



Identification of Phenol-Degrading *Nocardia* Sp. Strain C-14-1 and Characterization of Its Ring-Cleavage 2,3-Dioxygenase

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Abstract

An aerobic bacterial strain C-14-1 was isolated from an acrylic fiber wastewater. The strain was found to belong to *Nocardia* sp. according to morphological, physiological and its 16S rRNA gene sequence. This strain was able to degrade both alkanes and succinonitrile such as phenol. Catechol 2,3-dioxygenase (C23O) gene was found and amplified with the designed primers from the total DNA of C-14-1. The result of Southern blot indicated that there is only one C23O gene in the genome of C-14-1.

Keywords: Biodegradation, Broad-spectrum, Phenol, Catechol 2,3-dioxygenase gene

1. Introduction

Phenol is an aromatic molecule containing hydroxyl group attached to the benzenoid ring structure. The origin of phenols in the environment is both anthropogenic as well as xenobiotic (Hirayama, K. K., 1994; Bobdziewicz, J., 1998; Kumaran, P., 1997). Phenol is a major pollutant present in several types of industrial wastewater, such as that from coal refineries, phenol manufacturing pharmaceuticals, industries of resins, paints, dyes, petrochemicals, and textiles, and pulp and paper mills (Ahmed, 1995; Kumar, A., 2004; Bandhyopadhyay, K., 2001). It acts as a substrate inhibitor in the biotransformation (Hill, G. A., 1975). WHO has prescribed a concentration of 1 µg/L as the guideline concentration for drinking water. Thus, elimination of phenol effectively is necessary to preserve the environment and the health of human beings.

It is currently removed by costly and inefficient chemical or physical methods. Biological degradation has been utilized as an alternative, since it has low associated costs and leads to complete mineralization (Tay, S. T., 2005). The ability to degrade phenol and other phenolic compounds is widespread in microorganisms. Several bacterial strains belonging to the species of *Pseudomonas*, *Bacilli*, *Klebsiella*, *Ochrobactrum*, *Rhizobia*, etc. are reported for phenol degradation (Chitra, S., 1995; Balasankar, T., 2000).

As the complex composition of industrial wastewater, microorganisms with broad-spectrum degrading ability are urgently to be found to avoid antagonism and other adverse effect during biological degradation. In this paper, we report here the identification and characterization of phenol degrading bacteria isolated from the acrylic fiber wastewater. In previous study we get that this strain can degrade not only alkanes but succinonitrile. Physiological and biochemical features are used to characterize C-14-1, and phylogenetic analysis based on 16S rRNA gene is used to reveal genetic relationship of the isolate with other *Nocardia* strains. A catechol 2,3-dioxygenase is found and cloned from C-14-1 with designed primers firstly. A new way of studying bacteria broad-spectrum degrading ability and constructing genetically engineered bacterial can be provided through this paper.

2. Materials and methods

2.1 Chemicals

Most of the chemicals used were either from Sigma, USA or from Fluka Chemika, Switzerland. Phenol crystals (99%

purity) were obtained from Shanghai reagent factory. The experimental procedure for liquid phenol preparation was developed according to Sambrook et al. (Sambrook, J., 1989). Nutrient broth and other chemicals were obtained from commercial supplies.

2.2 Physiological and biochemical characteristics of C-14-1

Seven important physiological and biochemical features of C-14-1 were used to characterize the strain C-14-1. The colony character and bacterium configuration were observed by microscope. The analysis included dying, aerobic test, catalase activity, oxidation zymolysis of glucose, etc. All tests were done in duplicate.

2.3 16S rRNA amplification and phylogeny analysis of 16S rRNA gene

Strain C-14-1 was incubated in TSB and the genomic DNA was extracted using a DNA extraction reagent after fission by bacteriolysis. The 16S rRNA gene was amplified using primers FP (5'-GGTGTAGCGGTGGAATGCGCAGAT-3') and RP (5'-CGAGCTGACGACAACCATGCACCAC-3'). 50 μ L of PCR volume consisted of 10 pg of total DNA, 25 μ L 2 $\times$ Reaction buffer, 4 μ L of 2.5mM each dNTP, 1 μ L of 20mM FP, and RP, 0.5 μ L of 5 U LA Taq polymerase and adding ddH₂O making the volume up to 50 μ L. The PCR was conducted at 95 $^{\circ}$ C predenaturing for 5 min and the 30 cycles (94 $^{\circ}$ C denaturing 0.5 min, 58 $^{\circ}$ C annealing 0.5 min and 72 $^{\circ}$ C extending 0.5 min), finally 72 $^{\circ}$ C extending 7 min again. The PCR amplification product was purified using the PCR purification kit (geneworks). After purification, the PCR product was sequenced by Shanghai Sangon Company.

2.4 Cloning and sequencing of catechol 2,3-dioxygenase gene

PCR was performed to amplify the C23O structural gene. The sequences of the primers were designed on the basis of strain (GenBank ID AP006618) FP: (5'-CTGGGTGATGCCGTGCTT-3') and RP: (5'-CTCTGGGAGGCCGAGAAAT-3'). 50 μ L of PCR volume consisted of 10 pg of total DNA, 25 μ L 2 $\times$ Reaction buffer, 4 μ L of 2.5mM each dNTP, 1 μ L of 20mM FP, and RP, 0.5 μ L of 5 U LA Taq polymerase and adding ddH₂O making the volume up to 50 μ L. The PCR was conducted at 95 $^{\circ}$ C predenaturing for 5 min and the 30 cycles (94 $^{\circ}$ C denaturing 0.5 min, 58 $^{\circ}$ C annealing 0.5 min and 72 $^{\circ}$ C extending 0.5 min), finally 72 $^{\circ}$ C extending 7 min again.

2.5 Phenol degradation

The effect of the different substrate concentrations (0, 100, 200, 400, 600, 800, 1000mg/L) on the phenol degradation by C-14-1 was examined. The growth medium was a kind of inorganic medium incubated at 35 $^{\circ}$ C, under a 220 rpm shaking rate and a pH of 7.0.

2.6 Analytical procedures

Growth of the organisms was recorded by monitoring the optical density (OD) of the culture in a 752-N UV spectrophotometer at 460 nm. Phenol was estimated spectrometrically using 4-aminoantipyrine as per standard procedure (APHA, 1992).

3. Results and discussion

3.1 Physiological and biochemical characteristics of C-14-1

The physiological, biochemical characteristics of C-14-1 were given in Table 1. It had several mycolic acids which were notable feature of *Nocardia* sp. The colony was observed round shape by using microscope, Φ 0.5~1.0mm, orange, smooth wet and low-raised surface. Bacterium configuration was hypha in medium Φ 0.5~0.8 μ m, some branched, broken into globularity within 52h.

3.2 16S rRNA gene phylogeny of C-14-1

16S rRNA sequences analysis was a fast and accurate method to identify C-14-1 phylogeny position. Part-length (about 391bp) 16S rRNA genes were sequenced and shown in Fig. 1 (GenBank ID EU579437). We found that the strain C-14-1 was classified in the *Nocardia* genera, the similarities between C-14-1 and *N.amamiensis* (AB275164), *N.pneumoniae* (AB108780) and *N.inohanensis* (DQ659908) were 97.0, 97.0 and 96.0, respectively. Combined with the physiological and biochemical characteristic results, C-14-1 was identified to be *Nocardia* sp.

3.3 Detection of catechol 2,3-dioxygenase genes in C-14-1

A PCR product encoding the C23O gene from C-14-1 was successfully obtained with the expected size and cloned into the pGEM-T vector. A recombinant plasmid containing a 402bp insert with the correct orientation, which was transformed into *E. coli* DH5 α for heterologous expression. Part-length (about 402bp) C23O genes were analyzed and sequenced and shown in Fig. 2 and Fig.3 (GenBank ID EU586325). The result hinted there might be some C23O genes in genomic DNA of C-14-1. In order to confirm how many copies of C23O were in C-14-1, southern blot with the labeled PCR product by Digoxin as the probe was carried out. Five kinds of restriction enzymes (*Kpn*I, *Nde*I, *Not*I, *Pac*I and *Eco*R I) were used to thoroughly digest genomic DNA of C-14-1. Probe labeling and efficiency determination and southern blot to locate the homologous DNA fragments in the genome of the strain C-14-1 were shown in Fig. 4

and Fig. 5. As shown in Fig. 5, the strain C-14-1 had only one C23O gene in its genomic DNA.

3.4 Effect of substrate concentration

C23O was known one of key enzymes in benzene ring refusion. C-14-1 was capable of using phenol as carbon source and its ring cleavage was found at 2,3-position in follow-up study. Seven different initial phenol concentrations were used (Fig. 6). It was shown that as the initial concentration of phenol increase the degradation rate increase to a value of 3.27 h^{-1} then started to decrease with further increasing the concentration of phenol. This was attributed to the fact that phenol was essentially toxic to microorganisms and cells were inhibited with further increase in the phenol concentration. The phenol degradation experienced two phases from the figure. The first was stagnant phase in which the phenol degradation was quite slow. In the second quick degrading phase, phenol was rapidly degraded. It conferred that microorganisms usually needed a period of time to adapt the reaction system. But in case the degrading reaction

begun after the adapting period, the strain could degrade the substrate quickly. This phenomenon was reported in some other studies (Wang, J. S., 1993).

4. Conclusion

The *Nocardia sp.* strain C-14-1 isolated from the acrylic fiber wastewater could well degrade phenol while it also had high strength alkanes and succinonitrile degrading ability. C-14-1 belonged to the *Nocardia* genus according to 16S rRNA gene analysis, and it was closely related to *N.amamiensis*. One catechol 2,3-dioxygenase gene was found in its genomic DNA. The phenol degradation rate was 100.0 and 42.2% with an initial concentration of 800 and 1000mg/L within 28h.

References

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Table 1. The physiological and biochemical characteristics of C-14-1

characteristics	C-14-1
Gram’s stain	+
Aerobic	+
Catalase activity	+
Glucose oxidase	+ (produce alkaline)
Acid resisting dye	-
Colony character	Colony round in shape, Φ0.5~1.0mm, orange, smooth wet and low-raised surface, fringe hypha like root hair, no aerial hypha
Bacterium configuration	Hypha in medium Φ0.5~0.8μm, some branched, broken into globularity within 52h, amount of globularity and short haulm formed in anaphase

GGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAA
GCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTG
CCGTAGCTAACGCATTAAGCGCCCCGCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGG
CGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCGGACCGCCCCAGAGATGGGGTTTCCTTG
TGGTCGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCG

Figure 1. The sequence of 16S rRNA fragment of C-14-1

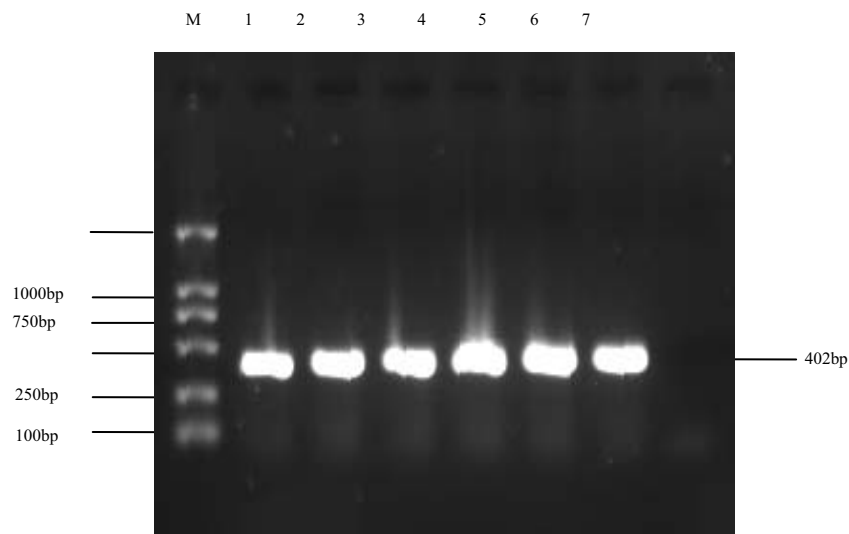


Figure 2. PCR identification photograph of the recombined plasmid pGEM-T-C14 C23O (M: DL2000 DNA Marker, Lane 1 to 6 are pGEM-T-C14 C23O PCR amplified DNA, 7: NTC)

CTGGGTGATGCCGTGCTTGTCGGGACCTGCCTCGATCTGAATGTCGTAGTCCCGGAACATCTCGACCGGTCTGCGAGGTGCTGGCCGG
TGCCGTAGTAGAACGCGAGGTGGTGGAGCTTGCCGTGTCCGCCGGTCATGTCACGCATGCAGGCGACCTCGTGACCGAGCAGGTTCTGA
ACTCATCCAGGCGCCGATCTCCACGCCGCCGTCCACCACGCGTTTCGGTCTGCGGAATCCGAGGTGCCGCTCGAACGAGTCCTTACCC
GCGGTCACGTCGGAGGACATGAGGTTTCAGGTGATCGATCCGCTTGACCGGAATACCCTGGAGCGGCTTCTTCGACGGACGCGACAGGA
TCTTGCTCTGCAGCTCTGCGGGGCGACGTATTTCTCGGCCTCAATCCC

Figure 3. The sequence of catechol C23O fragment of C-14-1

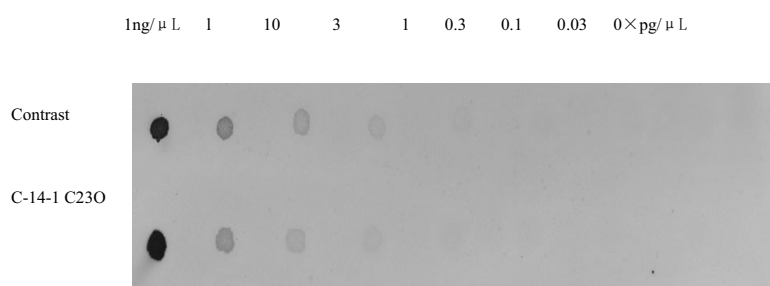


Figure 4. Result of probe labeling and efficiency determination

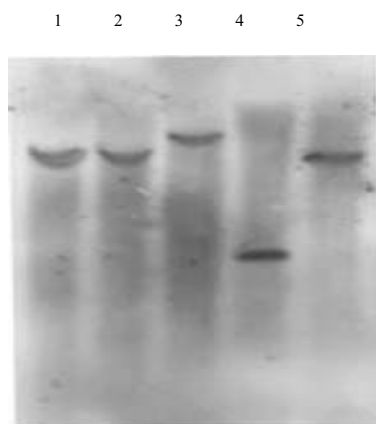


Figure 5. Southern blot to locate the homologous DNA fragments in the genome of the strain C-14-1 (The genomic DNA of strain C-14-1 were digested with the restriction enzymes *Kpn*I, *Nde* I, *Not* I, *Pac* I, *Eco*R I corresponding to lane 1, 2, 3, 4 and 5 respectively)

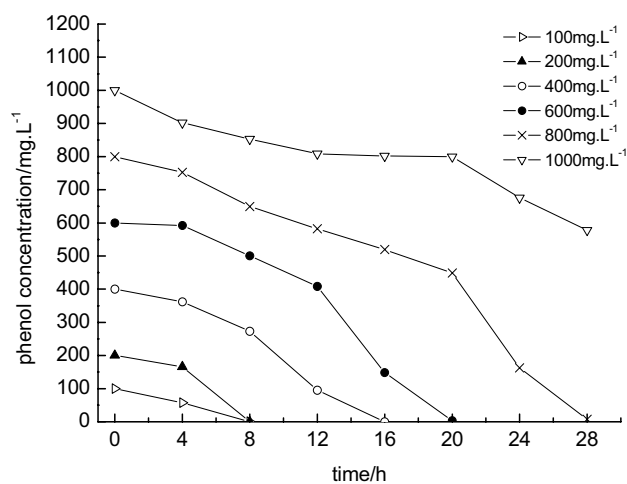


Figure 6. Phenol biodegradation of different temperature by C-14-1