Cloning of Catechol 2,3-Dioxygenase Gene and Construction of a Stable Genetically Engineered Strain for Degrading Crude Oil

Yun Xie · Feng Yu · Qi Wang · Xin Gu ·
Wuling Chen

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Abstract *Pseudomonas putida* strain BNF1 was isolated to degrade aromatic hydrocarbons efficiently and use phenol as a main carbon and energy source to support its growth. Catechol 2,3-dioxygenase was found to be the responsible key enzyme for the biodegradation of aromatic hydrocarbons. Catechol 2,3-dioxygenase gene was cloned from plasmid DNA of *P. putida* strain BNF1. The nucleotide base sequence of a 924 bp segment encoding the catechol 2,3-dioxygenase (C23O) was determined. This segment showed an open reading frame, which encoded a polypeptide of 307 amino acids. C23O gene was inserted into NotI-cut transposon vector pUT/mini-Tn5 (Km^r) to get a novel transposon vector pUT/mini-Tn5-C23O. With the helper plasmid PRK2013, the transposon vector pUT/mini-Tn5-C23O was introduced into one alkanes degrading strain *Acinetobacter* sp. BS3 by triparental conjugation, and then the C23O gene was integrated into the chromosome of *Acinetobacter* sp. BS3. And the recombinant BS3-C23O, which could express catechol 2,3-dioxygenase protein, was obtained. The recombinant BS3-C23O was able to degrade various aromatic hydrocarbons and n-alkanes. Broad substrate specificity, high enzyme activity, and the favorable stability suggest that the BS3-C23O was a potential candidate used for the biodegradation of crude oil.

Keywords Catechol 2,3-dioxygenase · Biodegradation · pUT/mini-Tn5 · Genetically engineered microorganisms

Introduction

Environmental contamination by oil and its derivatives has become a serious problem in this century [1]. Nowadays more and more pollutions and hereby toxicity to biological system are increasing with the rapid development of exploitation, production, and transport of crude oil on the earth [2]. Biodegradation by naturally occurring populations of microorganisms is a major mechanism of oil removal from the environment [3]. Crude oil is composed of a wide range of hydrocarbons, including saturated compounds and polynuclear aromatic hydrocarbons, some of which are suspected carcinogens [4]. Different components of crude oil are degraded at different rates. The ability of a number of microorganisms to degrade phenol has therefore been studied to provide efficient strains for bioremediation processes [5].

Soil microorganisms such as *Pseudomonas putida* have the ability to aerobically catabolize a wide range of aromatic hydrocarbons via suites of specialized catabolic enzymes [6]. The genes responsible for the meta-ring cleavage of aromatic hydrocarbons have been extensively studied in various microorganisms [7, 8]. Catechol 2,3-dioxygenase transforms catechol by meta-cleavage to 2-hydroxymuconate semialdehyde.

Many strains were shown to acquire the ability to degrade various toxic compounds during their evolution, but the rate and efficiency of removal of these compounds by bacteria are mostly low, because they evolve on the basis of environmental fitness rather than degradation efficiency [9]. The strain *Acinetobacter* sp. BS3 was found to degrade alkanes in crude oil very efficiently and it was capable of producing bio-surfactant. *P. putida* strain BNF1 was isolated to degrade aromatic hydrocarbons efficiently and use phenol as a main carbon and energy source to

Y. Xie · F. Yu · Q. Wang · W. Chen (✉)
Biology Department, Northwestern University, Xi'an, China
e-mail: wuling_chen@126.com

X. Gu
Sinochem Chemical Industry Park, Taicang, China

Table 1 Bacterial strains and plasmids used in this work

Strains and plasmids	Description	Reference
BS3-C23O	Genetically engineered strain, Str ^r	This work
<i>E. coli</i> S17-1 (λ pir)	Host strain for pUT/mini-Tn5, km ^r	Biovector, Inc
<i>E. coli</i> HB101	Host strain for helper plasmid pRK2013, Str ^r	TaKaRa Corp.
pUT-mini-Tn5	Derivative of Tn5 Km ^r , Amp ^r , oriR6 K	Biovector, Inc
pRK2013	Helper plasmid, km ^r	Biovector, Inc

support its growth. The efficiency of these strains may be improved by physiological adaptation or by genetic engineering.

In this study, the *C23O* gene was cloned from the aromatic hydrocarbons-Degrading *P. putida* BNF1 and sequenced. Then the *C23O* gene was integrated into the chromosome of BS3 by the transposon mini-Tn5 to extend the degrading capability of *Acinetobacter* sp. BS3. Our data suggest that the recombinant strain BS3-C23O with favorable stability, could degrade alkanes and aromatic hydrocarbons simultaneously and was a potential candidate used for the biodegradation of crude oil.

Materials and Methods

Bacterial Strain, Plasmid and Culture Conditions

Aromatic hydrocarbons-degrading strain *P. putida* NA3 (GenBank accession number EF100617) and alkanes-degrading strain *Acinetobacter* sp. BS3 (GenBank accession number JX992849) were used in this study. Strains were cultured aerobically at 30 °C in mineral salt medium [10] with phenol (mixed oil) as the sole carbon source. The plasmid pGEM T-Easy (Promega Corp.) was used as cloning and sequencing vector and *Escherichia coli* JM109 (Promega Corp.) was used as a host strain for the recombinant plasmid DNA. *E. coli* cells were grown at 37 °C on Luria–Bertani (LB) medium. Other bacterial strains and plasmids used in this study are listed in Table 1.

Cloning and Sequencing of Catechol 2,3-Dioxygenase Gene

Plasmid DNA was isolated by the alkali lysis method as described by [11]. A pair of specific primers PSCA23St and PSCA23End [12] were used based on the nucleotide sequence of *P. putida* plasmid NAH7 *C23O* (GenBank accession number M17159). PCR amplification was carried

out using rTaq DNA polymerase (TaKaRa). The PCR products were purified with the Cycle-Pure Kit (Omega) and then ligated into the pGEM-T Easy vector and used to transform *E. coli* JM109 cells. White colonies on LB plates containing 50 $\mu\text{g ml}^{-1}$ ampicillin, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) and isopropyl- β -D-thiogalactopyranoside (IPTG) were picked, and recombinant DNA was extracted and purified by Wizard plus plasmid DNA purification kit [13]. The nucleotide sequence analysis of the selected clones was determined with a model ABI 310 sequencer (Applied Biosystems, CA, USA). Protein sequence analysis was performed using the software DNAMAN and BLAST program (NCBI).

Construction and Identification of Recombinant Expression Vector

A specific PCR amplification was redesigned to obtain the complete catechol 2,3-dioxygenase gene *C23O*. The forward primer 23OF was 5'-ATTTGCGGCCGCAGGTGCTCGGTTTCTATCTGTTTA-3' and the reverse primer 23OR was 5'-ATTTGCGGCCGCATGGTCTTGCCGTGAGTGTTTA-3'. The underlined sequence in each primer was a NotI site. Amplification was performed in the PCR buffer using about 20 ng of the BS3 plasmid DNA as a template, thermal cycler temperature controller program was set as follows: 95 °C for 5 min, 30 s, 68 °C for 30 s, 72 °C for 1 min, 10 min, and 4 °C forever. The PCR product was digested by NotI for 24 h and then inserted into NotI-cut transposon vector pUT/mini-Tn5 (Km^r) to form a novel transposon vector pUT/mini-Tn5-C23O (Fig. 1). pUT/mini-Tn5-C23O was transformed into *E. coli* S17-1 (λ pir) by electroporation and the positive cloning was picked.

Fig. 1 Map of plasmid pUT-mini-Tn5-C23O

Triparental Conjugation

The recipient (*Acinetobacter* sp.BS3), donor (*E. coli* S17-1 λ pir harboring the suicide vector pUT/mini-Tn5-C23O), and helper (*E. coli* HB101 with pRK2013) strains were grown individually in LB overnight with the concentrations of $30 \mu\text{g ml}^{-1}$ for streptomycin, $50 \mu\text{g ml}^{-1}$ for kanamycin. After incubation of the recipient at 42°C for 15 min to temporarily inactivate its restriction systems, 2 ml recipient was mixed with 3 ml of the donor and 3 ml of the helper. Cells were collected by centrifugation at 10,000 rpm at room temperature for 3–5 min, after washing three times with saline, the cells were resuspended to remove the antibiotics in fresh LB, and spotted on bacterial membrane of LB solid plate. After overnight incubation at 30°C , cells were washed off the plate and resuspended in 1 ml of LB, and serial dilutions were plated on selective LB medium containing $50 \mu\text{g ml}^{-1}$ streptomycin, $50 \mu\text{g ml}^{-1}$ kanamycin and $60 \mu\text{g ml}^{-1}$ phenol. To pick the positive transformants, C23O enzyme activity was visually assayed by spraying pyrocatechol solution ($0.2\text{--}0.5 \text{ mol l}^{-1}$) on the conjugants grown on the LB plates. Positive transformants could make colony of bacteria on the plates turn yellow colour.

Enzyme Activity and Substrate Specificity Assay

Acinetobacter sp.BS3 and the recombinant strain BS3-C23O were grown to LB medium, after overnight incubation in a shaking flask (150 r min^{-1}) at 30°C , harvested by centrifugation at 10,000 rpm for 10 min at 4°C and supernatant was extracted, then washed twice with 10 mM potassium phosphate buffer (PH7.5). The bacterial pellets were resuspended in the same buffer and then sonicated with a cell disruptor followed by centrifugation at 12,000 rpm for 30 min to obtain supernatant as cell extract. Catechol 2,3-dioxygenase (C23O) activity was measured

according to the method described by [14]. One unit of enzyme activity was defined as the amount of enzyme that converts $1 \mu\text{mol}$ of substrate to ring-cleavage product per minute at 25°C . And protein concentration was determined by the Bradford method [15]. The representative aromatic compounds were selected, including 2,4-dichlorophenol, pentachlorophenol, naphthalene, anthracene and phenanthrene as the sole carbon and energy source to make selective medium. The phenol induced and non-induced strain BS3 and BS3-C23O were inoculated into the plate for qualitative observation of bacterial growth.

Alkanes Biodegradation Activity and Growth Characteristic Assay

The growth and biodegradation rate (BR) of strain *Acinetobacter* sp.BS3 and recombinant strain BS3-C23O were tested in the mineral medium containing 2 g l^{-1} mixed oil as sole carbon and energy source. Oil concentration was determined according to the gravimetric method (National Standard CJ/T57-1999, China). Oil-biodegradation rate (%) was defined as the amount of oil degraded versus the amount of initial oil. Oil biodegradation rates were measured every 7 days and up to 28 days. Activated cells were inoculated in a shaking flask (180 r min^{-1}) at 30°C . Growth was monitored by measuring absorbance at 600 nm at regular intervals of 4 h using 756MC-type spectrophotometer (Shanghai Spectrum, China).

The Genetic Stability Testing of the Engineered Strain

Precultures of the engineered strain grown in LB at $1 \times 10^8 \text{ CFU ml}^{-1}$ were simultaneously enumerated on both non selective (LB) media and selective (50 mg l^{-1} kanamycin) cultured at 30°C . The stability of the strain was determined by periodical assay for the phenol-degrading activity of the successive plating strains on both selective ($5 \mu\text{g ml}^{-1}$ kanamycin) and nonselective media for 30 days.

Results and Discussion

Nucleotide sequence of catechol 2,3-dioxygenase from strain BNF1

An amplified DNA fragment of $\sim 900 \text{ bp}$ (Fig. 2) was cloned in pGEMT Easy vector and sequenced. The nucleotide sequence of C23O was found to be composed of 924 bp as shown in Fig. 3. Analysis of the nucleotide sequence revealed an open reading frame (ORF) encoding 307 amino acids. The deduced amino acid sequence of C23O from *P. putida* BNF1 exhibited 98 % identity with

Fig. 2 Identification of C23O gene after PCR M, DNA Marker; lines 1 and 2, PCR products of C23O gene

1 ATG AAC AAA GGT ATA ATG CCG CCG GGC CAT GTG CAG CTG CGT GTA 45
 1 Met Asn Lys Gly Ile Met Arg Pro Gly His Val Gln Leu Arg Val 15
 46 CTG GAC ATG AGC AAG GCC CTG GAA CAC TAC GTC GAG TTG CTG GGC 90
 16 Leu Asp Met Ser Lys Ala Leu Glu His Tyr Val Glu Leu Leu Gly 30
 91 CTG ATC GAG ATG GAC CCG GAC GAC CAG GGC CGT CTC TAT CTC AAG 135
 31 Leu Ile Glu Met Asp Arg Asp Asp Gln Gly Arg Val Tyr Leu Lys 45
 136 GCT TGG ACC GAA GTG GAT AAA TTC TTC GTG GTG CTA GGC GAG GCT 180
 46 Ala Trp Thr Glu Val Asp Lys Phe Ser Val Val Leu Arg Glu Ala 60
 181 GAC GAA CCG GGT ATG GAT TTT ATG GGT TTC AAG GTT GTG GAT GAG 225
 61 Asp Glu Pro Gly Met Asp Phe Met Gly Phe Lys Val Val Asp Glu 75
 226 GAT GCT CTC CCG CAA CTG GAG CCG GAT CTG ATG GCA TAT GGC TGT 270
 76 Asp Ala Leu Arg Gln Leu Glu Arg Asp Leu Met Ala Tyr Gly Cys 90
 271 GCC GTT GAG CAG CTA CCC GCA GGT GAA CTG AAC AGT TGT GGC CCG 315
 91 Ala Val Glu Gln Leu Pro Ala Gly Glu Leu Asn Ser Cys Gly Arg 105
 316 CCG GTG CCG TTC CAG GCC CCC TTC GGG CAT CAC TTC GAG TTG TAT 360
 106 Arg Val Arg Phe Gln Ala Pro Ser Gly His His Phe Glu Leu Tyr 120
 361 GCA GAC AAG GAA TAT ACT CGA AAG TGG GGT TTG AAT GAG CTC AAT 405
 121 Ala Asp Lys Glu Tyr Thr Gly Lys Trp Gly Leu Asn Glu Val Asn 135
 406 CCC GAG GCA TGG CCG CCG CAT CTG AAA GGT ATG GCG GCT GTG CGT 450
 136 Pro Glu Ala Trp Pro Arg Asp Leu Lys Gly Met Ala Ala Val Arg 150
 451 TTC GAC CAT TGC CTA CTG TAT GGC GAC GAA TTG CCG GCG ACC TAT 495
 151 Phe Asp His Cys Leu Leu Tyr Gly Asp Glu Leu Pro Ala Thr Tyr 165
 496 GAC CTG TTC ACC AAG GTG CTC GGT TTC TAT CTG GCG GAA CAG GTG 540
 166 Asp Leu Phe Thr Lys Val Leu Gly Phe Tyr Leu Ala Glu Gln Val 180
 541 CTG GAC GAA AAT GCG ACC CCG GTC GCG CAG TTT CTC AGT CTG TCG 585
 181 Leu Asp Glu Asn Gly Thr Arg Val Ala Gln Phe Leu Ser Leu Ser 195
 586 ACC AAG CCC CAC GAC GTG CCC TTC ATT CAC CAT CCG GAA AAA GGC 630
 196 Thr Lys Ala His Asp Val Ala Phe Ile His His Pro Glu Lys Gly 210
 631 CCG CTC CAT CAT GTG TCC TTT CAC CTC GAA ACC TGG GAA GAC TTG 675
 211 Arg Leu His His Val Ser Phe His Leu Glu Thr Trp Glu Asp Leu 225
 676 CTT CCG CCC GCG GAC CTG ATC TCC ATG ACC GAC ACC TCG ATC GAT 720
 226 Leu Arg Ala Ala Asp Leu Ile Ser Met Thr Asp Thr Ser Ile Asp 240
 721 ATC GCG CCA ACC CCG CAC GCG CTC ACT CAC GCG AAG ACC ATC TAC 765
 241 Ile Gly Pro Thr Arg His Gly Leu Thr His Gly Lys Thr Ile Tyr 255
 766 TTC TTC GAT CCG TTC GGT AAC CCG AAC GAA GTG TTC TCG GCG GGA 810
 256 Phe Phe Asp Pro Ser Gly Asn Arg Asn Glu Val Phe Cys Gly Gly 270
 811 GAT TAC AGC TAC CCG GAT CAC AAA CCG GTG ACC TGG ACC ACC GAC 855
 271 Asp Tyr Ser Tyr Pro Asp His Lys Pro Val Thr Trp Thr Thr Asp 285
 856 CAG CTG GCG AAG GCG ATC TTT TAC CAC GAC CGT GTG CTC AAC GAA 900
 286 Gln Leu Gly Lys Ala Ile Phe Tyr His Asp Arg Val Leu Asn Glu 300
 901 CCA TTC ATG ACC GTG CTG ACC TGA 924
 301 Arg Phe Met Thr Val Leu Thr End

Fig. 3 Nucleotide sequence of the *C23O* gene encoding catechol 2,3-dioxygenase gene from *Pseudomonas putida* BNF1

that of TOL plasmid in *P. putida* mt-2 [16] and 97 % identity with those of *P. putida* (GenBank accession number DQ364064) and *Pseudomonas* sp. S-47 [17].

Identification of *C23O* Gene Expression Vector After Enzyme Digestion

After digestion with NotI, the complete catechol 2,3-dioxygenase gene *C23O* sequence was inserted into the NotI site of the pUT/mini-Tn5 to form the recombinant

Fig. 4 Construction of pUT/mini-Tn5-*C23O*. M₁, lambda DNA/*Hind*III molecular weight marker. 1: pUT/mini-Tn5. 2: pUT/mini-Tn5-*C23O*. 3: pUT/mini-Tn5-*C23O* digested by restriction with NotI

Table 2 The activity of catechol 2,3-dioxygenase in strain BS3 and BS3-*C23O*

Strains	Activity (U mg protein)	
	Supernatant	Cell extract
BS3	0.0825	0.4310
BS3- <i>C23O</i>	0.0914	0.4873

transposon pUT/mini-Tn5-*C23O* (Fig. 4). pUT/mini-Tn5-*C23O* was transformed into *E. coli* S17-1 (λ pir) by electroporation.

Construction of Engineering Strain

One conjugant endowed with the catechol 2,3-dioxygenase activity was picked. Specific PCR amplification was performed to check the *C23O* gene from the conjugant. The result showed that the *C23O* gene could be amplified from the genome of the conjugant, while the *C23O* gene could not be amplified from the genome of *Acinetobacter* sp. BS3 by PCR. And the plasmid detection experiment showed that the conjugant did not carry any plasmid. All results indicated that the *C23O* gene had been inserted into the chromosome of strain BS3.

Enzyme Activity and Substrate Specificity Assay

Acinetobacter sp. BS3 and the recombinant strain BS3-*C23O* were grown to LB medium, after overnight incubation in a shaking flask (150 r min⁻¹) at 30 °C, supernatant and cell extract were extracted and determined for catechol

Table 3 Growth of strains BS3 and BS3-C23O on aromatic compounds

Substrates	Growth of strain BS3		Growth of strain BS3-C23O	
	Uninduced cells	Induced cells	Uninduced cells	Induced cells
2,4-Dichloropheno	+	+	+	+
Pentachloropheno	–	+	+	+
Naphthalene	–	+	–	+
Anthracene	–	–	–	–
Phenanthrene	–	–	–	+

+ colony growth could be observed, – colony growth could not be observed

Fig. 5 Biodegradation rate of mixed oil by strain *Acinetobacter* sp. BS3 and recombinant BS3-C23O **Fig. 6** Growth curve of BS3 and BS3-C23O in LB medium

2,3-dioxygenase activity. As shown in Table 2, both strains showed high enzyme activity and the C23O activity of cell extract was much higher than that of supernatant, so the catechol 2,3-dioxygenase was endoenzyme.

In order to assay substrate specificity of the two strains, we selected representative aromatic compounds, including 2,4-dichlorophenol, pentachlorophenol, naphthalene, anthracene and phenanthrene as the sole carbon and energy source to make selective medium. The phenol induced and non-induced strain BS3 and BS3-C23O were inoculated into the plate for qualitative observation of bacterial growth. As shown in Table 3, the strain BS3 could only utilize 2,4-dichlorophenol before inducing and could utilize pentachlorophenol and naphthalene after inducing. And the strain BS3-C23O could utilize both 2,4-dichlorophenol and pentachlorophenol before inducing and could also utilize naphthalene and phenanthrene. So the strain BS3-C23O showed broader substrate.

Biodegradation Activity and Growth Characteristic Assay

Growth and biodegradation rate of strains BS3 and BS3-C23O, respectively, using mixed oil as sole carbon and energy source in LB medium were tested at 30 °C with continuous shaking (150 r min⁻¹). As shown in Fig. 5 and 6, both strains showed high ability to utilize mixed oil as sole carbon and energy source. The BR was up to 50 %, when strains were cultured with mixed oil after 14 days. The BR was close to 80 % after 28 days. The growth curve of the two strains under the experimental conditions was illustrated in Fig. 6. It showed that both strains had similarly s-type growth curve, and the absorbance could achieve 2.0. Therefore, C23O gene integrated into the genome of BS3 just enhanced the function of the initial strain, but did no harm to the growth.

The Genetic Stability Testing of the Engineered Strain

The engineering strain was successively cultured for 30 days on both selective (5 µg ml⁻¹ kanamycin) and non-selective LB plate. Spectrophotometric method analyzed the C23O activity and alkanes biodegradation activity. These results showed that whether the selective conditions were supplemented into medium or not, the enzyme activity and biodegradation activity of BS3-C23O was nearly the same level of that of BS3, which indicated that the C23O gene successfully integrated into the genome of BS3 by homologous recombination had the high genetic stability.

Conclusions

In the present study, a novel catechol 2,3-dioxygenase gene (C23O) was cloned from the plasmid DNA of *P. putida* BNF1. The C23O was a aromatic hydrocarbons-degrading

gene. *C23O* was sequenced and analyzed, and then, integrated into the genome of BS3 to construct engineering strain for biodegrading crude oil. The recombinant strain BS3-*C23O* displayed a broad substrate specificity, high enzyme activity, and favorable stability. These characteristics were very important for handling environmental pollution. All the results indicated that the stable genetically engineered strain was a very potential candidate for the degradation of oil residues in natural environment. The further studies for its practical application were ongoing, such as wash solutions and oil field experiment.

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Conflict of interest The authors declare that there are no conflicts of interest.

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