Location and PCR analysis of catabolic genes in a novel *Streptomyces* sp. 
DUT_AHX capable of degrading nitrobenzene

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Abstract

A novel strain of *Streptomyces* sp. DUT_AHX was isolated from sludge contaminated with nitrobenzene and identified on the basis of physiological and biochemical tests and 16S ribosomal DNA (rDNA) sequence analysis. The optimal degradation conditions were as follows: temperature 30°C, pH 7.0–8.0, shaking speed 150–180 r/min, and inocula 10% (V/V). The strain, which possessed a partial reductive pathway with the release of ammonia, was also able to grow on mineral salts basal (MSB) medium plates with 2-aminophenol, phenol, or toluene as the sole carbon source. Furthermore, the enzyme activity tests showed crude extracts of nitrobenzene-grown DUT_AHX contained 2-aminophenol 1,6-dioxygenase activity. The 17-kb plasmid was isolated by the modified alkaline lysis method and was further cured by sodium dodecyl sulphate (SDS) together with 37°C. As a result, the cured derivative strain DUT_AHX-4 lost the 2-aminophenol 1,6-dioxygenase activity. The results suggested that the catabolic genes encoding the nitrobenzene-degrading enzymes were plasmid-associated. Moreover, the plasmid DNA was amplified with degenerate primers by touchdown PCR and an expected size fragment (471 bp) was generated. The Blast results revealed that the gene encoding a 157 amino acid polypeptide was 39%–76% identical to YHS domain protein. The further examination of the plasmid would demonstrate the molecular basis of nitrobenzene catabolism in *Streptomyces*, such as regulation and genetic organization of the catabolic genes.

Key words: Streptomyces; nitrobenzene; degradation; plasmid curing; PCR

Introduction

Bacterial biodegradation has been extensively characterized in Gram-negative bacteria, such as *Pseudomonas* species capable of degrading organic compounds, but is less reported in Gram-positive bacteria. Comparative studies have indicated that there are often differences between biodegradation processes in Gram-positive and Gram-negative bacteria (Dean-Ross et al., 2001). *Streptomyces*, Gram-positive bacteria, have a higher G+ content (more than 70%) than nearly all other organisms. Thus, *Streptomyces* is unique in biodegradation (Goszczynski, 2001). *Comamonas*, Gram-positive bacteria, have a higher G+ content (more than 70%) than nearly all other organisms. Thus, *Streptomyces* is unique in biodegradation (Goszczynski et al., 2001; Iwagami et al., 2000; Ishiyama et al., 2004). *Streptomyces* are one of the most abundant microbial genera in soil and are well known for their ability to produce biologically active small molecules, such as antibiotics. However, they have been largely ignored for their biodegradation capabilities.

Nitroaromatic compounds are used in the production of dyes, plastics, pharmaceuticals, and pesticides. Many of these compounds are highly toxic even at low concentrations. Nitrobenzene is one of the top 50 industrial chemicals produced in the United States (Storck et al., 1996) and listed as a priority by the U.S. Environmental Protection Agency (Keith and Telliard, 1979).

Nitrobenzene has been proven to be biodegradable in the last decade. Aerobic degradation of nitrobenzene involves two major pathways, a widespread partial reductive pathway characterized by the release of ammonia (Nishino and Spain, 1993; Park et al., 1999) and an oxidative pathway characterized by the release of nitrite (Nishino and Spain, 1995). In the partial reductive pathway, as illustrated in *Pseudomonas pseudoalcaligenes* JS45 (Nishino and Spain, 1993), *Pseudomonas putida* HS12 (Park et al., 1999) and *Comamonas* sp. CNB-1 (Wu et al., 2006), the degradation of nitrobenzene leads to the formation of 2-aminophenol. In the oxidative pathway described in *Comamonas* sp. strain JS765 (Nishino and Spain, 1995), nitrobenzene is converted to catechol.

*Streptomyces* have been reported to degrade many aromatic compounds including phenol (Antai and Crawford, 1983), benzoate (Grund et al., 1990), pentachlorophenol (Zaborina et al., 1997), and lignin (Iwagami et al., 2000; Nishimura et al., 2006) via classic aromatic catabolic pathways. However, mineralization of nitrobenzene by *Streptomyces* has not been reported previously. We describe the isolation and characterization of a novel
Streptomyces sp. DUT_AHX which is able to utilize nitrobenzene as the sole carbon source and the evidences that catabolic genes are plasmid-associated.

1 Materials and methods

1.1 Isolation and growth of bacteria

Mineral salts basal (MSB) medium, which contained 1 mmol/L nitrobenzene dissolved ultrasonically, was used for microbial growth and degradation studies. The MSB medium contained the following (per liter of distilled water): 7 g Na₂HPO₄·12H₂O, 1 g KH₂PO₄, 10 mg CaCl₂·2H₂O, 1 mg FeCl₃, 20 mg MgSO₄·7H₂O, 1 g (NH₄)₂SO₄, pH 7.0.

To screen nitrobenzene-degrading microorganisms, sludge samples from a nitrobenzene-manufacturing facility (Jinan, China) were incubated in the carbon-free MSB medium supplemented with nitrobenzene. After 2 months of enrichment, samples were spread on agar plates with nitrobenzene as the sole carbon source. Individual colonies were transferred into MSB medium. After 48 h of incubation at 30°C, the cultures were spread on MSB medium agar plates with nitrobenzene and some isolates were screened by repeatedly streaking culture on new plates from a single colony.

1.2 Effect of different environmental factors

Degradation of nitrobenzene by growing cells was carried out in MSB medium with 1 mmol/L nitrobenzene for 24 h, at different temperature (20–40°C), pH (4.0–10.0), shaking speeds (0–200 r/min), and inocula (1%–15%, V/V). The control experiment was that cell-free culture was grown with nitrobenzene and culture with autoclaved cells was grown in MSB medium with nitrobenzene. The degradation rate (Rd, %) was calculated according to Eq.(1):

\[ R_d = \left( C_A - C_B \right) \times 100 / C_A \]  

where, \( C_A \) is the nitrobenzene concentration of 1 mmol/L and \( C_B \) (mmol/L) is the nitrobenzene concentration of the cultures of strain DUT_AHX incubated for 24 h. All experiments were conducted thrice and each with triplicate.

1.3 Identification of isolates

The microorganism isolated from the enrichment culture was identified by the standard procedures described in Bergey’s Manual of Systematic Bacteriology (Buchanan and Gibbons, 1984). A PCR was also performed to amplify the 16S ribosomal DNA (rDNA) of the strain. One individual colony was dissolved in 50 µl ultrapure water, boiled for 10 min and centrifuged at 12,000×g for 10 min at 4°C. The supernatant fluids were used as PCR templates. The 27f and the 1522r universal primers (Lane, 1991) were used to amplify the 16s rDNA by PCR. The DNA templates were first subjected to an initial denaturation step for 5 min at 94°C. The 30 cycles consisted of a 40 s denaturation step at 94°C, 40 s annealing step at 55°C and 1.5 min extension step at 72°C. A final 10 min extension at 72°C was included. The fragments generated were purified using the TaKaRa Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa Biotechnology, Dalian, China), and then were directly sequenced on an ABI model 3730XL automatic DNA analyzer using BigDye Terminator V3.1 Kit (Applied Biosystems, USA). The 16s rDNA sequence analysis was performed with the BLAST program at the National Center for Biotechnology Information (NCBI, USA). The phylogenetic analysis was performed using the MEGA 3.1 software after multiple alignment of data by CLUSTAL X 1.8 software. Distances and clustering were calculated with the neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 resamplings.

The ability to utilize other aromatic substrates was examined by growing on MSB medium plates with tested substrates (50 mg/L) as the sole carbon source. The plates were incubated at 30°C for 48 h. The tested substrates included 2-aminophenol, aniline, benzoic acid, benzene, catechol, phenol, picolinic acid, and toluene.

1.4 Preparation of cell extracts

For experiments with induced cells, cultures containing nitrobenzene (1 mmol/L) in MSB medium (200 ml) were grown for 48 h at 30°C with shaking (150 r/min). These cultures were inoculated into 2 L of MSB medium containing nitrobenzene (1 mmol/L) and incubated at 30°C with shaking (150 r/min). After 24 h, 1 mmol/L nitrobenzene was again added to the cultures and incubation was continued for 30–35 h until most of the nitrobenzene disappeared. Cultures grown in LB without nitrobenzene served as the control.

Cells were harvested by centrifugation (8,000×g) for 10 min at 4°C, washed twice with fresh 0.02 mol/L phosphate buffer (pH 7.2), and then resuspended in the same buffer. For preparation of crude extracts, cells were disrupted by an ultrasonic disruptor CPX750 (20% amplitude, Cole-Palmer, USA) and centrifuged (60,000×g) for 60 min at 4°C. The pellets were discarded, and the supernatant fluids were stored on ice until used.

1.5 Enzyme assays

2-Aminophenol 1,6-dioxygenase activity was measured by monitoring the increase in the absorbance of the 2-aminophenol ring cleavage product at 380 nm (Nishino and Spain, 1993). Catechol 2,3-dioxygenase activity was determined by measuring the increase in the absorbance of the catechol ring cleavage product at 375 nm. Reaction mixtures contained 2-aminophenol or catechol (0.3 µmol), sodium phosphate (29.5 µmol, pH 7.2), and cell extracts (0.2–0.4 mg of protein) in a final volume of 3 ml at room temperature.

1.6 Plasmid curing

For curing plasmids by sodium dodecyl sulphate (SDS), an overnight culture of the strain in LB medium at 30°C was used to inoculate fresh LB (1% inoculum, V/V) containing SDS (40, 60, 80, 100, and 120 mg/L) (El-Mansy et al., 2000). After overnight growth at 37°C, the culture...
medium was again used to inoculate fresh LB containing SDS. Following the fourth subculture, the culture medium was diluted and plated on LB agar plates. After 24 h incubation under aerobic conditions at 30°C, all LB plates were marked and then were duplicated on fresh LB agar plates and MSB medium agar plates with nitrobenzene as the sole carbon source. Colonies, which were incapable of growth on the selective plates, were picked and examined for the loss of plasmids. The cultures without SDS served as the control. The ability of the cured derivatives to utilize other aromatic substrates was also tested by growing on MSB medium plates with tested substrates (50 mg/L) as the sole carbon source. The 2-aminophenol 1,6-dioxygenase activity and catechol 2,3-dioxygenase activity were further analyzed.

1.7 DNA isolation

Rapid plasmid isolation from the strain and its derivatives was done by the modified alkaline lysis method (Voskuil and Chambliss, 1993). Chromosomal DNA isolation from the strain was performed by the modified SDS method (Sambrook and Russell, 2001). The DNA profiles were visualized by 0.7% (V/V) agarose gel electrophoresis. DNA was cloned into pMD19-T Vector (TaKaRa Biotechnology, Dalian, China). The purified DNA was used as PCR templates. Touchdown PCR method (Don et al., 1991; LeVano-Garcia et al., 2005) was used. The GeneAmp® PCR 9700 thermal cycler (Applied Biosystems, USA) was programmed for touchdown PCR, in which denaturation at 94°C for 5 min before addition of LA Taq (TaKaRa Biotechnology, Dalian, China) was followed by 20 cycles of denaturation at 94°C for 50 s, annealing at 55–45°C for 50 s and extension at 72°C for 1 min; the initial annealing temperature of 55°C was decreased by 0.5°C every cycle. The 15 cycles of denaturation at 94°C for 50 s, annealing at 45°C for 50 s and extension at 72°C for 1 min was immediately performed. The PCR ended with an extra extension of 10 min at 72°C. The fragments generated were analyzed by 1% (W/V) agarose gel electrophoresis and purified using the TaKaRa Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa Biotechnology, Dalian, China). The purified DNA was cloned into pMD19-T Vector (TaKaRa Biotechnology, Dalian, China).

Nucleotide sequences were determined on an ABI model 3730XL automatic DNA analyzer using BigDye Terminator V3.1 Kit (Applied Biosystems, USA). The BLAST network services at NCBI were used for nucleotide and amino acid sequence homology searches.

1.9 Analytical methods

Nitrobenzene was determined by measuring the maximum absorbance at 268 nm using an UV-visible spectrophotometer V-560 (JASCO, Japan) via the Beer-Lambert law. The relationship between absorbance and concentration was linear in the nitrobenzene concentration range of 0–30 mg/L. Cell concentration was measured at 600 nm and converted to the cell dry weight (1 OD600 = 0.2115 g cell dry weight/L, R² = 0.9903). Nitrite and ammonia releases were measured by standard methods (Gerhardt et al., 1994). Total organic carbon (TOC) was determined using a TOC-5000 analyzer (Shimadzu, Japan). Protein was measured by Bradford assay (Bradford, 1976) with bovine serum albumin as the protein standard.

2 Results and discussion

2.1 Characterization of Streptomyces sp. DUT_AHX

After 2 months of enrichment, six strains able to utilize nitrobenzene as the sole carbon and energy source were isolated. The isolate selected for further study which possessed the best nitrobenzene degradability in six strains, was a Gram-positive, aerobic bacterium. Colonies cultivated on solid MSB medium were smooth, rounded, lichenoid, and achromatous. The aerial mycelia grew gradually from white to yellowish-white. Fig.1 shows the scanning electron micrograph picture of strain DUT_AHX. In addition, this microorganism could utilize galactose, sucrose, maltose, cellobiose, fucose, rhamnose, D-xylose, L-arabinose, D-ribose, D-mannite, succinic acid, and citric acid but did not utilize lactose, starch, L-rhamnose, erythritol, adonitol, and inositol. It possessed a nitrreductase activity and did not form sulfureted hydrogen. The strain was identified as a Streptomyces and designated strain DUT_AHX on the basis of these phenotypic properties and the 16S rDNA gene sequence anlaysis (GenBank No. DQ409080). The closest phylogenetic relative of DUT_AHX was Streptomyces albidoflavus (16S rDNA gene sequence identity was 99%, Fig.2).

![Fig. 1 Scanning electron micrograph of Streptomyces sp. DUT_AHX.](image-url)
2.2 Growth and degradation of nitrobenzene

Nitrobenzene-grown cells of *Streptomyces* sp. DUT_AHX were washed to remove nitrobenzene and cultured in 0.02 mol/L phosphate buffer (pH 7.2) containing 1 mmol/L nitrobenzene. As shown in Fig. 3, the concentration of nitrobenzene rapidly decreased with culture time and cell density gradually increased. Ammonia was detected in the culture medium, but the amount of released ammonia was not stoichiometric to that of nitrobenzene consumed. This seemed to be due to the fact that a significant amount of released ammonia was taken up by the microorganism as a nitrogen source for cell growth (Park et al., 1999). There were no changes in optical density in control cultures incubated without nitrobenzene. No nitrite release was detected in the culture medium that degraded nitrobenzene. It seemed that *Streptomyces* sp. DUT_AHX possessed a partial reductive pathway (Nishino and Spain, 1993).

When strain DUT_AHX was cultivated in MSB medium with nitrobenzene, the optimal pH, temperature, shaking speed, and inocula were 7.0–8.0, 30°C, 150–180 r/min, and 10% (V/V), respectively, for cell growth and nitrobenzene degradation (Fig. 4). The strain took about 72 h to degrade 99% of 1 mmol/L nitrobenzene and over 98% TOC was removed at the optimal conditions but the nitrobenzene concentration did not decrease in the control experiment. This indicated nitrobenzene was mineralized by strain DUT_AHX.

Extracts of cells grown on nitrobenzene in MSB medium exhibited 2-aminophenol 1,6-dioxygenase activity but not catechol 2,3-dioxygenase activity (data not shown). Extracts of cells grown in LB medium showed none of two enzyme activities. This suggested that degradable enzymes were induced by nitrobenzene.

2.3 Utilization of other substrates

Strain DUT_AHX was examined for growth on MSB medium plates with a variety of aromatic substrates. The strain could utilize nitrobenzene, 2-aminophenol, phenol, toluene, aniline, benzoic acid, and picolinic acid (data not shown).

2.4 Plasmid curing

When the concentration of SDS in LB was 100 mg/L or greater, no growth was observed. The sublethal concentration of SDS was 80 mg/L and a killing rate of 99% was observed. After 80 mg/L SDS treatment, a serial dilution was made and plated on LB agar plates and then screened by plating on MSB medium agar plates with nitrobenzene. A mutant named strain DUT_AHX-4 that lost the ability to utilize nitrobenzene for growth was isolated. As can be seen from Fig. 5, the strain DUT_AHX-4 lost its indigenous plasmid. It didn’t utilize nitrobenzene, 2-aminophenol, phenol, toluene, aniline, benzene, catechol, benzoic acid, or picolinic acid as the sole carbon source. Moreover, it lost the 2-aminophenol 1,6-dioxygenase activity (data not shown). This indicated that the strain DUT_AHX-4 lost the ability of degrading aromatics.

Elimination of plasmid DNA was an important step in identifying the phenotypic traits encoded by a given plasmid. SDS, which is an anionic detergent, has been widely used as a plasmid curing agent (El-Mansi et al., 2000). Elevated growth temperature had been successfully used in curing plasmids of some strains (Morrison et al., 1983; Ghosh et al., 2000).

Many catabolic pathways involved in the degradation of aromatic compounds have been found to be encoded on catabolic plasmids, and biochemical and genetic studies of these catabolic plasmids have been extensively investigated. All the catabolic genes of the nitrobenzene catabolic pathway of *P. putida* HS12 were found to be located on two plasmids, 59.1-kb pNB1 and 43.8-kb pNB2 (Park and Kim, 2000). Genes involved in nitrobenzene and 4-chloronitrobenzene degradation in *Comamonas* sp. strain CNB-1 were located on a newly isolated 89-kb plasmid, pCNB1 (Wu et al., 2006). The catabolic genes which were involved in nitrobenzene and 4-chloronitrobenzene mineralization in *Pseudomonas putida* strain ZWL73 were located in an approximately 100-kb transmissible plasmid, pZWL73 (Zhen et al., 2006). However, most of these plasmids were found in *Pseudomonas*. In this study, a novel 17-kb plasmid isolated by the modified alkaline lysis method was detected in *Streptomyces*. Moreover, the catabolic genes for the degrading nitrobenzene might...
be located in the plasmid or might be coded by the plasmid and the chromosome. The study of the plasmid in strain DUT_AHX might form the molecular basis of nitrobenzene catabolism in Streptomyces.

2.5 Screen genes encoding ring-hydroxylating dioxygenase

Two intensive DNA bands were amplified by touchdown-PCR. One was about 2,000 bp and the other which was expected size on 1% (W/V) agarose gel was approximately 500 bp (Fig.6). DNA sequencing of the expected fragment revealed a 471 base sequence which codes for a 157 amino acid polypeptide. This exhibited 39%–76% overall identity to YHS domain protein which was found in copper transporting ATPases, some phenol hydroxylases and in a set of uncharacterized membrane proteins. The deduced amino acid sequences of 48 comparable amino acid residues had 39% identity to
YHS domain protein from *Syntrophobacter fumaroxidans* MPOB, whereas the deduced amino acid sequences of 90 comparable amino acid residues had 76% identity to YHS domain protein from marine actinobacterium PHSC20C1. This suggested that there might be a ring-hydroxylating dioxygenase system in strain DUT_AHX. In general, low-stringency touchdown PCR conditions could be found to ensure that the consensus-degenerate hybrid oligonucleotide primer could anneal to the target genomic sequence in the vicinity of the insertion site and generate a PCR product.

### 3 Conclusions

The isolated strain DUT_AHX capable of mineralizing nitrobenzene was a member of the genus *Streptomyces* by physiological and biochemical tests and 16S rDNA sequence analysis. The optimal degradation conditions were as follows: temperature 30°C; pH 7.0–8.0; shaking speed 150–180 r/min; inocula 10% (V/V). The strain could utilize nitrobenzene, 2-aminophenol, phenol, and tolulene as the sole carbon source. The crude extracts of nitrobenzene-grown cells showed 2-aminophenol 1,6-dioxygenase activity. Furthermore, the experiments of plasmid curing revealed that some catabolic genes involved degrading nitrobenzene were apparently plasmid-associated. Also, the 471 bp DNA encoding ring-hydroxylating dioxygenase was amplified by touchdown PCR using degenerate primers and had 39%–76% overall identity to YHS domain protein. The enzyme analysis and sequencing of plasmid need to be further studied for a full understanding of nitrobenzene catabolism in strain DUT_AHX.

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### References


