

## Biodegradation of pyridine and quinoline by two *Pseudomonas* strains

Xia Yi<sup>1\*</sup>, Jiang Zhong<sup>2</sup>

<sup>1</sup> Department of Life and Environmental Science, Kashi Normal College, Kashi 844009, China

<sup>2</sup> Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China

**Abstract** [Objective] To study the degradation of pyridine and quinoline by two *Pseudomonas*. [Methods] Based on the analysis of 16S rRNA gene sequence homology and the intergenic spacer region sequence, the two isolates were identified. The degradation capability of pyridine and quinoline was determined according to spectrophotometry and Electrospray Ionisation/Mass Spectrometry (ESI/MS). The degrading plasmids were detected by plasmid curing and the possible degrading genes were also cloned. [Results] The two isolates were identified as *Pseudomonas* and nominated XJUHX-1 and XJUHX-12. The two *Pseudomonas* were tolerant with pyridine and quinoline and two and four possible metabolites were detected in the culture medium containing quinoline and pyridine, respectively. The degrading capability of curing plasmids was lower than the crude isolates. The gene segments coding for the NADH (acceptor) reductase component OxoR for quinoline degradation and nitrogenase reductase (NifH) of denitrification for pyridine degradation were amplified from the genome of XJUHX-1 and XJUHX-12, both were cloned and expressed in *E. coli* BL 21 producing recombinant proteins with molecular mass of 43 kDa and 16 kDa. [Conclusion] The two isolates could degrade pyridine and quinoline respectively.

**Keywords:** pyridine, quinoline, ESI/MI, *Pseudomonas*, plasmid curing, degrading gene

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Quinoline and pyridine and their derivatives are important representatives of heterocyclic compounds. They are generated by the mining industry, coal tar- and oilshale-processing operations, wood-preserving facilities, and chemical manufacturing plants. They are soluble in water and easily transported to groundwater because of their heterocyclic structure. Quinoline or

pyridine-containing waste is greatly harmful to human health and environmental quality because of their toxicity and nauseous odor. Several quinoline-degrading bacteria, such as *Moraxella* sp.<sup>[1]</sup>, *Pseudomonas* sp.<sup>[2]</sup>, *Rhodococcus* sp.<sup>[3]</sup>, *Desulfobacterium indolicum*<sup>[4]</sup>, *Burkholderia pickettii*<sup>[5]</sup>, *Comamonas* sp.<sup>[6]</sup> and white rot fungus<sup>[7]</sup>, have

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\* Corresponding author. Tel: +86-998-2891282; Fax: +86-998-2893543; E-mail: yixia0110@163.com

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been identified. Studies of quinoline-degradating strains were mainly focused on the mechanism of transformation of quinoline and bioaugmentation for pollution treatment<sup>[8-12]</sup>. The microorganisms degrading pyridine, such as *Pseudomonas* sp.<sup>[13]</sup>, *Paracoccus* sp.<sup>[14-15]</sup>, *Shinella zoogloeoides* BC026<sup>[16]</sup> have been reported, too. The metabolic pathways of quinoline and pyridine-degradation were tracked by GC/MS qualitative analysis and the degradation capability of microorganisms was detected by HPLC quantitative analysis. Zhang et al. (2009) investigated the feasibility of using pyridine-degrading bacteria the microbial fuel cell (MFC), from which electricity was generated. They proved that pyridine may be used as the MFC fuel in practical applications of wastewater treatment<sup>[17]</sup>.

Kumarb (2003) and Ma Y et al (2006) studied the large plasmids of polycyclic aromatic hydrocarbon-degrading bacteria carrying large plasmids responsible for pyridine degradation, especially<sup>[13,18]</sup>. Kaiser et al. (1996) presented information concerning the microbial metabolism of pyridine, quinoline, acridine and their derivatives under aerobic and anaerobic conditions with an emphasis on metabolic pathways<sup>[9]</sup>. The metabolic pathways of quinoline-degradation are diversified, but the first steps of degrading are similar. The quinoline-degradation genes encoding 2-oxo-1, 2-dihydroquinoline 8-monoxygenase and quinoline 2-oxidoreductase were cloned and expressed, and the activity of enzymes were also detected<sup>[19-21]</sup>. The quinoline 2-oxidoreductase genes (*gorMSL*) were successfully expressed and the Qor preteins were also analyzed<sup>[22]</sup>. Carl put forward a gene cluster of quinoline-degradation to elucidate the genetic organization and regulation of the catabolic pathway<sup>[23-24]</sup>. It showed that *oxoR* and *oxoO* located in upstream and downstream, respectively. And there were six ORFs and three ORFs between *oxoR* and *gorMSL* and between *gorMSL* and *oxoO*, respectively. The gene *oxoS* which was involved in quinoline-dependent transcriptional activation was in the upstream of *oxoO*. The genes involved in pyridine-degrading have also been studied, which included

nitrous oxide reductase (*NosZ*), nitrogenase reductase (*NifH*), cytochrome *cd<sub>1</sub>*-containing nitrite reductase (*NirS*), and Cu-containing nitrite reductase (*NirK*) of denitrification<sup>[25-26]</sup>.

In this study, two *Pseudomonas* strains XJUHX-1 and XJUHX-12, capable of degrading quinoline or pyridine respectively, were identified based on 16S rRNA gene sequence homology and ISR. And their degradation ability and possible metabolic products of quinoline and pyridine were studied. Two of the genes involved in degradation of quinoline and pyridine were cloned and expressed successfully.

## 1 Materials and methods

### 1.1 Chemicals and Enzymes

Quinoline and pyridine were from Sinopharm Chemical Reagent Co., Ltd and Jinshanting New Chemical Reagent Co., Shanghai, respectively. Mytomycin C was from Roche Hoffmann-La Roche Co., Ltd. Antibiotics used was ampicillin (100 µl/ml). pMD18-T Easy vector and enzymes used in this study were purchased from TaKaRa company.

### 1.2 Bacteria strains and plasmids

*Pseudomonas* strain XJUHX-1, XJUHX-12 and XJUHX-16 were the kindly gifts from Erkin Rahman in Xinjiang University, which were isolated by selective enrichment with quinoline and pyridine as the carbon source, respectively. *E. coli* BL21 was used for recombinant protein production with pET28a(+). *E. coli* strain JF1125 was used as host cell for molecular cloning. Plasmid pMD18-T Easy vector was used to T/A cloning of PCR products. DNA sequencing was done by the Boshang Biological Company of Shanghai.

### 1.3 Media and growth conditions

The Luria-Bertani (LB) medium<sup>[27]</sup> was used for bacterial enrichment and maintenance. Mineral salt medium (MSM) was described by Kilbane (2005) and Wang et al. (2004)<sup>[28,51]</sup>.

### 1.4 Biodegradation of quinoline and pyridine

The experiments were conducted in 250ml Erlenmeyer flasks containing 100ml MSM with a

different concentration of quinoline (400 – 2400 mg/l) or pyridine (200 – 1200 mg/l). The initial inoculum was the overnight culture of XJUH1 and XJUH2 (about 0.1 OD<sub>602</sub>). All flasks were sealed with sealfilm and shaken at 30°C, 180 rpm, and sampled periodically. The samples were filtered with 0.22 μm membrane and analyzed for the concentration of quinoline and pyridine. And OD<sub>602</sub> values were measured to represent the biomass of the culture.

### 1.5 ESI/MS analysis

Possible metabolites of quinoline and pyridine degradation were analyzed with ESI/MS. The cultures were centrifuged (6000 × g, 10 min, 20°C) and the supernatants were stored at 4°C. 5 μl of sample was analyzed in a positive mode (+ESI). The ion source conditions were: temperature of dry heater 180°C, nebulizer 1.0 bar, dry gas 8.0 l/min, capillary: 4500 V, end plate offset – 500 V. Transition of m/z 21 – 600 was used for detection of the degradation of quinoline and pyridine.

### 1.6 Plasmid curing

Plasmid curing was done with the mytomycin C curing method<sup>[29]</sup>. The nearly toxic level of Mitomycin C (2 μg/ml) was used in the curing. The plasmids were extracted according to Kado CI et al (1981)<sup>[30]</sup>.

### 1.7 Bacterium identification by 16S rRNA gene sequence

Total bacterial DNA was extracted by the CTAB method<sup>[31]</sup>. Partial fragments of the 16S rRNA gene were amplified with the following primer pairs, P1 (5′ – 3′) AGA GTT TGA TCC TGG CTC AG, P2 (5′ – 3′) AAG GAG GTG ATC CAG CCG CA. PCR was performed with an initial denaturation of the template DNA at 94°C for 5 min, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1.5 min, followed by a final extension at 72°C for 7 min and then kept at 4°C.

### 1.8 PCR of 16S – 23S rRNA intergenic spacer region

The 16S – 23S rRNA intergenic regions were amplified according to Daffonchio et al. (1998)<sup>[32]</sup> using primers proven to be useful for species, which were ISR1 (5′-GAAGTCGTAACAAGG-3′) and ISR2

(5′-CAAGGCATCCACCGT-3′).

### 1.9 Quinoline and pyridine degradation genes

Extraction of the total DNA from the strain was done as described above. In the genome, two gene fragments, one encoded a component of the NADH (acceptor) reductase, OxoR, for quinoline degradation, and the other encoded nitrogenase reductase (NifH) for pyridine degradation, were amplified with the primers reported by Roßsch et al (2002)<sup>[25]</sup> for *nifH*, and Pr (GCAGgaattcggcagttcattgcagatcca) and Pr (ATAGggatcccactgagggtcagcactca) for *oxoR* gene, respectively.

The *oxoR* was amplified with PrimerSTAR™ HS DNA Polymerase. And *nifH* gene was obtained with Taq by Touch-Down PCR as described by Roßsch et al (2002)<sup>[25]</sup> and the pre-denaturation temperature was set at 97°C before adding Taq. The PCR products were separated by agarose (1.0%) gel electrophoresis. The target DNA fragment was purified using GenClean gel extraction kit (Generay Biotech, China) and cloned into the pMD18-T Easy vector. The ligation products were transformed into competent *E. coli* JF1125, and then the gene sequences of the *oxoR* and *nifH* were determined. The sequences were analysed and submitted in NCBI website.

### 1.10 DNA sequencing and sequence analysis

The program FASTA was used to search for similar sequences, Mega4 was used to construct the phylogenetic tree.

### 1.11 Purification of NifH and OxoR proteins

The recombinant plasmids harboring the *oxoR* and *nifH* genes were introduced into *E. coli* BL21. The transformants were cultured at 30°C with shaking overnight until OD<sub>602</sub> = 0.1, then IPTG (final concentration 0.4 mM) were added to induce enzyme to express for 2 h at 30°C. And the method of purification proteins was according to the His-tag fused protein protocols of Merck Co., Ltd. SDS-PAGE was performed according to the method of Laemmli (1970)<sup>[33]</sup> with the concentrations of polyacrylamide being 15% and 5% (w/v) in separation and condensation gels, respectively. The proteins staining solution

contained Coomassie blue R-250 ( 0.05% , w/v ) in 10% ( w/v ) acetic acid and 50% ( w/v ) methanol. Destaining solution was H<sub>2</sub>O/methanol/acetic acid ( 88 : 5 : 7 ) .

### 1.12 Nucleotide sequence accession number

The sequence *oxoR* and *nifH* genes were submitted to the NCBI database under accession number HM345922 and HM345921.

## 2 Results

### 2.1 Biodegradation of quinoline and pyridine

The growth of strain XJUXH-1 and XJUHX-12 in MSM containing different initial concentrations of quinoline or pyridine , respectively , were shown in Fig. 1 and 2. The growth of two strains can be divided into three phases , lag phase , exponential phase and stationary phase.

The quinoline removal was by the way of bioabsorption and biodegradation ( Fig 1 ) . After absorption , the growth of strain XJUHX-1 stayed at the

lag phase because the bacterium needed to adapt to the available carbon source , nitrogen source and the new growing environment. So , strain XJUHX-1 grew slowly during the lag phase. And the lag phase lasted for average 5 – 8 hours for different concentrations , and for the low quinoline concentration ( 400 and 800mg/l ) it was also close to 5 hours. Biodegradation was following the bioabsorption and the isolate was in exponential phase. And the quinoline degradation rate was faster for the concentration of 400 , 800 and 12000mg/l. For all the treated quinoline concentrations , the stationary phase started at about 30 hours.

Pyridine degradation was mainly by bioabsorption ( Fig 2 ) . The exponential phase and the stationary phase of pyridine degradation were shorter and longer than that of quinoline degradation , respectively. And the optimum degradation concentration was 400mg/l.

According to Fig. 1 and Fig. 2 , strain XJUHX-1 was able to use quinoline and strain XJUHX-12 was able to use pyridine as the sole carbon source and nitrogen source.

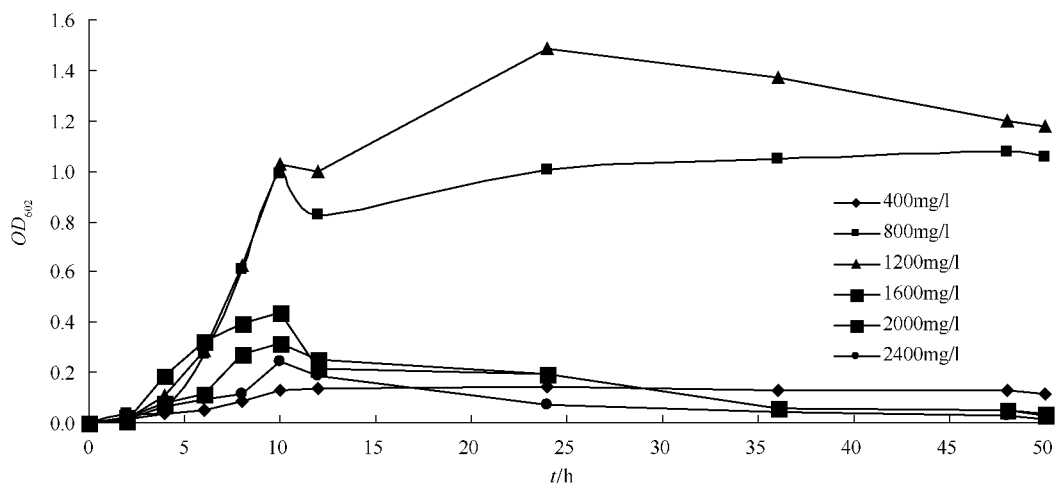


Fig. 1 The growth curves of XJUHX-1 using quinoline as substrate.

### 2.2 The analysis of possible metabolic products by ESI/MS

To obtain the possible metabolic products of quinoline by strain XJUHX-1 and pyridine degradation by strain XJUHX-12 , ESI/MS analysis was applied. The results showed that two kinds of products of 2H-

pyrano-2-one [ 3 , 2B ]-5H-6-pyridone and 2 , 8-dihydroxyquinoline were the possible products from quinoline degradation. Meanwhile , four compounds , 1 , 4-dihydropyridine , 2-hydroxypyridine , 4-( N-formyl ) -aminobut-3-enoic acid and 2 , 3 , 6-trihydropyridine were the possible degrading products

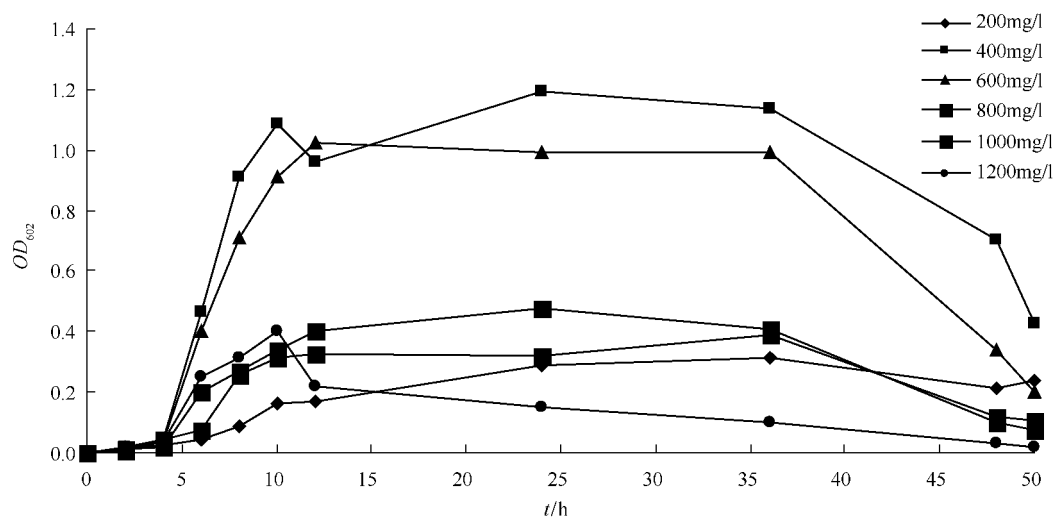


Fig. 2 The growth curves of XJUHx-12 using pyridine as substrate.

from pyridine degradation. According to the previous reports the possible metabolic pathway, 1, 4-dihydropyridine was the first product and 4-(N-formyl)-aminobut-3-enoic acid was one of the second possible products for pyridine degradation. 2-Hydroxypyridine was produced by pyridine hydroxylation, and the precursor of 2, 3, 6-trihydropyridine. And the two data of 1, 4-dihydropyridine was detected and analyzed by adding  $H^+$  and  $Na^+$ .

### 2.3 Plasmid isolation and curing

Plasmid curing was done by mytomycin C on the two isolates to check whether the pyridine and quinoline-degradation genes were located on the plasmid or on the chromosomal DNA. And the DNA fragments were obtained from the two isolates. It showed that the crude plasmids of the two isolates were cured by mytomycin C (Fig. 3). The plasmid-cured XJUHx-1 and XJUHx-12 did not grow on MSM medium with either pyridine or quinoline, respectively, suggesting that the genes encoding degradation enzymes might be localized on plasmids rather than chromosome in strain XJUHx-1 and XJUHx-12.

### 2.4 Identification of the isolates

The fragments of 1441bp and 1448bp of the partial 16S rRNA gene of XJUHx-1 and XJUHx-12

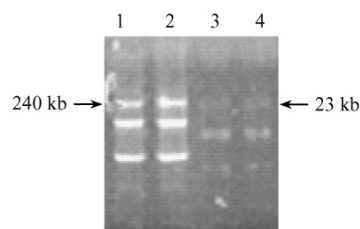


Fig. 3 Electrophoresis of the curing and the crude plasmid by mytomycin C. And 1, 2, 3 and 4 indicated plasmids from XJUHx-1, XJUHx-12, XJUHx-1 and XJUHx-12, respectively. The two latter were treated by mytomycin C.

were amplified by PCR and sequenced. The phylogenetic tree was constructed after comparing the 16S rRNA gene sequence of strains XJUHx-1 and XJUHx-12 and those of representative strains of *Pseudomonas* (Fig. 4). The two isolates formed a tight cluster with *Pseudomonas* sp. The result indicated that both of the 16S rRNA sequences of strain XJUHx-1 and XJUHx-12 shared 99% identity with *P. putida* and *P. geniculata*, respectively, suggesting that they are members of *Pseudomonas*. But strain XJUHx-1 and XJUHx-12 were not at the same branch with *P. putida* and *P. geniculata*, respectively.

### 2.5 16S - 23S rRNA gene intergenic spacer region

The amplified length polymorphism of the high variable parts of conservative genomic regions revealed typically large 16S - 23S intergenic regions in both *Pseudomonas* sp. compared with each other. The

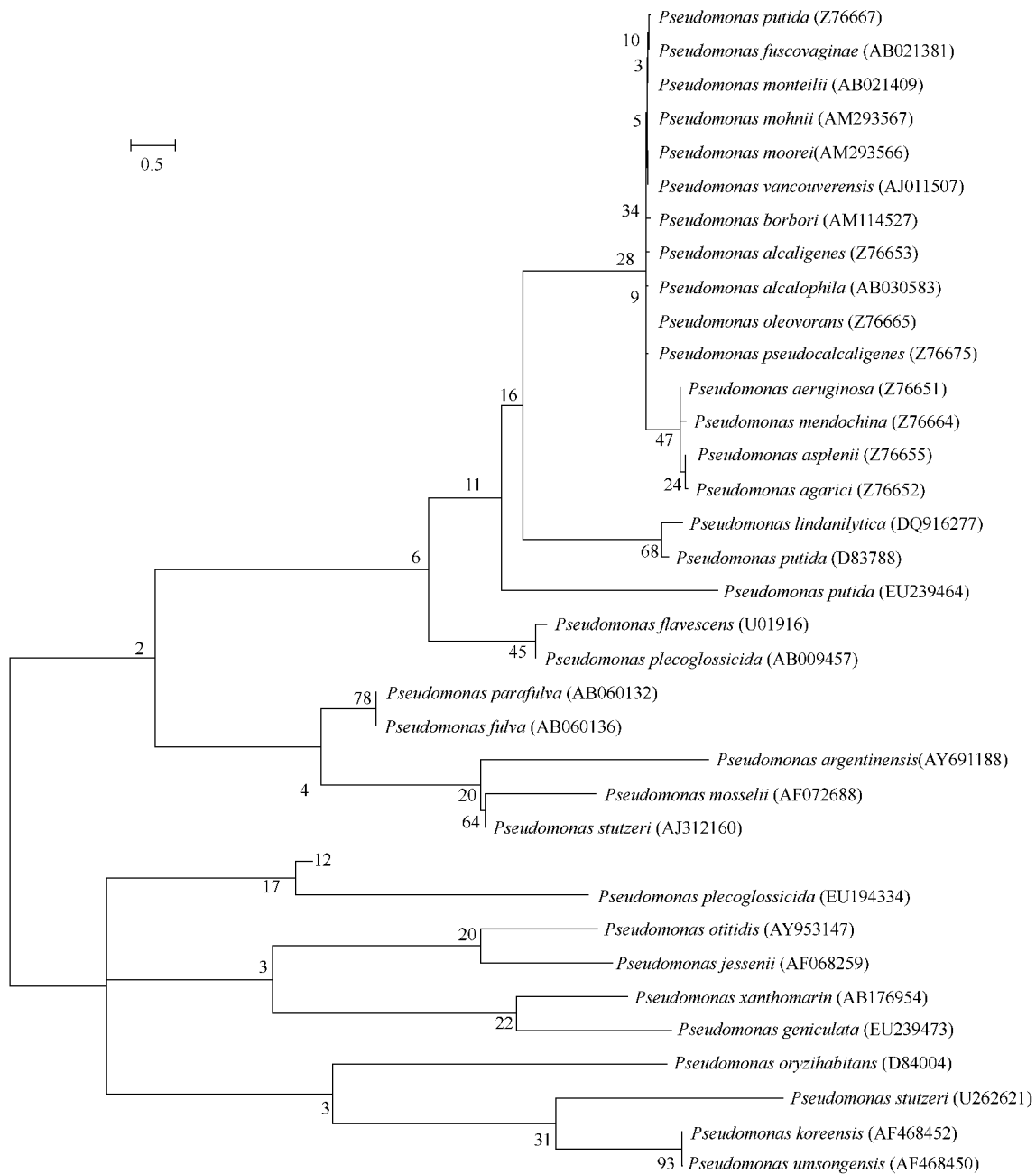


Fig. 4 Phylogenetic tree showing the similarity of the two isolates XJUH-X1 and XJUH-X2 to the members of *Pseudomonas* sp. of their closest relatives. Evolutionary distances were calculated by the neighbour-joining method<sup>[34]</sup>. Numbers in parentheses represent the sequences' accession number in GenBank. Numbers at the nodes represent the confidence level from 1000 replicate bootstrap samplings. Bar 1 substitutions per nucleotide.

analysis revealed the existence of a polymorphism affecting the size and number of ISR ( Fig. 5 ). And the arrow indicated the absence of *E. coli* and the existence of three major bands between 250 and 750 bp in XJUH-X1 , XJUH-X2 and XJUH-X6

( EU194334 ). The common band of ISR suggested the possibility that the two isolates belonged to the same genus. And the different band patterns also showed that the three strains discriminated into different subspecies and this result was consistent with the result

of the analysis of 16S rRNA gene sequence. So, the two isolates of XJUHX-1 and XJUHX-2 were classified and identified as *Pseudomonas* sp.

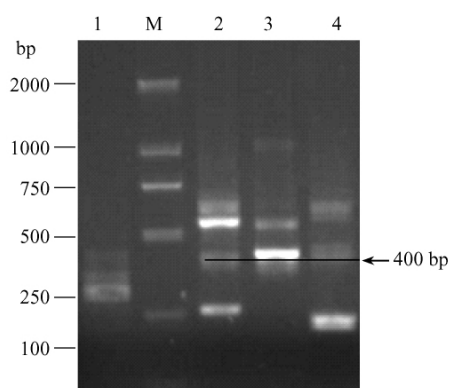


Fig. 5 2% agarose gel of intergenic spacer region (ISR) PCR patterns. Lanes: 1, *E. coli*; 2, XJUHX-1; 3, XJUHX-2; 4, XJUHX-16; M, Marker 2000 with the size of 2000, 1000, 750, 500, 250, 100

## 2.6 Amplification and sequence analysis of *nifH* and *oxoR* genes

The fragments of *nifH* gene (331bp) and *oxoR* (1077bp) were amplified and analyzed (Fig. 6). And the sequences were submitted to NCBI database under accession number HM345921 and HM345922, respectively. And the alignment results showed *oxoR* gene of strain XJUHX-1 and *nifH* of strain XJUHX-2 shared 98% identity with the *oxoR* gene of *P. putida* (Y12654) and with the partial sequence of the dinitrogenase reductase Fe protein gene (*nifH*) (AJ223993) of an uncultured bacterium clone CB907H79, respectively.

## 2.7 Alignment of the deduced amino acid sequence of NifH and OxoR

The phylogenetic trees of NifH and OxoR proteins from bacterial strains were constructed (Fig. 7). The OxoR protein of strain XJUHX-1 was at the different subbranch as that of the other strains. It suggested that the OxoR protein of XJUHX-1 was distant from that of *P. putida* (Y12654), *P. aeruginosa* (D844146) and *P. putida* (AB004059).

The NifH protein of strain XJUHX-2 was grouped into the same class of nitrogenase iron protein, and it

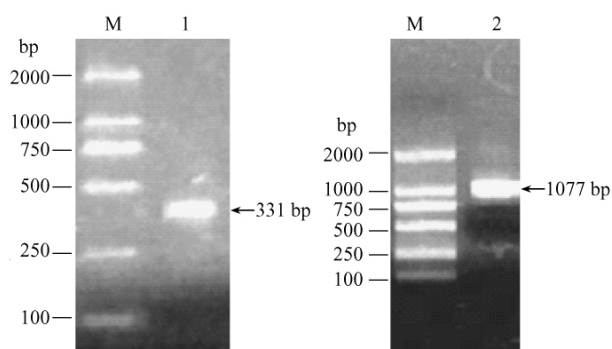


Fig. 6 Agarose gel (1%) of PCR *oxoR* and *nifH* genes amplification products. 1: gene fragment for *nifH*; 2: gene fragment for *oxoR*; M, Marker 2000.

was at the same branch with the NifH protein of clone B34 (AF099794) and clone B18 (AF099790).

## 2.8 Purification of NifH and OxoR proteins

After IPTG induction, the expression of recombinants NifH and OxoR proteins were detected by SDS-PAGE. Two expected 14kDa and 35kDa proteins were obtained from cell extracts of *E. coli* BL21.

## 3 Discussion

The biodegradation of pyridine and quinoline by strain XJUHX-1 and XJUHX-2 was investigated. Based on the analysis of 16S rRNA gene sequence and ISR, XJUHX-1 and XJUHX-2 were identified as *Pseudomonas* sp. These two strains could grow in MSM utilizing either pyridine or quinoline as the sole carbon, nitrogen and energy sources. By spectrophotometry, plasmid curing experiments and the recombination of *oxoR* and *nifH* genes, it indicated that the two isolates could degrade quinoline and pyridine, respectively.

According to ESI/MS, it was clear that there were two and four possible metabolic products during the degradation of quinoline and pyridine by strain XJUHX-1 and XJUHX-2, respectively. So, the possible degradation pathways were similar to that proposed by Schwarz et al (1989)<sup>[35]</sup> and Shukla et al. (1984, 1986)<sup>[36,2]</sup> for quinoline degradation, and by Shukla et al. (1986)<sup>[37]</sup> and Watson et al. (1975)<sup>[38]</sup> for pyridine degradation. It showed that the samples picked up in different cultural time produced

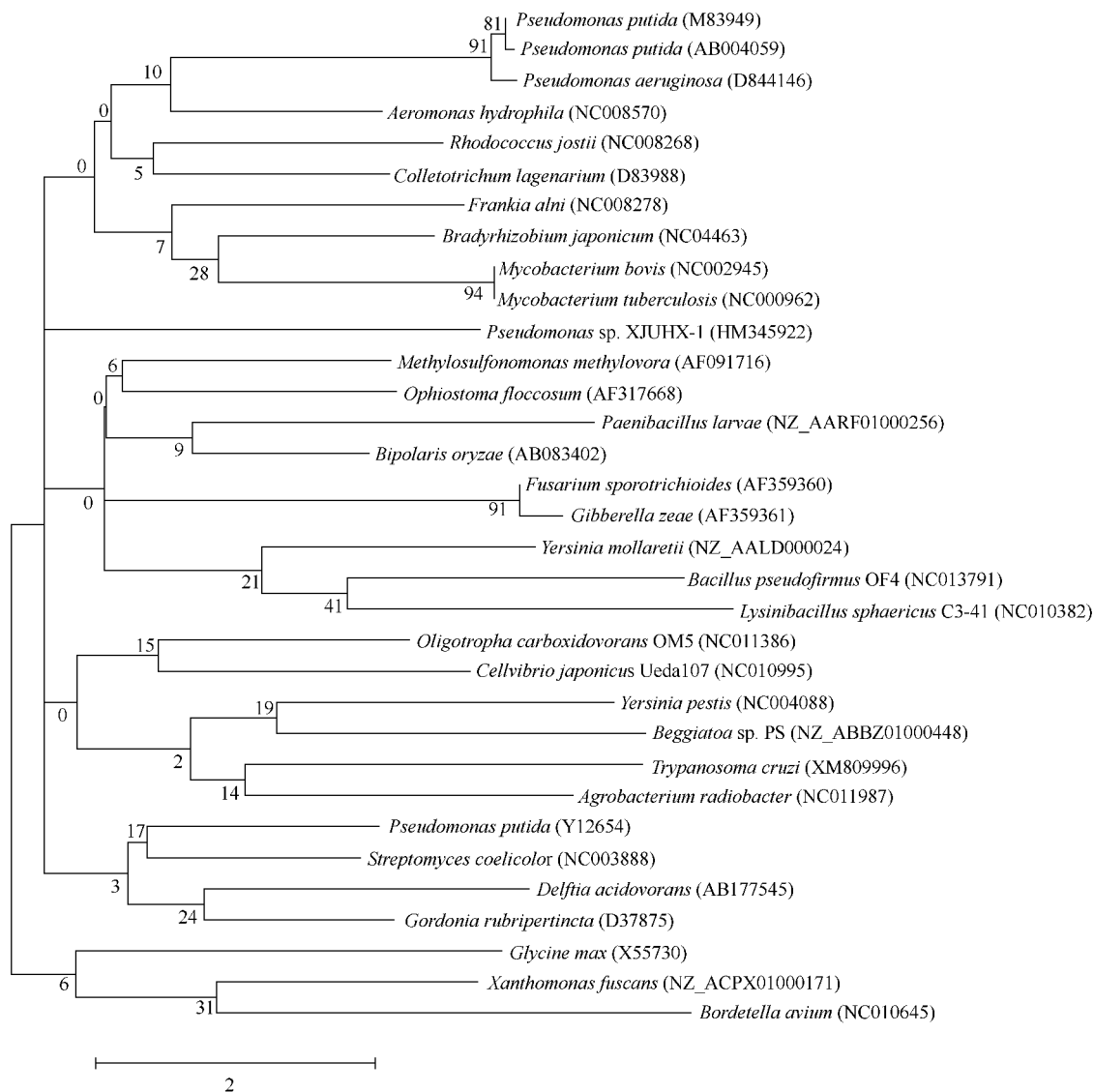


Fig. 7 Phylogenetic tree of OxoR proteins. Numbers in parentheses represent the sequences' accession number in GenBank. Numbers at the nodes represent the confidence level from 1000 replicate bootstrap samplings. Bar 2 substitutions per nucleotide.

differernt products. So , the metabolism was so fast that the possible intermedium degrading products could not be full detected perhaps due to the biological reaction was sensitive and subtle.

Many bacteria can transform and degrade pyridine and quinoline , most of which are *Pseudomonas* sp. And in many studies , extra carbon and mixed bacterial strains were exploited to augment the biodegradation of

pyridine and quonoline<sup>[14 , 16 , 39]</sup> . So , the further study will be needed , to know whether the media of different co-substrates such as glucose , ammonium chloride and trace elements have different effects on biodegradation of quinoline and pyridine by these isolates and whether the mixed bacterial strains shall be used to perform the degradation. And the degrading genes of quinoline and pyridine should also be exploited.



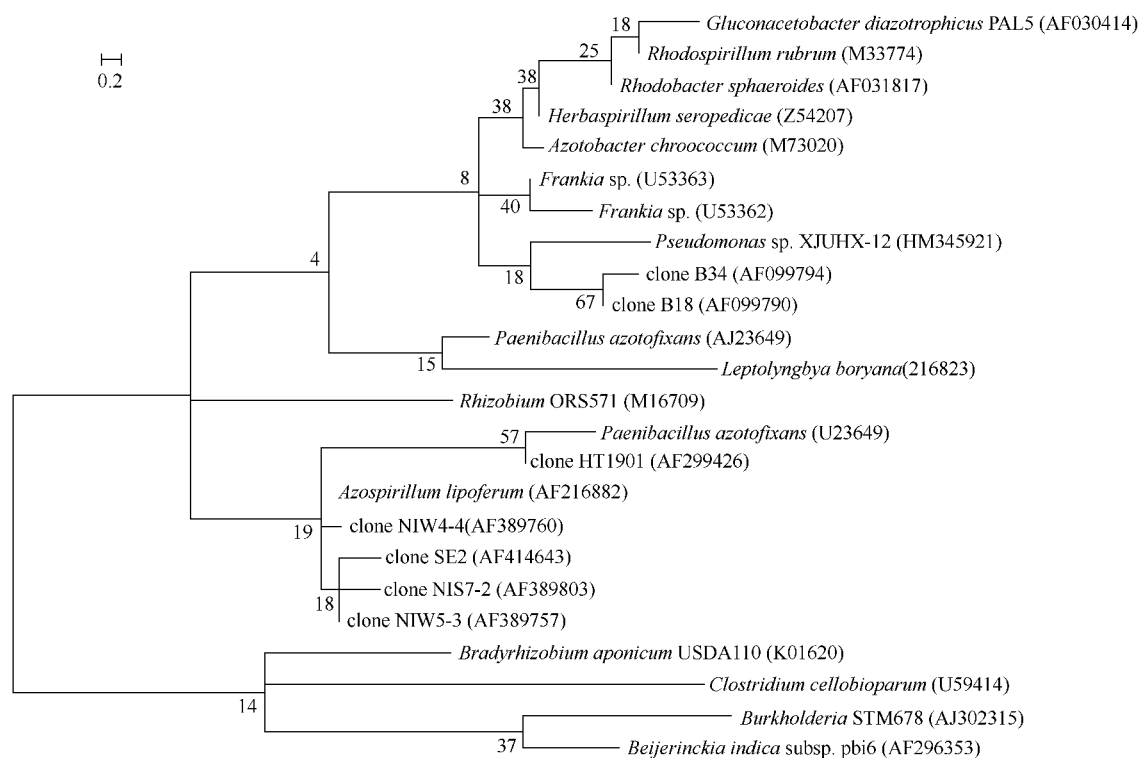


Fig. 8 Phylogenetic tree of NiH proteins. Numbers in parentheses represent the sequences' accession number in GenBank. Numbers at the nodes represent the confidence level from 1000 replicate bootstrap samplings. Bar 0.2 substitutions per nucleotide.

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## 两株假单胞菌对吡啶和喹啉的生物降解

易霞<sup>1</sup>, 钟江<sup>2</sup>

<sup>1</sup>喀什师范学院生命与环境科学系 喀什 844009

<sup>2</sup>复旦大学生命科学学院微生物学与微生物工程系, 上海 222043

**摘要** 【目的】探究 2 株假单胞菌(*Pseudomonas*) 对吡啶和喹啉的降解。【方法】基于 16S rRNA 序列同源性和基因间区分析, 对分离菌株进行分类鉴定。通过分光光度法和电喷雾电离质谱法(Electrospray Ionisation/Mass Spectrometry, ESI/MS) 确定分离菌株对吡啶和喹啉的降解性能。通过质粒消除验证降解质粒的存在, 同时克隆了可能的降解基因。【结果】鉴定结果表明, 两株分离细菌隶属于 *Pseudomonas*, 并将其命名为 XJUHX-1 和 XJUHX-12。降解数据表明 2 株菌株分别耐受吡啶和喹啉, 同时分别检测到 4 种和 2 种吡啶和喹啉的可能降解产物。结果还表明, 消除质粒后的菌株对吡啶和喹啉的降解能力降低。扩增的编码 NADH 还原酶部分的降解喹啉 *oxoR* 基因和编码硝酸还原酶的降解吡啶的 *nifH* 基因, 同时在 *E. coli* 中表达了 43kDa 和 16kDa 的蛋白。【结论】2 株 *Pseudomonas* 具有降解吡啶和喹啉的能力。

**关键词:** 吡啶, 喹啉, 生物降解, ESI/MS, *Pseudomonas*, 质粒消除, 降解基因

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作者简介: 易霞(1979-), 女, 新疆人, 讲师, 理学硕士, 主要从事资源微生物方面的研究。Tel: +86-998-2891282; Fax: +86-998-2893543;

E-mail: yixia0110@163.com

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