Ecotoxicology and Environmental Safety 138 (2017) 39–46

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Enhancement of microbial 2,4,6-trinitrotoluene transformation with increased toxicity by exogenous nutrient amendment

Shih-Hsiung Liang[a], Duen-Wei Hsu[a], Chia-Ying Lin[a], Chih-Ming Kao[b], Da-Ji Huang[c], Chih-Ching Chien[d], Ssu-Ching Chen[e], Isheng Jason Tsai[f], Chien-Cheng Chen[a,*]

[a] Department of Biotechnology, National Kaohsiung Normal University, Kaohsiung, Taiwan
[b] Institute of Environmental Engineering, National Sun Yat-sen University, Kaohsiung, Taiwan
[c] Department of Environmental Resources Management, Chia Nan University of Pharmacy & Science, Tainan, Taiwan
[d] Graduate School of Biotechnology and Bioengineering, Yuan Ze University, Chung-Li, Taiwan
[e] Department of Life Sciences, National Central University, Taoyuan, Taiwan
[f] Biodiversity Research Center, Academia Sinica, Taipei, Taiwan

ARTICLE INFO

Keywords:
2
4
6-trinitrotoluene (TNT)
Transformation
Bioremediation
Cometabolism
Nitroreductase
Toxicity

ABSTRACT

In this study, the bacterial strain *Citrobacter youngae* strain E4 was isolated from 2,4,6-trinitrotoluene (TNT)-contaminated soil and used to assess the capacity of TNT transformation with/without exogenous nutrient amendments. *C. youngae* E4 poorly degraded TNT without an exogenous amino nitrogen source, whereas the addition of an amino nitrogen source considerably increased the efficacy of TNT transformation in a dose-dependent manner. The enhanced TNT transformation of *C. youngae* E4 was mediated by increased cell growth and up-regulation of TNT nitroreductases, including NemA, NfsA and NfsB. This result indicates that the increase in TNT transformation by *C. youngae* E4 via nitrogen nutrient stimulation is a cometabolism process. Consistently, TNT transformation was effectively enhanced when *C. youngae* E4 was subjected to a TNT-contaminated soil slurry in the presence of an exogenous amino nitrogen amendment. Thus, effective enhancement of TNT transformation via the coordinated inoculation of the nutrient-responsive *C. youngae* E4 and an exogenous nitrogen amendment might be applicable for the remediation of TNT-contaminated soil. Although the TNT transformation was significantly enhanced by *C. youngae* E4 in concert with biostimulation, the 96-h LC50 value of the TNT transformation product mixture on the aquatic invertebrate *Tigriopus japonicas* was higher than the LC50 value of TNT alone. Our results suggest that exogenous nutrient amendment can enhance microbial TNT transformation; however, additional detoxification processes may be needed due to the increased toxicity after reduced TNT transformation.

1. Introduction

The massive use of military and industrial explosives (i.e., trinitrotoluene and related compounds) has seriously contaminated a vast area of soil and groundwater (Khan et al., 2012). The remediation of TNT-contaminated sites to sustain the safety and quality of environmental ecosystems has become an urgent need. Various traditional physical-chemical procedures for ex situ soil slurry reactors, soil incineration, landfilling, soil composting, and thermal desorption have been established for the remediation of TNT-contaminated soils; unfortunately, these procedures are very cost-intensive. Recently, biological-based remediation has been considered as an ecologically compatible approach to detoxify areas contaminated with TNT (Erkelens et al., 2012; Kalderis et al., 2011; Weisse, 2008). Bioremediation technologies primarily focus on microbial degradation and phytoremediation and provide a promising low-cost prospective to clean up TNT-contaminated soil (Panz and Miksch, 2012; Stenuit and Agathos, 2010).

Although some microorganisms have evolved oxidative and reductive pathways to transform TNT (Smets et al., 2007), TNT is less susceptible to oxidative microbial degradation due to the electron deficiency of the aromatic ring (Heiss and Knackmuss, 2002). In contrast to oxidation, the reduction of TNT's nitro groups is a ubiquitous and fundamental reaction for the microbial transformation of TNT. Reductive pathways rely on either the reduction of the nitro groups to hydroxyamino/amino derivatives or the reduction of the aromatic ring via hydride addition to form a monohydride-Meisenheimer complex or a dihydride-Meisenheimer complex, resulting

* Corresponding author.
E-mail address: cheng@nknu.edu.tw (C.-C. Chen).

http://dx.doi.org/10.1016/j.ecoenv.2016.12.012
Received 26 July 2016; Received in revised form 18 November 2016; Accepted 8 December 2016
Available online 22 December 2016

in the release of nitrite (Symons and Bruce, 2006; Williams et al., 2004). Reactive nitroso and hydroxylamino intermediates can further react with the condensated azoxy-dimers and acetyl derivatives of TNT. Under strictly anaerobic conditions, monoaminodinitrotoluene (ADNT) is further reduced to 2,4,6-triaminotoluene (2,4,6-TAT), which is highly reactive and can polymerize or irreversibly bind to the organic soil matrix (Thiele et al., 2002).

Bacterial nitroreductases have been shown to catalyze the breakdown of nitroaromatic compounds. These enzymes fall into two main families: oxygen-insensitive nitroreductases and old yellow enzymes (OYEs). Some oxygen-insensitive nitroreductases have been intensively studied, such as NfsA and NfsB from *Escherichia coli* (Zenno et al., 1996a, 1996b), PnrA and PnrB from *Pseudomonas putida* (Caballero et al., 2005b), and NitA and NitB from *Clostridium acetobutylicum* (Kutty and Bennett, 2005). The OYEs are classified into type I and type II and mediate the NADPH-dependent addition of one or two hydride ions to the aromatic ring, resulting in the formation of Meisenheimer-complexes, which is often accompanied by the release of nitrite (Kim and Song, 2005; Pak et al., 2000). The well-characterized OYE members include XenA - XenF of *P. putida* KT24406, XenB from *Pseudomonas fluorescens* (Pak et al., 2000), PETN reductase from *Enterobacter cloacae* PB28, NemA reductase from *E. coli* (Miura et al., 1997) and YqjM from *Bacillus subtilis* (Fitzpatrick et al., 2003).

Many bacteria that are capable of degrading TNT have been selected from different niches (e.g., TNT-contaminated soil, where the presence of the energetic compound is favorable for the enrichment of an indigenous bacterial consortia that readily degrades and utilizes TNT for growth either as a nitrogen source or as a carbon and energy source (Singh et al., 2012; Symons and Bruce, 2006)). A specific purpose for the screening of TNT-degrading strains is bioaugmentation, which is defined as a technology for improving the degradative capacity of contaminated areas via the addition of competent microorganisms (Mrozik and Piotrowska-Seget, 2010). Additionally, supplementary exogenous nitrogen and carbon sources can stimulate microbial TNT transformation in solution (Cho et al., 2009). The biostimulation of the indigenous consortia for TNT degradation in contaminated soil was also observed with amendments such as fertilizers, organic biosolids, green/animal manures and molasses (Makris et al., 2010; Muter et al., 2012).

Previous studies reported that TNT and its metabolites exhibited a highly persistent, toxic and mutagenic potential on all living organisms (Muter et al., 2012; Rylott et al., 2011). The accumulation of TNT can reach very high concentrations near/on the soil surface (Erkelens et al., 2012), and the high bioavailability readily results in toxic effects on the biota of contaminated sites. In this study, *C. youngae* strain E4 was isolated from TNT-contaminated soil. The effect of exogenous nutrient sources on TNT transformation, the cell numbers and nitroreductase expression by *C. youngae* E4 were analyzed. A pilot-scale experiment to assess TNT transformation was conducted using a TNT-contaminated soil slurry via biostimulation combined with *C. youngae* inoculation. Finally, *T. japonicas* was used to investigate the biotoxicity of the TNT metabolites transformed by *C. youngae*.

2. Materials and methods

2.1. Isolation and identification of TNT-degrading bacteria

A one-gram soil sample collected by the grab sampling technique following the soil sampling guideline (Soil Sampling Method, S102.62B, National Institute of Environmental Analysis, Taiwan Environmental Protection Administration) from a TNT-contaminated site located in southern Taiwan (Kaohsiung, Taiwan) was inoculated into Luria-Bertani (LB) broth and aerobically cultured at 30 °C with gentle agitation. The culture medium (10 μL) was streaked onto a LB agar plate. After growth overnight at 30 °C, colonies with different morphologies were selected and inoculated into LB broth and then into TNT-NFG mineral salts medium. The TNT transformation capacity of each bacterial isolate in TNT-NFG (nitrogen-free glucose salt) medium was assessed by observation of the turbidity and optical density at 600 nm. Pure cultures obtained from the enrichment process were subjected to initial identification by determination of their 16 S rRNA gene sequences. The 16 S rRNA gene sequences were analyzed as previously described by Chen et al. (2001). The almost-complete 16 S rRNA gene sequence (1.4 kb) was compared with the sequences available from the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). The TNT-NFG defined medium contained $K_2HPO_4{\cdot}2H_2O$ (50 mM), KH_2PO_4 (100 mM), $MgSO_4{\cdot}7H_2O$ (1 mM), $CaCl_2$ (0.1 mM), glucose (0.8%, w/v) and TNT (100 mg/L). Glucose, as a carbon source in this case, was passed through a 0.22-μm filter and added to the NFG medium to the desired concentrations (w/v). All chemicals used in this study were commercially available chemicals of reagent or analytical grade.

2.2. Determination of TNT biotransformation by bacterial cultures

C. youngae E4 was initially cultured in M9 minimal medium until mid-log phase (OD_{600} of 0.5). A cell pellet from 1 mL of a M9 bacterial culture was centrifuged, washed twice with NFG medium, and subsequently inoculated into 100 mL of TNT-NFG medium complemented with glucose (0.8%, w/v) and various concentrations of nitrogen nutrients, NH_4Cl (0.025%, 0.05%, 0.1%, 0.2%) or yeast extract and tryptone hydrolysate equivalent to 0%, 0.5%, 1%, and 2% of the LB broth. Then, the bacteria were cultured at 30 °C with agitation for 72 h. An experiment set with 2% LB or 0.2% NH_4Cl amendment but no bacterial inoculation served as the abiotic control. The TNT concentrations in the suspension at successive time intervals were determined by HPLC as described below. Three independent experiments were conducted.

The amount of TNT in the culture medium was determined by high-performance liquid chromatography (HPLC) according to EPA (US Environmental Protection Agency) Method 8330 A. During the growth process, 1 mL of the culture medium was collected by centrifugation at successive time intervals. The supernatants were collected after passing through a 0.22-μm filter and stored at 4 °C. TNT present in the supernatants was identified and quantified by HPLC analysis using pump L-2130 equipped with a L-2450 UV–vis diode array detector (DAD) (Hitachi, Japan). The DAD has variable wavelength in the UV–vis range (200–700 nm). The experiment was conducted at ambient temperature using a Thermo™ Acclaim Explosives E2 column (5 μm, 4.6 × 250 mm) (Thermo Fisher Scientific, PA, USA) with methanol/water (50:50 v/v) as the mobile phase and a flow rate of 1 mL/min. The sample injection was 20 μL, and the detection was performed at 254 nm and 30 °C. The combined stock solution NAIM-833E purchased from Ultra Scientific (N. Kingstown, RI, USA) was used as an analytical mix standard.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

C. youngae E4 cultured in M9 medium (OD_{600} 0.5) was collected, washed and inoculated at 100-fold dilutions into 100 mL of TNT-NFG medium containing glucose (0.8%, w/v) with/without 0.02% tryptone hydrolysate and 0.01% yeast extract. The cells were harvested 24, 48 and 72 h after inoculation. Degenerate primer pairs were designed based on the conserved nucleotide sequences of the nitroreductase genes *nemA*, *nfsA* and *nfsB* from related bacterial species (Table 2). PCR was employed to amplify a partial DNA fragment of each nitroreductase using the chromosomal DNA extracted from *C. youngae* E4 as the template, and the partial DNA fragments were sequenced. To determine the nitroreductase transcription levels, total RNA was isolated using the Ambion TRIzol reagent according to the manufacturer's recommendations (Invitrogen, USA). The relative quantities of mRNA from the bacteria were assessed by RT-PCR. One microgram of total RNA from each sample was used to generate first-strand cDNA using random hexamers (Invitrogen, USA) and the M-MLV reverse transcriptase