol in LB were grown for 24 h at 30℃ with shaking (150 rpm). Cells were harvested by centrifugation and washed twice with fresh phosphate buffer (0.2 M, pH 7.0) before use in subsequent experiments. Uninduced cells were grown overnight in LB medium.

*Analytical Methods*

Phenol concentration was determined at a 12 h interval by HPLC and HPLC analysis was performed on an Agilent Eclipse XDB-C8 column (Agilent 1100 series). With ethanol and water (70:30, v/v) as the mobile phase, flow rate was 1.0 mL/min. Compounds were monitored at a UV A254 with a G1313A ALS diode array detector (Agilent 1100 series). TOC concentration was monitored by a TOC Combustion Analyzer (Apollo 9000 Tekmar-Dohrmann, USA) at a 12 h interval.

*Chemicals*

If mentioned specifically, all chemicals were of analytic purity and commercially available.

## RESULTS

*Isolation and Identification of Phenol-degrading Bacteria*

After one month of enrichment, several strains of bacteria able to use phenol as the sole carbon and energy source were isolated. Two strains, designated as strain ZD 4-1 and strain ZD 4-3, were selected for further study. They were all gram-negative rods, and strain ZD 4-1 was flagellate while ZD 4-3 was not. On the basis of their characteristic and the 16S rDNA sequence analysis, strains ZD 4-1 and ZD 4-3 were identified as *C. testosteroni* and *P. aeruginosa*, respectively.

*Growth on Phenol*

Using the mineral salts medium containing 250 mg/L phenol as the sole source of carbon and energy, strain ZD 4-1 grew well between pH 6.0 and 10.0 and achieved its optimal growth at about pH10.0, while ZD 4-3 obtained its optimal growth at about pH 7.0 and its pH growth scope was very narrow (Fig.1). On the other hand, the concentration of residual phenol was very compatible with the growth of the bacterial strains. In addition, strain ZD 4-3 was able to grow with the phenol concentration up to 1 000 mg/L, but strain ZD 4-1 could only tolerate the phenol concentration up to 500 mg/L. These data showed that strain ZD 4-1 owned a wider pH tolerant ability than strain ZD 4-3, but in terms of phenol concentration, strain ZD 4-3 grew at a higher phenol concentration than ZD 4-1 did.

*Growth on Aromatic Compounds*

As for cultures inoculated with uninduced cells, strain ZD 4-1 only grew on 2,4-dichlorophenol and *p*-nitrophenol (PNP), while strain ZD 4-3 grew on 2,4-dichlorophenol, PNP and phenanthrene. After induction with phenol, strain ZD 4-1 was able to grow on 2,4-dichlorophenol, PNP, naphthalene, anthracene and phenanthrene when naphthalene, anthracene and phenanthrene were provided as solid crystals to cultures on agar plates, while strain ZD 4-3 only grew on pentachlorophenol (PCP), 2,4-dichlorophenol, PNP and phenanthrene (Table 1).

*Activity of Catechol Dioxygenases*

To obtain information concerning the catabolism of phenol by strains ZD 4-1 and ZD 4-3, the specific activities of catechol dioxygenases were determined in crude extracts obtained

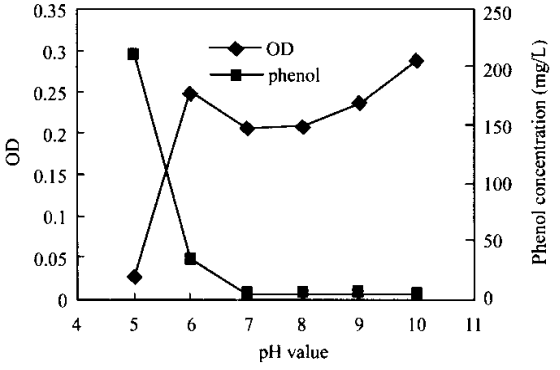


FIG. 1(a). Growth of strain ZD 4-1 on phenol at different pH value.

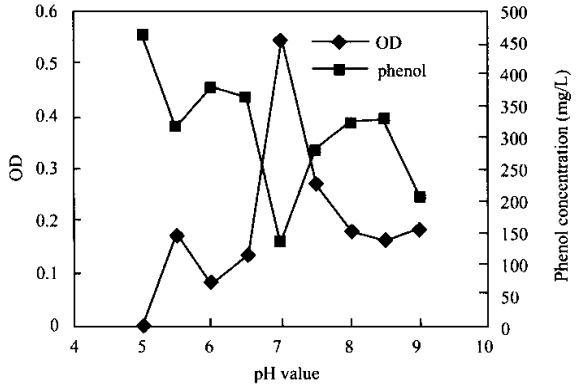


FIG. 1(b). Growth of strain ZD 4-3 on phenol at different pH value.

TABLE 1

Growth of Strains ZD 4-1 and ZD 4-3 on Aromatic Compounds

| Substrates | Growth of ZD 4-1 | | Growth of ZD 4-3 | |
|---|---|---|---|---|
| | Uninduced Cells | Induced Cells | Uninduced Cells | Induced Cells |
| 2,4-dichlorophenol | + | + | + | + |
| *p*-nitrophenol | + | + | + | + |
| Pentachlorophenol | − | − | + | + |
| Phenanthrene | − | + | − | + |
| Naphthalene | − | + | − | − |
| Anthracene | − | + | − | − |

*Note*: + represents the growth was observed; − represents the growth could not be observed.

from these two strains (Table 2). These data revealed that only C12O activity was detected in strain ZD 4-1 rather than in strain ZD 4-3. In contrast, C23O only occurred in strain ZD 4-3, rather than in strain ZD4-1.The phenomenon indicated that strain ZD 4-1 cleaved phenol via *ortho*-pathways while strain ZD 4-3 via *meta*-pathways. On the other hand, after induction, the activity of C23O was 85 times as high as that before induction and the C12O was 15 times of that. According to the activities of catechol dioxygenases, the dioxygenases, both C12O and C23O, were strongly inducible enzymes because their activities were dependent on their inducers.

TABLE 2

Specific Activities of Catabolic Enzymes in Crude Extracts of Strains ZD4-1 and ZD 4-3

| Strain and Enzymes | | Activity* (μmol $min^{-1}$ mg $Protein^{-1}$) | |
|---|---|---|---|
| | | Before Induction | After Induction |
| ZD 4-1 | C12O | 0.1406 ± 0.1108 | 2.1392 ± 0.0986 |
| | C23O | ND | ND |
| ZD 4-3 | C23O | 0.1181 ± 0.0698 | 10.058 ± 0 |
| | C12O | ND | ND |

*Note*: *All data shown were obtained from at least three independently performed experiments.

*Ring Cleavage of Catechol*

The catabolism products of phenol by strains ZD 4-1 and ZD 4-3 were scanned by uv-vis spectrophotometrically (Fig. 2). A characteristic absorption maximum of 2-hydroxymuconic semialdehyde (HMS) at 375 nm appeared on the curve of strain ZD 4-3, but it was not observed on the curve of strains ZD 4-1, suggesting that HMS is formed during the catabolism process of phenol. Therefore, it could be deduced that strain 4-3 cleaved the phenol via *meta*-pathways, while strain ZD 4-1 metabolized the phenol via *ortho*-pathways. This was in agreement with the results of enzymes assay.

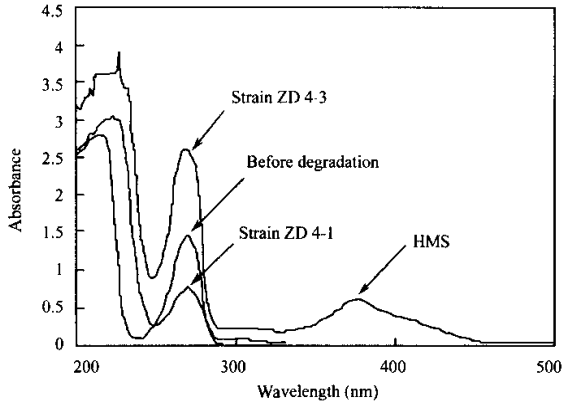


FIG. 2. The UV absorbance curves of two strains ZD 4-1 and ZD 4-3.

*Phenol Biodegradation and Mineralization*

In order to further compare the biodegradation characteristics of *ortho*-pathways and

*meta*-pathways by strains ZD 4-1 and ZD 4-3, attempts were made to investigate the biodegradation efficiency and mineralization extent of phenol. The results (Fig. 3) showed that at the phenol concentration of 250 mg/L, strain ZD 4-3 converted phenol quickly. The phenol disappeared completely after 24 h, but in the culture of ZD 4-1 it disappeared after 48 h. In terms of TOC removal, strain ZD 4-3 also exhibited higher removal ability than ZD 4-1. These data were also compatible with the biomass increase of strain ZD 4-1 and ZD 4-3 in the same cultures. It is noteworthy that strain ZD 4-3 attained its maximum biomass at 12 h and then decreased slightly because the phenol, the sole source of carbon and energy, was exhausted. Contrary to that, the biomass of strain ZD 4-1 increased more slowly. It is clear that strain ZD 4-3 bearing a *meta*-pathway in phenol metabolism was more efficient than strain ZD 4-1 metabolizing phenol via *ortho*-pathways in phenol biodegradation. The results were confirmed by the activities of catechol dioxygenases previously in this paper.

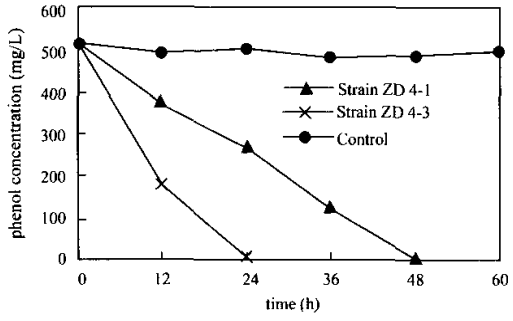


FIG. 3 (a). Phenol biodegradation by strains ZD 4-1 and ZD 4-3.

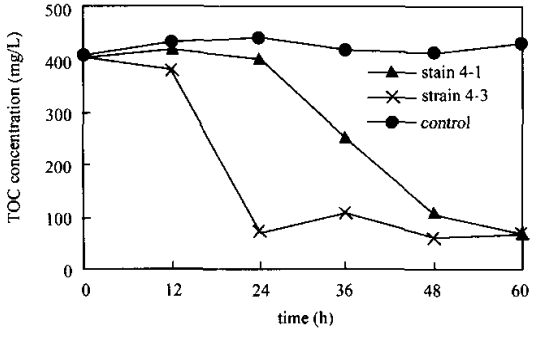


FIG. 3 (b). TOC removal by strains ZD 4-1 and ZD 4-3.

*Preliminary Gene Location*

The curing experiment was carried out to determine whether the enzymes responsible for *meta*-pathways and *ortho*-pathways were chromosomally or plasmid encoded in the bacterial strains. After treatment by acridine orange 50 mg/L, the strain ZD4-3 was able to grow on the PMS agar plate, while ZD 4-1 could not grow on PMS medium any longer. This

result indicated that the genes responsible for the *meta*-cleavage in strain ZD4-3 were located on the chromosome, and the genes encoding the *ortho*-pathways were plasmid encoded. It also could be observed that strain ZD4-1 could not grow on PMS after being transferred on LB medium several times, but ZD 4-3 could do it. This suggests that the gene of C12O may be located on the plasmid and the gene of C23O on the chromosome.

## DISCUSSION

Due to the toxicity and carcinogenicity of aromatic compounds to living organisms, much attention has been paid to their biodegradation in natural environment. Many researchers suggested that construction of genetically engineered bacteria for bioremediation was a promising method to attenuate the harm of these xenobiotic compounds[13-15]. So, investigation of the biochemistry and genetic mechanisms of the pollutants biodegradation in the bacterial strains is extremely necessary.

Bacterial strains ZD 4-1 and ZD 4-3 were selected for further research for their fast phenol biodegradation. The identification results showed that strains ZD 4-1 and ZD 4-3 belonged to *C. testosteroni* and *P. aeruginosa*, respectively. Many different biodegradation characteristics were observed between these two strains when they grew on phenol and other aromatic compounds. The strain ZD 4-1 was able to biodegrade phenol at a wide range of pH scope, while ZD 4-3 at a narrow one, suggesting that the former could tolerate a strong pH fluctuation. The results of aromatic compounds utilization revealed that although these two strains were capable of using a variety of aromatic hydrocarbon pollutants, strain ZD 4-1 metabolized not only the compounds with simple ring, but also the two-cyclic compounds, while strain ZD 4-3 could not use them except from phenanthrene. The greatest surprise was that strain ZD 4-3 could use phenanthrene instead of naphthalene and anthracene as the sole source of carbon.

In order to clarify the metabolism pathways of phenol by strains ZD 4-1 and ZD 4-3, the intermediates of phenol metabolism as well as activities of catechol 1,2- dioxygenase and catechol 2,3-dioxygenase were detected. The results revealed that phenol was metabolized by different ways in these two strains, in which strain ZD 4-3 degraded phenol via *meta*-pathways and strain ZD 4-1 via *ortho*-pathways. According to the intermediates detection and key enzymes assay, the metabolic routes carried in bacterial strains ZD 4-1 and ZD 4-3 were proposed for phenol biodegradation (Fig. 4).

To our knowledge, the *meta*-pathways and *ortho*-pathways are carried in the same bacterial strain in many cases. In their recent paper, Heinaru[12] *et al.* also demonstrated that many phenolic compounds degraders harbored two types of catabolism way. But it was demonstrated that the aromatic compounds degrading bacteria might own only one type of metabolism way. This was somewhat an unexpected result.

Given that information, it is interesting to investigate the degradation efficiency and mineralization extent of the two different metabolism ways in these strains. To date, little is known about the biodegradation efficiency and mineralization extent by the two types of degradation way. To explore that problem, biodegradation and mineralization tests were carried out. The results revealed that phenol degradation by *meta*-pathways was faster than by *ortho*-pathways though the mineralization extent by the two types of metabolism way were equal. This suggested that the *meta*-pathways and *ortho*-pathways owned by strains ZD 4-3 and ZD 4-1 could mineralize the phenol completely.

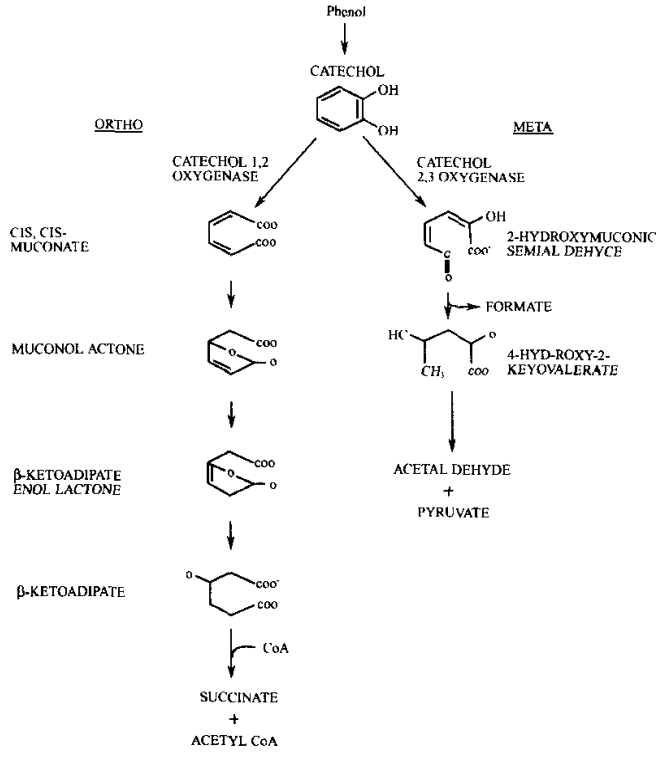


FIG.4. The putative pathways of phenol biodegradation by strains ZD 4-1 and ZD 4-3.

It is known that the enzymes taking part in the degradation of hydrocarbons in *P. putida* are all inducible[16]. The results reported here also demonstrate that both C12O and C23O are all inducible and the activities of catechol dioxygenases are strongly dependent on the inducers.

Generally, genes encoding the *meta*-pathways in aromatic compounds metabolism are carried on plasmids, and the genes responsible for the *ortho*-pathways are chromosomally encoded[17]. But in this paper, our initial results showed that the genes of *meta*-cleavage are carried on chromosome, while the genes responsible for *ortho*-cleavage are located on plasmid. This is somewhat interesting although this should be confirmed by further experiments such as Southern Hybridization with corresponding gene probes.

Catechol dioxygenases are key enzymes in many bacterial pathways for the degradation of aromatic compounds, and the reactions catalyzed by these enzymes are the rate-limiting steps for the decomposition of some aromatic compounds such as *p*-xylene/*p*-toluate and 3-chlorotoluene/s-chlorobenzoate[18-20]. The results described in this paper show that the *meta*-pathways harbored in *P. aeruginosa* ZD 4-3 are of higher efficiency than the *ortho*-pathways in *C. testosteroni* ZD 4-1. However, the former can tolerate narrower pH fluctuation and its growth ability on other aromatic compounds is inferior to the latter. So we suggest that it is possible to construct efficient aromatic compounds-degrading bacterium by transferring the genes encoding the *meta*-pathways in strain ZD 4-3 into strain ZD 4-1. In

this way, it will enhance the degradation ability of engineered bacterial strain.

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