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ORIGINAL PAPER

Chlorothalonil degradation by *Ochrobactrum lupini* strain TP-D1 and identification of its metabolites

Xiu-Zhen Shi · Rong-Jun Guo · Kazuhiro Takagi · Zuo-Qing Miao · Shi-Dong Li

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Abstract Chlorothalonil (2, 4, 5, 6-tetrachloroisophthalonitrile, TPN) has been widely used as a wide-spectrum fungicide in China and other countries, and is considered to be an important soil and water contaminant. Here we report the isolation and characterization of a novel TPN-degrading bacterial strain TP-D1 from a heavily TPN-polluted soil in Henan Province, China, and identified it as a strain of *Ochrobactrum lupini* based on 16S rRNA gene sequence analysis and its morphological, biochemical, and physiological characteristics. Strain TP-D1 could degrade 90.4 and 99.7% of TPN after 4- and 7-day incubation in mineral salt broth with 50 mg TPN l^{-1} and in autoclaved soil with 50 μg TPN g^{-1}, respectively. Two new metabolites, methyl 2, 5, 6-trichloro-3-cyano-4-methoxy-benzoate (metabolite C) and methyl 3-cyano-2, 4, 5, 6-tetrachlorobenzoate (metabolite D), were detected besides previously reported 4-hydroxy-2, 5, 6-trichloroisophthalonitrile (TPN-OH, metabolite A). This result suggests that the cyano-group in TPN could be converted into amide groups by strain TP-D1, and reveal the biodegradation mechanism of TPN in soil.

Keywords Chlorothalonil (TPN) · Metabolic pathway · *Ochrobactrum lupini* · Biodegradation

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Abbreviations

BHC	Hexachlorcyclonhexane
CCHT	1-Carbamoyl-3-cyano-4-hydroxy-2, 5, 6-trichlorobenzene
c.f.u.	Colony forming units
CTB	3-Cyano-2, 4, 5, 6-tetrachlorobenzamide
DDT	Dichloro-diphenyl-trichloroethane
DTTC	1, 3-Dicarbamoyl-2, 4, 5, 6-tetrachlorobenzene
ECD	Electron capture detector
GC	Gas chromatography
HPLC	High performance liquid chromatography
LB	Luria-Bertani
LC-ESI-MS	Liquid chromatography-electrospray ionization mass spectrometry
LC-APCI-MS	Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry
MS	Mineral salt
NA	Nutrient agar
PCNB	Pentachloronitrobenzene
SPE	Solid-phase extraction
TPN	Chlorothalonil; 2, 4, 5, 6-tetrachloroisophthalonitrile
TPN-OH	4-Hydroxy-2, 5, 6-trichloroisophthalonitrile

Introduction

Chlorothalonil (2, 4, 5, 6-tetrachloroisophthalonitrile, TPN) is a widely used wide-spectrum fungicide. It controls plant fungal diseases by reacting with cellular thiols and inhibiting fungal respiration (Godard et al. 1999). According to the US Geological Survey of 1997 and

Chlorothalonil-Pesticide Use Statistics of 2008 (http://pesticideinfo.org/Detail_ChemUse.jsp?Rec_Id=PC34550), TPN use was common in the United States; 4.8×10^6 kg TPN was produced each year and applied to more than 50 crops. In China, TPN was usually used to control greenhouse vegetable diseases, and diseases of fruits, rice and wheat. Its production reached 8×10^6 kg each year (New pesticide 2002), but was far from the demand of 2006 and 2007 (Sun 2008). Fungicide residue has been reported in vegetables and fruits (Wang et al. 2005; Xiao et al. 2007), soil and water (Cox 1997), groundwater (Winkler et al. 1996) and in locations as remote as the surface microlayer and fog of the Chukchi Arctic ecosystem (Chernyak et al. 1996). The half-life of TPN in soil is 1–2 months (US Environmental Protection Agency 1986). It may remain in soil for 100 days (Takagi et al. 1991), or one year after repeated application (Motonaga et al. 1996). Though TPN has no acute toxicity, it is still harmful to non-target organisms including soil microbes (Sigler and Turco 2002; Suyama et al. 1993) and terrestrial and aquatic vertebrates (Cox 1997), and has been classified as a "probable human carcinogen" by US EPA (Cox 1997).

TPN can be degraded chemically and microbiologically (Roberts and Hutson 1999). Till now, about 11 kinds of TPN metabolites have been detected (Chaves et al. 2008). In soil and water, the main metabolites were 4-hydroxy-2, 5, 6-trichloroisophthalonitrile (TPN-OH), 1-carbamoyl-3-cyano-4-hydroxy-2, 5, 6-trichlorobenzene (CCHT), and 1, 3-dicarbamoyl-2, 4, 5, 6-tetrachlorobenzene (DTTC) (Roberts and Hutson 1999; Rouchaud and Roucourt 1988), and they were considered to be converted from dechlorination (Sato and Tanaka 1987) and oxidation/hydration of the cyano (–CN) groups in TPN (Putnam et al. 2003; Roberts and Hutson 1999; Rouchaud and Roucourt 1988). Among them, TPN-OH is the most toxic, around thirty times more toxic than its parent compound TPN (Cox 1997; Kenneth and Siegel 1981), more persistent in soil by binding to soil particles (Motonaga et al. 1998), and more readily dissolved in water to lead to second pollution. Therefore, it is important to clarify the biodegradation pathway of TPN to develope new bioremediation strategies for TPN-pollution, especially by a culturable microorganism.

It has been reported that a carbon supplement was necessary for in vitro TPN degradation by bacteria (Katayama et al. 1991; Sato and Tanaka 1987). However, *Flavobacterium* NL0-1 (Katayama et al. 1991) and bacterium TB I (Motonaga et al. 1996) have been reported to degrade TPN without nutrient supplement, and two new strains, *Ochrobactrum* sp. CTN-11 (Liang et al. 2010) and *Lysobacter ruishenii* CTN-1 (Wang et al. 2010a), also showed the same capacity. TPN metabolites degraded by these bacteria were identified to be TPN-OH (Liang et al. 2010; Motonaga et al. 1996) and methylthiotrichloroisophthalonitrile (Katayama et al. 1997). These two products were considered to be derivatives of TPN with replacement of the chlorine atom at the 4-position (Katayama et al. 1992, 1997). However, detection of CCHT and DTTC in soil (Putnam et al. 2003; Roberts and Hutson 1999; Rouchaud and Roucourt 1988) suggested the possibility of other metabolic pathways to degrade TPN by pure culture. Herein, we report the isolation of a bacterium that could effectively degrade TPN without any nutrient supplement and the identification of two new metabolites resulted from the –cyano group conversion of TPN.

Materials and methods

Chemicals and media

TPN powder (effective component >95.0%) was provided by Ruize Pesticide Company, Henan, China. Standard chlorothalonil (>99.6%) was purchased from National Research Center of China for Certified Reference Materials (Beijing, China). Nutrient agar (NA) (Fang 1996) and mineral salt (MS) agar (1.5 g K_2HPO_4, 1.0 g NH_4NO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, and 15 g agar per liter water) were used to isolate TPN-degrading bacteria from soil.

Isolation of TPN-degrading bacteria

Soil sample was collected from a field close to a chlorothalonil-producing factory in Henan, China. One gram of soil sample was placed into liquid NA medium supplemented with 50 mg TPN l^{-1} and incubated at 28°C for 4 days with constant agitation of 180 rev min^{-1}. Five milliliters of the culture was subjected to subculture in the same fresh medium with TPN of 50–600 mg l^{-1} in 50 mg l^{-1} increments. One hundred microliters of suspension from each subculture was spread on NA plates containing different concentrations of TPN and incubated at 28°C for 3–7 days. Colonies growing vigorously or producing a clear zone on NA plates were purified and stored at 4°C on NA plate or slant containing 50 mg TPN l^{-1}. In order to identify their modes of utilization of TPN, these isolates were cultured on MS plates (pH 7.0) containing 50 mg TPN l^{-1}. Each strain had triplicate plates. The strain showing the highest TPN-degrading ability, denoted TP-D1, was selected for further testing.

Identification of strain TP-D1

Strain TP-D1 was characterized based on morphological features and physiological and biochemical properties after incubation on NA plates for 48 h according to Bergey's

Manual of Determinative Bacteriology (Holt et al. 1994). Molecular identification of strain TP-D1 was performed as described below. Strain TP-D1 was incubated in Luria-Bertani (LB) medium at 30°C for 24 h, and cells were collected by centrifugation and washed twice with sterile distilled water. Total DNA was extracted following the standard procedure (Sambrook and Russell 1998). Oligonucleotide primers 27F (5′-AGAGTTTGATCCTGGTCA G-3′) and 1492R (5′-TACGGCTACCTTGTTACGACT-3′) (Mincer et al. 2002; Zeng et al. 2008) were employed to amplify the 16S rRNA gene region. PCR reactions were carried out with a thermocycler (PTC-200; MJ Research, Waltham, MA, USA) under the following conditions: 5 min at 95°C, 1 min at 94°C, 31 cycles of 40 s at 95°C, 45 s at 54°C, 2 min at 72°C and one final step of 10 min at 72°C. The PCR product was purified on 1% agarose gel using Gel Extraction Kit (TIANGEN, Beijing, China), ligated into pMD18-T vector (TaKaRa, Ostu, Japan), and transformed into *Escherichia coli* DH5α competent cells. Positive transformants were screened on LB plates containing 80 mg X-Gal ml^{-1}, 0.5 mM IPTG and 50 mg ampicillin l^{-1}, and sequenced. ClustalX (version 1.8) (Thompson et al. 1997) was used to align the 16S rRNA gene sequence of strain TP-D1 with that of reference species from the GenBank database. A phylogenetic tree was constructed using Mega 4.0 (Tamura et al. 2007).

Degradation of TPN in MS broth

Cells of strain TP-D1 were collected from NA plates after incubation at 30°C for 1–2 days and suspended in 10 ml MS broth (pH 6.8). The density of the bacterial suspension was adjusted to approximately 1×10^9 c.f.u. ml^{-1}. Bacterial suspension was diluted 100 times into 50 ml MS broth containing 50 mg TPN l^{-1}, and incubated at 30°C and 180 rev min^{-1} for 4 days. To determine the degrading ability of strain TP-D1 in MS broth, samples were collected at days 1, 2, 3 and 4, respectively, and the amount of TPN was determined by gas chromatography (GC) with an electron capture detector (ECD). Fifty milliliters of MS broth was poured into a 250-ml flask to evaporate acetone and water using a Rotavapor R-215 (Buchi, Postfach, Switzerland) under reduced pressure. The residue was dissolved in 25 ml *m*-xylene, and 1 ml of the solution was diluted with acetone to an appropriate concentration for GC analysis. Column DB-5 (30 m × 0.25 mm × 0.25 μm) was used, and the operation conditions were as follows: the oven was heated up to 100°C and maintained for 2 min, then increased to 210°C at a rate of 20°C min^{-1}; the temperature of injection port and detector port were maintained at 240 and 250°C, respectively; nitrogen carrier gas flow rate was 29 ml min^{-1}; and injection volume was 1.5 μl. The recovery rate was 90.7–108.8%. Bacteria-free MS broth containing TPN was used as blank control and the experiment was carried out in triplicate. The degrading ability of strain TP-D1 was determined as the percentage of TPN degraded.

Degradation of TPN in soil

The degrading ability of strain TP-D1 in soil was also determined. Dry natural soil was passed through a 60-micron sieve and autoclaved at 160°C for 1.5 h. TPN solution was sprayed onto 10 g of the soil in a Petri dish to the final concentration of 50 μg g^{-1} dry soil. A bacterial suspension of strain TP-D1 was mixed with the TPN-containing soil at 10^7 c.f.u. g^{-1} dry soil. Plates were incubated at 28°C in darkness for 7 days, and soil moisture was maintained at 20% by adding distilled water when needed. The amounts of TPN in soil samples were monitored at day 0, 1, 3, and 7, respectively. Each treatment had triplicates, and bacteria-free soil with TPN was used as control.

Extraction and analysis of TPN in soil was conducted following the protocol of Motonaga et al. (1996) with some modifications. The concentration of NaCl and the volume of hexane were 5% and 100-ml, respectively. The residue of hexane evaporation was dissolved in 10 ml 100% methanol and sonicated for 3 min. Two milliliters of the extracts were analysed by HPLC under the following conditions: column, C18 reversed phase Shim-Pack CLC-ODS (150 × 4.6 mm); mobile phase, acetonitrile and 0.1% acetic acid buffer at 65:35 (vol/vol); flow rate, 1 ml min^{-1}; injection volume, 50 μl; room temperature for column performance; and u.v. wavelength at 232 nm. Recovery rate of TPN from the soil was from 83.5 to 101.2%.

Strain growth and metabolite production in modified MS broth

In order to avoid the possible utilization of acetone as carbon by strain TP-D1, TPN was pre-dissolved in acetone at 50 mg l^{-1}, and the acetone in the flasks was evaporated before adding the modified MS broth. The modified MS broth containing 1.5 g K_2HPO_4, and 0.5 g KH_2PO_4 per liter water (pH 6.8) was used to avoid the influence of chloride anion and other inorganic ions in MS broth. The bacterial suspension of strain TP-D1 mentioned above (1×10^9 c.f.u. ml^{-1}) was diluted 100 times into 50 ml modified MS broth and incubated at 30°C and 180 rev min^{-1} for 3 days. Samples were taken at 0, 12, 24, 48, 56, 72 h of incubation, and the amount of cells were counted as c.f.u. ml^{-1}. TPN-free modified MS broth was used as blank control and the experiment was carried out in triplicates.

To detect the metabolites, TPN was pre-dissolved in acetone, bacterial suspension of 10^7–10^8 c.f.u. ml^{-1} was incubated in the modified MS broth at 30°C and 180 rev

min^{-1} in darkness for 6 days, and samples were taken at day 0, 2, 4 and 6 for TPN metabolite analysis. Each sample had triplicates. After methanol extraction, an aliquot of 10 ml sample was extracted using solid-phase extraction disks (OASIS® HLB; Waters Corporation, Milford, MA, USA) after pH adjustment as described previously (Chaves et al. 2008). A total of 1 ml extract was used for analysis by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS). HPLC-MS (2695-ZQ4000; Waters) equipped with a u.v. detector (2487; Waters) and electrospray ion source was used to detect the TPN metabolites under the following conditions. LC-MS conditions were: column, Xterra C18 (2.1 mm × 100 mm × 3.5 μm); column temperature, 35°C; u.v. detector, 230 nm; mobile phase, acetonitrile (added 0.1% formic acid) 10% (0–0.5 min)–95% (0.5–12 min); and flow rate, 0.3 ml min^{-1}. MS conditions were: mass scanning range (*m*/*z*), 80–600; mass detector, a quadrupole mass spectrometer; source temperature, 100°C; cone voltage, 30 V; capillary voltage, 3,000 V; desolvation gas flow rate, 500 l h^{-1}; and desolvation temperature, 300°C.

Results

Isolation of TPN-degrading bacteria

Two strains had ability to degrade TPN on NA plates. Growth tests on MS plates indicated that TPN could be degraded without other nutrient supplement. Strain TP-D1 showing higher TPN-degrading ability (with a clear zone of around 2 mm-in-diameter on MS plates) was purified and used for further testing.

Identification of strain TP-D1

Morphological and biochemical tests indicated that the cells of strain TP-D1 were rod-shaped, 1.6 × 0.75 μm in dimension, motile with one or two polar flagella, non-spore-forming, aerobic, gram-negative, and non-fluorescent. Colonies on NA plate were circular, smooth with regular margins, milk-white and mucilaginous with 2.0 mm in diameter after 3 days of incubation. The isolate was positive to oxidase and catalase reactions. It can utilize arginine, ornithine, lysine, citrate, urea, sucrose, fructose, xylose, mannitol, inositol and glucose. It was negative to esculin hydrolysis, the Kovacs Indole, ONPG, H_2S, Voges-Proskauer, methyl red, and nitrate reduction tests.

The full-length 16S rRNA gene sequence of strain TP-D1 (1,404 bp, GenBank accession no. EF998851) was compared with the related sequences in the GenBank, and its phylogenetic tree was constructed. Strain TP-D1 exhibited high sequence similarity with bacteria belonging to the genus *Ochrobactrum* (Fig. 1). Similarity calculations indicated that strain TP-D1 was closely related to *O. lupini* (99.8%), *O. anthropi* (99.8%), and *O. tritici* (98.9%).

Comparison of the representative physiological and biochemical characteristics of strain TP-D1 with other close relatives (Table 1) indicated that strain TP-D1 was more similar to *O. lupini* except for the contrary reaction of esculin. Thus strain TP-D1 was identified as a strain of *O. lupini*.

Degrading ability of strain TP-D1 in MS broth and soil

TPN in MS broth was detected by GC and showed a peak at 9.3 min. Calculation based on peak areas showed that the degrading efficacy increased when incubation was prolonged and reached 90.4% after 4-day incubation (Fig. 2a). The degrading efficiency of strain TP-D1 in autoclaved soil was determined by HPLC. TPN was degraded of 95.0% after 3-day incubation and 99.7% after 7 days (Fig. 2b).

Strain growth and metabolite production in modified MS broth

The population of strain TP-D1 in modified MS broth increased gradually from 0 to 60 h, but decreased at 72 h (Fig. 3).

Four TPN metabolites were detected by HPLC after a 2-day incubation. Among them, peak A had a retention time at 6.74 min (Fig. 4a, a-1) and was detected by LC-ESI-MS at negative mode (Fig. 4a, a-3). Peak B, C and D were detected at 7.75, 8.12 and 8.55 min (Fig. 4a, a-1), respectively, at positive mode (Fig. 4a, a-2). The *m*/*z* of each metabolite was listed in Table 2. During the degradation process, the range of metabolites varied a lot. At day 0, only TPN (Peak E) was detected at negative mode. At day 2, four metabolites (peak A, B, C, and D) were detected. At day 6, only peak A was detected either by LC-ESI-MS or by LC-APCI-MS. In the sample of blank control only TPN was detected.

The metabolite represented by peak A was the major degradation product of TPN. The mass spectra of three chlorine compounds at *m*/*z* 244.5, *m*/*z* 248.6, and *m*/*z* 250.6 (Fig. 4b, b-1) were identical to TPN-OH based on the analysis of LC-ESI-MS and LC-APCI-MS at negative mode. Due to the easy substitution of the *para*-chlorine of TPN, metabolite A might also be TPN-OH. Peak B represented a minor metabolite (Fig. 4b, b-2). From its isotope clusters for four chlorine atoms, peak B should be a cyano derivative of TPN. In combination with its *m*/*z*, it was deduced to be 3-methoxycarbonyl-2, 4, 5, 6-tetrachlorobenzeneacetamide. Metabolite C was deduced to be methyl

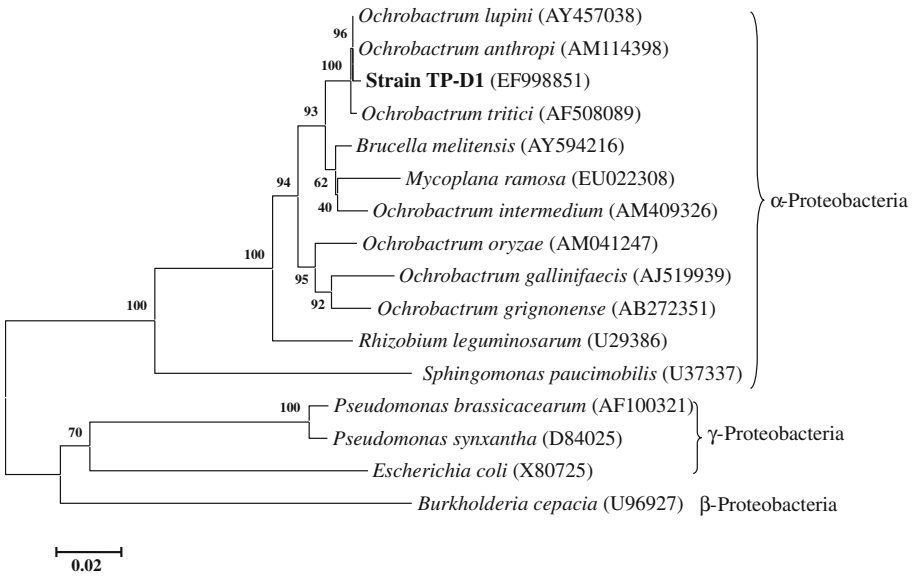

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain TP-D1 and representative species of the genus *Ochrobactrum* and related genera. This tree was constructed with the neighbor-joining method using MEGA 4.0. GenBank accession numbers are shown in parentheses. The significance of each branch is indicated by a bootstrap value for 1,000 subsets. *Bar*, 2 nt substitutions per 100 nt

Table 1 Comparison of strain TP-D1 with *O. lupini*, *O. anthropi* and *O. tritici*

Characteristics	Strain TP-D1	*O. lupini*	*O. anthropi*	*O. tritici*
Growth at 40°C	−	W	V	+
Growth at pH 10	+	+	ND	−
Assimilation of Citrate	+	+	−	−
Nitrate reduction	−	−	+	+
Hydralyzation of Esculin	−	+	−	−
Utilization of D-Mannose	+	+	+	−
Nitrogen fixation	+	+	−	−
Reaction to Polymyxin B	R	R	S	S
References	This study	Trujillo et al. (2005)	Holmes et al. (1988)	Lebuhn et al. (2000)

W weak; *V* variable, *ND* not determined; + positive; − negative; *R* resistant; *S* sensitive

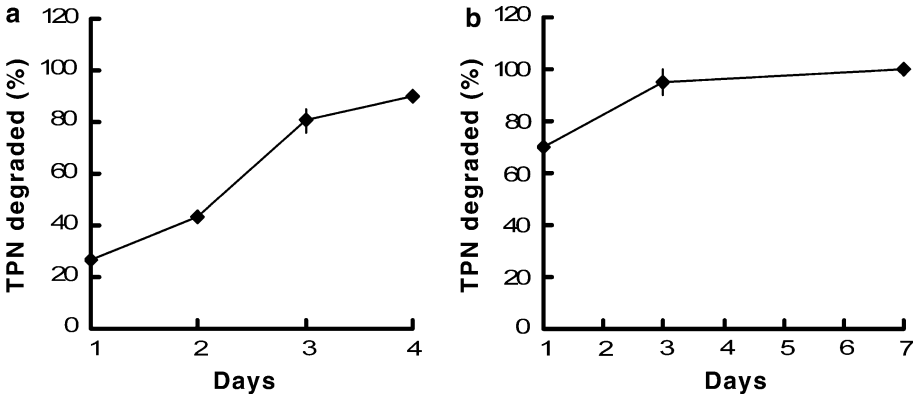

Fig. 2 Degradation efficiency of TPN by strain TP-D1 in mineral salt broth at 30°C (**a**) and in autoclaved soil at 28°C (**b**). Each value in the panel represents the mean ± SD

2, 5, 6-trichloro-3-cyano-4-methoxy-benzoate from its isotope clusters of three chlorine atoms and its *m*/*z* (Fig. 4b, b-3). Metabolite D was assumed to be converted from hydration of the cyano- group of TPN, and might be methyl 3-cyano-2, 4, 5, 6-tetrachlorobenzoate (Fig. 4b, b-4). And metabolites C and D might be esterified from CCHT and CTB in the presence of formic acid during the analysis procedure.

In conclusion, the metabolites of TPN degraded by strain TP-D1 in modified MS broth might be TPN-OH, methyl 2, 5, 6-trichloro-3-cyano-4-methoxy-benzoate, and methyl 3-cyano-2, 4, 5, 6-tetrachlorobenzoate. It was

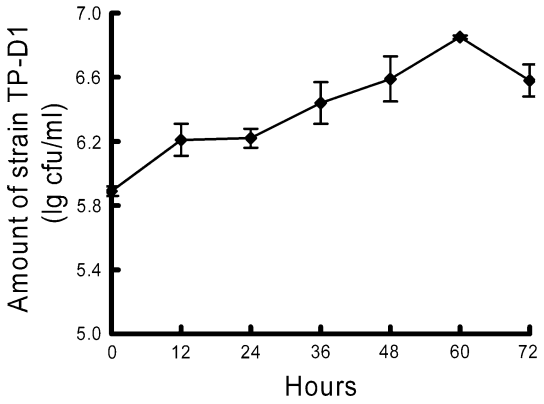

Fig. 3 The growth of strain TP-D1 in modified MS broth containing 50 mg TPN l^{-1} at 30°C. Each value in the panel represents the mean ± SD

proposed that TPN was degraded by strain TP-D1 via direct hydroxylation of its chloro- group (Pathway I), hydration of its cyano- group (Pathway II), or both (Pathway III) (Fig. 5).

Discussion

Bacterial degradation of TPN has been reported previously (Katayama et al. 1991; Sato and Tanaka 1987). TPN metabolites, DTTC and CCHT that were probably bioconverted from the oxidation of the cyano-group of TPN, have been detected in soil, suggesting the presence of some bacteria responsible for the oxidation process (Roberts and Hutson 1999; Rouchaud and Roucourt 1988). Herein we report on the isolation and characterization of a bacterial strain, denoted TP-D1, which can degade TPN without carbon supplement and produce two new metabolites besides TPN-OH. Compared with the reported TPN-degrading bacteria, strain TP-D1 had similar TPN-degrading ability with *Ochrobactrum* sp. CTN-11 (Liang et al. 2010), but stronger than that of *Flavobacterium* NL0-1 (Katayama et al. 1991) and TB I (Motonaga et al. 1996).

TPN-OH (Motonaga et al. 1996; Liang et al. 2010) and methylthiotrichloroisophthalonitrile (Katayama et al. 1997) have been reported to be TPN metabolites. In this study, we detected two hitherto unreported metabolites, methyl 2, 5, 6-trichloro-3-cyano-4-methoxy-benzoate (metabolite C) and methyl 3-cyano-2, 4, 5, 6-tetrachlorobenzoate (metabolite D). Metabolite B was not identified due to the limited information about the conversion of $–CONH_2$ group in TPN into $–CH_2CONH_2$ group. The $–COOCH_3$ group in metabolites B, C, and D might be esterified in the presence of formic acid during the analysis procedure instead of during the extraction process because the reaction conditions we were improper. During the detection procedure, we set up a blank control to avoid TPN conversion by chemicals during the extraction and analysis procedure, but no other metabolite except for TPN was detected (Table 2). It indicated that metabolites C and D derived from TPN degradation. We assume that metabolites C and D come from CCHT (Rouchaud and Roucourt 1988) and 3-cyano-2,4,5,6-tetrachlorobenzamide (CTB) (Szalkowski and Stallard 1977), respectively. CTB has been detected as a chemical hydrolysis product of TPN at pH 9.0 (Szalkowski and Stallard 1977); however, it is a former product of DTTC. DTTC and CCHT have been recovered from soil under field conditions, and are thought to be the biodegradation products under mild conditions (Rouchaud and Roucourt 1988). Although we did not detect CCHT and CTB in this study, detection of its downstream metabolites C and D suggested that TPN could be degraded by a specific bacterium through conversion of cyano- group of TPN, and CTB could be degraded microbiologically. Considering the presence of metabolite C, we proposed another degradation pathway besides the well-known TPN degradation pathways I and II (Putnam et al. 2003; Regitano et al. 2001; Roberts and Hutson 1999) (Fig. 5). In pathway II and III, the conversion of –cyano group of TPN was an important step, but no report has described the –cyano group breaking or substitution by degrading enzymes. Hitherto, only chlorine substitution of TPN by a hydrolytic dehalogenase (Wang et al. 2010b) and glutathione S-transferase (Kim et al. 2004) has been described. Isolation of strain TP-D1 and establishing its degradation pathway benefit our understanding of the TPN degradation mechanism.

Strain TP-D1 and strain TB I (Motonaga et al. 1996) could degrade TPN and grow slightly in MS broth. The reason might be that the medium was not purified enough and contained some carbon sources. However, their degrading metabolites were different, strain TB I only degraded TPN into TPN-OH. This difference might be ascribed to the detection method besides their different degrading ability. In this study, we used SPE, LC-APCI-MS and LC-ESI-MS techniques, which facilitate the analysis of amide degradation products (Chaves et al. 2008). During the whole degradation process, metabolites B, C and D could be detected at day 2, but were absent at day 6, suggesting that these metabolites were intermediates. However, no other metabolites were detected, probably due to the unsuitable detection method or the low concentrations of these metabolites. In this study, we also detected TPN-OH as a major metabolite (Table 2), and its presence might kill the cells and inhibit the growth of strain TP-D1 (Fig. 3) (Motonaga et al. 1996), consequently limiting the continuous conversion of cyano- group of TPN. Therefore, strain TP-D1 is not a safe bacterium to be used directly for bioremediation of TPN-polluted soil.

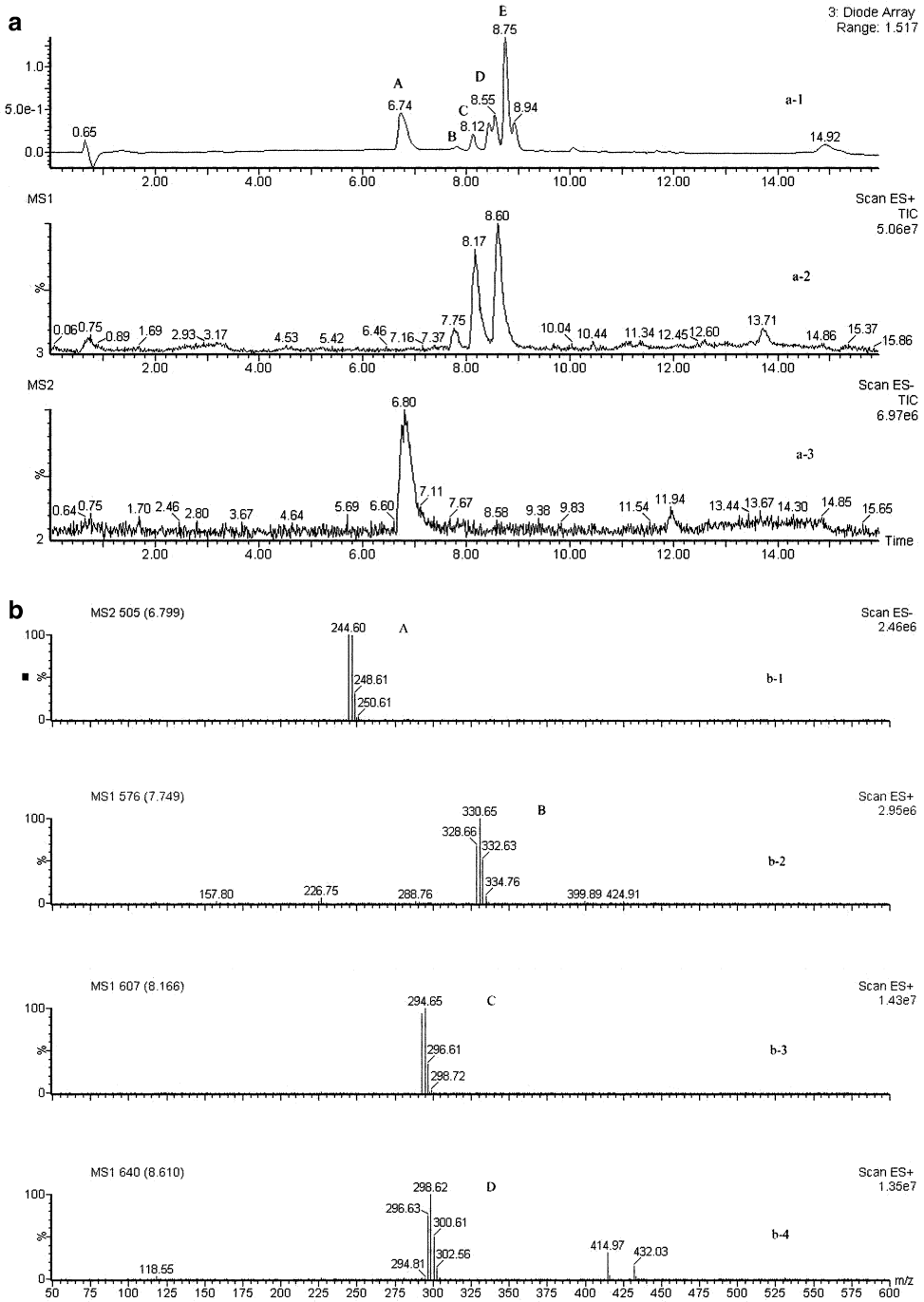

Fig. 4 HPLC, ion spectra and mass spectra of TPN metabolites degraded by strain TP-D1 in MS broth after incubation at 30°C for 2 days. Metabolites were extracted with methanol (V/V = 1:1), followed by SPE procedure and LC-ESI-MS analysis. **a** HPLC (*a−1*) and ion spectra of TPN and its metabolites detected with LC-ESI-MS (*a−2, a−3*). **b** Mass spectra of metabolites in (**a**)

Strain TP-D1 was classified to be *O. lupini* according to its 16S rRNA gene sequence and biochemical and physiological characteristics. Several *Ochrobactrum* strains have been reported to have the ability to degrade pesticide and organic chemicals, including TPN (Liang et al. 2010a), triazophos (Dai et al. 2005), parathionmethyl (Bai et al. 2004; Qiu et al. 2006) and aniline (Wei et al. 1998). However, even the same bacterial strain has different degrading ability to chemicals with similar groups. For example, *Ochrobactrum* strain mp_4 could degrade triazophos, methylparathion, phoxim, parathion and malathion, but not methamidophos (Dai et al. 2005). In order to know the degrading specificity of strain TP-D1, we tested it against several chemicals including pentachloronitrobenzene (PCNB), hexachlorocyclohexane (BHC), dichloro-diphenyl-trichloroethane (DDT), triazophos, parathionmethyl and aniline on MS agar plates at a concentration of 5 mg l^{-1}, and no bacterial growth or clear zone around the colonies was detected (unpublished data). This indicated that strain TP-D1 could not metabolize the tested chemicals as sole carbon

Table 2 TPN and its metabolites detected by LC-ESI-MS and LC-APCI-MS after degradation of strain TP-D1 at 30°C

Incubation period (days)	*m/z*[a]									
	Analysed by LC-ESI-MS					Analysed by LC-APCI-MS				
	A (NI)	B (PI)	C (PI)	D (PI)	E (NI)	A (NI)	B (PI)	C (PI)	D (PI)	E (NI)
0										244.7, 263.7
2	244.6	328.7	294.7	296.6	–	244.8	–	–	296.8	263.8
4	244.6	–	–	296.6	–	244.7	–	–	–	244.7
6	244.6	–	–	–	–	244.7	–	–	–	244.7

[a] A, B, C, D and E represents TPN-OH, 3-methoxycarbonyl-2,4,5,6-tetrachlorobenzeneacetamide, methyl 2,5,6-trichloro-3-cyano-4-methoxy-benzoate, methyl 3-cyano-2,4,5,6-tetrachlorobenzoate, and TPN, respectively

NI detected at negative mode; *PI* detected at positive mode; – no metabolites detected

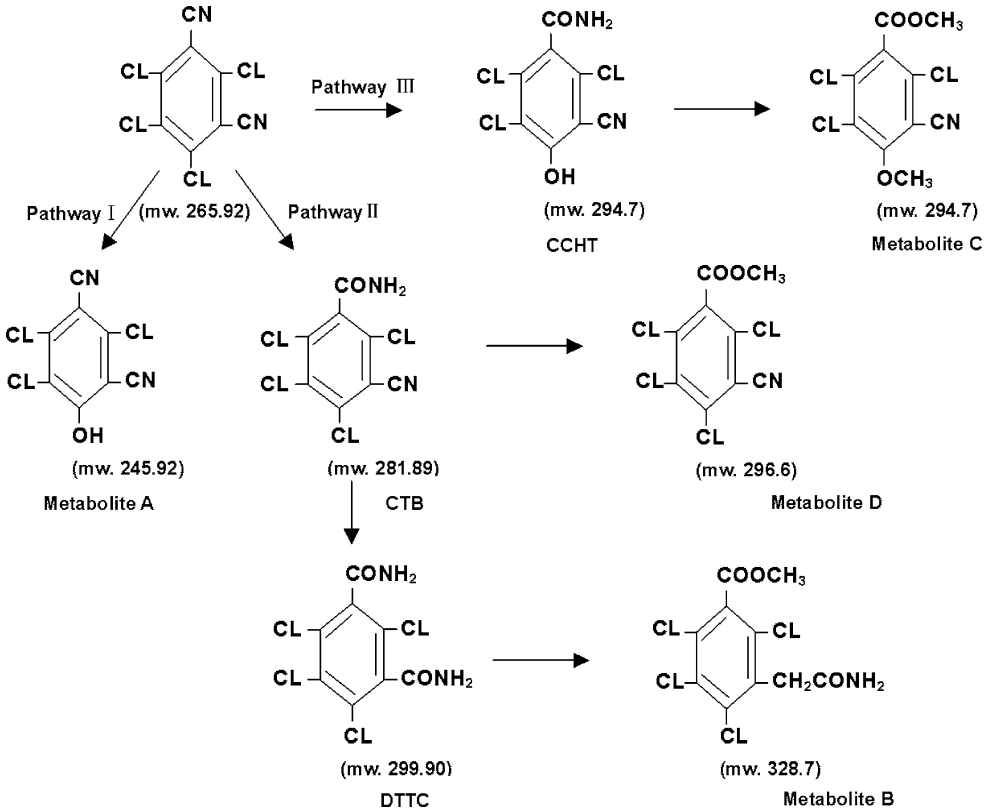

Fig. 5 Proposed metabolic pathways of TPN degraded by strain TP-D1 in modified MS broth containing 50 mg l^{-1} TPN. Pathway I showed the hydroxylation of chloro- group; Pathway II show hydration of its cyano- group; Pathway III showed both hydroxylation of the chloro- and hydration of cyano- group

source, or the ability of strain TP-D1 to degrade these chemicals was too weak to be observed. Further research on the degradation specificity of strain TP-D1 should be conducted in liquid broth.

TPN could be degraded by a hydrolytic dehalogenase, but the enzyme is specific. For example, the hydrolytic dehalogenase isolated from *Pseudomonas* sp. CTN-3 had no capacity to degrade some chloroaromatics including PCNB (Wang et al. 2010b). Further research on the degrading enzymes of strain TP-D1 should be conducted. Genes coding for these enzymes will be cloned, and their functions will be verified in future.

Conclusions

A novel TPN-degrading strain, *O. lupini* TP-D1, was isolated from heavily TPN-polluted soil. Strain TP-D1 had a strong ability to degrade TPN without nutrient supplement, and produced two new metabolites besides TPN-OH. We propose that strain TP-D1 could degrade TPN via another new metabolic pathway related to the conversion of –cyano group in TPN. Because TPN-OH was still the main metabolite of TPN degraded by strain TP-D1, our future objectives are to isolate other strains that are devoid of TPN-OH production, or to engineer strain TP-D1 not to produce TPN-OH.

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