Ann Microbiol (2015) 65:467–476
DOI 10.1007/s13213-014-0880-5

ORIGINAL ARTICLE

Biodegradation of 2,4-dinitrotoluene (DNT) by *Arthrobacter* sp. K1 isolated from a crude oil contaminated soil

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Received: 9 September 2013 / Accepted: 18 March 2014 / Published online: 10 April 2014

Abstract Laboratory experiments were performed to characterize and identify 2,4-dinitrotoluene (DNT)-degrading bacterial strains isolated from crude oil contaminated soil from a landfill dump site of a petroleum refinery in Mersin, Turkey. Fluorescent in situ hybridization (FISH) with *dntAa* probes encoding 2,4-DNT dioxygenase was used to detect 2,4-DNT-degrading bacteria. The direct FISH analysis of soil samples collected from a petroleum refinery showed very weak signals. Therefore, a selective enrichment culture technique using 2,4-DNT as the sole carbon source was then used to isolate DNT degrading bacteria. Following the culture enrichment procedure, the hybridization signals improved significantly in the isolated bacterial strains. Based on 16S rRNA sequences, the bacteria isolated from the soil samples were identified as *Arthrobacter* sp. Results from the batch biodegradation experiments indicate that the biodegradation rates of 2,4-DNT with this strain were highly dependent on environmental conditions such as pH and temperature, with optimum conditions obtained at 30 °C and pH ~7. A first-order kinetic model was able to accurately describe 2,4-DNT degradation rates under different environmental conditions (e.g., pH). The ability of *Arthrobacter* sp. for degrading 2,4-DNT was found to be plasmid-mediated through curing experiments. The size of the plasmid involved, referred to as pArK1, was estimated to be about 8.1 kb.

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Keywords Biodegradation · *Arthrobacter* · 2,4-Dinitrotoluene (DNT) · Plasmid · pArK1 · Fluorescence in situ hybridization (FISH)

Introduction

Nitroaromatic compounds (NACs) and polycyclic aromatic hydrocarbons (PAHs), constituents of crude oil and halogenated organic compounds, together constitute a large and diverse group of chemicals that are responsible for causing widespread environmental pollution (Jain et al. 2005). Nitroaromatic compounds have been released into the environment mostly due to various industrial activities involving explosive, pesticide, and dye production (Atlas 1981). 2,4-Dinitrotoluene (2,4-DNT), for instance, is produced as the major by-product during the synthesis of the explosive 2,4,6-trinitrotoluene (TNT). Due to its abundance, toxicity, and carcinogenic properties, 2,4-DNT is treated as a priority pollutant in several countries (Keith and Telliard 1979; Ortega-Calvo et al. 1999).

Many microorganisms can adapt their catabolic activities to make use of toxic organic pollutants as food sources, and thereby mineralize complex organic compounds to simpler compounds, carbon dioxide, and water (Singh et al. 2009). The presence of NACs in the environment creates intense selective pressure that can lead to the evolution of microorganisms capable of degrading a growing number of NACs (Jain et al. 2005). A number of bacterial strains can degrade 2,4-DNT by a pathway involving two oxygenase reactions that lead to the removal of the nitro-substituents. For example, 2,4-DNT is first oxidized to 4-methyl-5-nitrocatechol (MNC) by 2,4-DNT dioxygenase in *Burkholderia cepacia*. Then, monooxygenase and a quinone reductase can convert MNC sequentially to 2-hydroxy-5-methylquinone and 2,4,5-trihydroxytoluene (THT). Ring cleavage catalyzed by THT

oxygenase occurs at the meta-position (Johnson et al. 2002). The *dntA* and *dntD* genes, encoding DNT dioxygenase and 2,4,5-THT oxygenase, respectively, were cloned and characterized (Suen et al. 1996; Haigler et al. 1999).

Microbial communities from crude oil-contaminated soils have been shown to be capable of degrading NACs (Popescu et al. 2003). For instance, several *Arthrobacter* species constitute a large fraction of the aerobic microorganisms commonly found in soils and contaminated sites (Jain et al. 2005). They contribute to the cycles of elements, and can decompose a variety of contaminants, such as PAHs, pesticides, phenols, and polychlorinated biphenyls (Pohlenz et al. 1992; Pipke and Amrhein 1988; Hayatsu et al. 1999; Turnbull et al. 2001; Tixier et al. 2002; Widehem et al. 2002). Some strains of *Arthrobacter* sp. have been reported to grow using nitrophenol and 2,4,6-trinitrotoluene (TNT) (Tope et al. 1999; Qui et al. 2009). As stated above, although *Arthrobacter* sp. have previously been shown to successfully degrade various organic contaminants, to our knowledge, studies regarding the use of *Arthrobacter* sp. for the degradation of 2,4-DNT are not available in the literature. The purposes of the current study were to: (1) isolate and identify 2,4-DNT-degrading bacteria from soils contaminated with crude oil, and (2) determine the effects of environmental factors (e.g., pH, temperature) on 2,4-DNT biodegradation rates.

Materials and methods

Soil samples

Soil samples were collected from a crude oil-contaminated soil near a petroleum refinery located in the Mediterranean region of Turkey (36°49′27″N, 34°42′3″E). The samples (approximately 500 g) were transferred into sterile jars, and then transported to the laboratory for further analysis.

Culture media

Minimal Medium 9 (MM9) was used to isolate 2,4-DNT degrading bacteria. MM9 culture medium was composed of 60 g $NaH_2PO_4.7H_2O$, 30 g KH_2PO_4, 10 g NH_4Cl, 5 g NaCl, 0.52 g $MgSO_4$, and 2.5 mL mineral salt solution in 1,000 mL of distilled water. The mineral salt solution contained 0.05 g $ZnCl_2$, 0.03 g $MnCl_2$, 0.2 g $CoCl_2$, 0.02 g $NiCl_2$, 0.01 g Na-molybdate, and 0.01 g $CuCl_2$ in 1,000 mL distilled water. Yeast extract (0.5 g/L) was added to the MM9 media when the enrichment culture medium was prepared.

Oligonucleotide probe

Specific probes were designed for *dntAa* from the 2,4-DNT-degrading genes (Table 1). The probes were synthesized by Metabion (Germany) and Microsynth (Switzerland). The probe that targeted the *DntAa* gene was labeled with FITC (green). Nonspecific DAPI (4′,6-diamidino-2-phenylindole) (blue) dye was used to test cell viability. Cy3-labeled probes EUB338, which are complementary to a portion of the 16S rRNA genes conserved in the domain *Bacteria*, were used to visualize the entire bacterial population in the specimens (Martin et al. 2008). To control non-specific binding of EUB338, probe NON338 was used (Manz et al. 1992) (Table 1).

Isolation of 2,4-DNT degrading bacteria

A selective enrichment technique was used to isolate bacterial strains from the crude oil-contaminated soil samples. The soil sample (1 g) was added to 5 mL of sterile distilled water, and the suspension was centrifuged at 2,000 rpm for 10 min. Then, a 0.5-mL sample withdrawn from the supernatant was inoculated into 100 mL of MM9 culture medium containing 100 mg/L 2,4-DNT. The flasks were incubated at 30 °C with constant shaking at 150 rpm. The culture was continuously enriched at 5-day intervals. Growth strains inoculated onto MM9 agar containing 2,4-DNT were incubated at 30 °C for 5 days. Following incubation, the best breeding colony was selected. If necessary, the bacterium was also grown on Luria-Bertani medium.

Identification of bacteria by 16S rRNA analyses

16S rRNA sequencing was carried out using an ABI 3130XL Genetic Analyzer (PE Applied Biosystems). A Qiagen DNeasy Blood&Tissue Kit was used for the isolation of bacterial genomic DNA. The PCR was conducted using a reaction volume of 0.2× Taq buffer (Applied Biosystems), 1.5 mM $MgCl_2$, 0.25 mM dNTP (Fermentas), 0.03 U of AmpliTaq Polymerase (Applied Biosystems), 0.4 pmol (each primer), and 300 ng of bacterial genomic DNA. Amplification products were cleaned using a Macherey-Nagel NucleoSpin Extract II kit. Sequence data were classified with reference genomes using Molecular Evolutionary Genetics Analysis (MEGA) and its software MEGA5 (http://www.megasoftware.net). The phylogenetic tree was designated by using the minimum evolution tree method (Tamura et al. 2011).

High performance liquid chromatography (HPLC)

Culture turbidity measurements were obtained using a Perkin Elmer Lambda EZ 210 series UV–Vis spectrophotometer at 600 nm wavelength. Residual 2,4-DNT was analyzed by HPLC. A sample mixture was prepared by taking 0.7 mL of a sample and mixing it with 0.7 mL of acetonitrile. The resulting mixture was vortexed, and centrifuged at

Table 1 Oligonucleotide probes

Probe	Specificity	Sequence	Reference
646 F	*DntAa* gene	5′- FITC- AACTGGTAGTAGAACCCCTC-3′	(Snellinx et al. 2003)
1687 R	*DntAa* gene	5′-FITC- GGGGTTCACTCATGGCTTGG-3′	(Snellinx et al. 2003)
Eub338	Bacteria, 16S rRNA, position, 338–355	5′- CY3- GCTGCCTCCCGTAGGAGT-3′	(Martin et al. 2008)
Non338	Negative control	5′- CY3- ACTCCTACGGGAGGCAGC-3′	(Manz et al. 1992)

3,000 rpm for 5 min, and the supernatant was filtered through a 0.45-μm PIFE syringe filter unit (Gelman, MI, USA). The filtrate was analyzed for 2,4-DNT using a PerkinElmer Series 200 and a model 3 10 UV–Vis detector set at 254 nm. The mobile phase was methanol:water mixture (50:50, v/v), and 20-μL samples were injected into a ODS-2-C-18 column at 25 °C. The flow rate of the solvent was set to 1.0 mL/min (Park et al. 2003) The 2,4-DNT concentration in samples was then estimated based on calibration curves obtained using a standard.

Effects on temperature and pH on biodegradation of 2,4-DNT

The bacterial cells were incubated in MM9 medium containing 100 mg/L 2,4-DNT over a temperature range of 20–45 °C. The biomass growth and concentrations of 2,4-DNT were monitored on the 5th day of the experiments. The effects of pH on degradation rates were determined by incubating cultures containing 100 mg/L 2,4-DNT at pHs ranging from 4 to 9 using the experimental procedure outlined above. The pH of the culture medium was adjusted using 1 N HCl and 1 N NaOH. The biomass growth and concentrations of 2,4-DNT were continuously monitored for 10 days. The cultures were incubated at a desired temperature with constant shaking at 150 rpm.

Fluorescence in situ hybridization (FISH)

Bacterial cell samples harvested directly from soil samples and cells grown in MM9 medium with 100 mg/L 2,4-DNT culture were washed three times with 3× phosphate-buffered saline (PBS) containing 10 mM sodium phosphate buffer ($Na_2HPO_4{\cdot}7H_2O$) and 130 mM NaCl at pH 7.2. Following washing with PBS, the cells were fixed with 4 % paraformaldehyde at 4 °C for 3 h, and then stored in 1× PBS containing 50 % ethanol at −20 °C. Before analysis, a 250-μL fixed sample was re-suspended in 0.5 mL of 3× PBS, and then centrifuged at 13,000 rpm for 3 min. After the pellet was re-suspended in 0.5 mL of sterile-distilled water, a 10-μL sample was injected into each well on a slide, and then dried at 46 °C.

Next, 2 μL of lysozyme solution (lysozyme solution; 10 mg/L lysozyme in 50 mM Tris/HCl, pH 8.0) and 10 μL of 1× PBS were applied to the dried slides, followed by incubation for 5 min at 37 °C. The slides were rinsed with distilled water, and consecutively dehydrated in 50, 80, and 96 % ethanol for 2 min each, and then allowed to dry. To each well on the slides, 17 μL of hybridization solution and 3 μL of probe were added. In situ hybridization was performed in flasks at 46 °C for 4 h. After the hybridization, cells were stained with DAPI. Then, 10 μL of DAPI solution was added to each well, followed by incubation for 15 min at room temperature. Excess probes were washed off twice with washing buffer by heating at 48 °C for 7 min. As soon as the slides were dried at 46 °C, the cells were inserted into DABCO (1,4-diazabicyclo[2.2.2]octane) as antifading reagent (Amann 1995; Pernthaler et al. 2001). Microscopic observation was performed using an epifluorescence microscope (Olympus BX51). The option of different microscopic filters allows for the discrimination of nonspecific (DAPI) and specific (FISH) labeled cells. Image analysis was performed with DP-BSW software. Permeabilization and hybridization protocols or FISH were optimized for the bacterial strain. Hybridization was performed at different temperatures, and enzyme treatment was applied to enhance the uptake of probes by increasing cell membrane permeability.

Plasmid curing

The plasmid curing was performed as described by Trevors (1986). In brief, the culture was grown in nutrient broth containing ethidium bromide at the 500 μg/L concentration for 24 h at 30 °C. After the grown culture was plated on nutrient agar with and without 100 mg/L 2,4-DNT, the colonies were compared and analyzed.

Genomic DNA isolation and agarose gel electrophoresis

Total DNA of bacterial strain was isolated by using a Genomic DNA Isolation Kit (Fermentas). The DNA samples were transported in 0.7 % agarose gel at 60 V for 3 h. Following

staining with ethidium bromide, the band was observed on an UV-transilluminator. Molecular sizes of chromosomal and plasmid DNAs were determined with DNA size software.

Statistical analysis

The statistical analysis (correlation analysis) was performed using Microsoft Excel 2010. The software Scientist (Micromath) was used to fit related equations to the 2,4-DNT removal data as well as to test the statistical validity of model simulations.

Results

16S rDNA gene sequencing and phylogenetic analysis

Bacterial strains were isolated from soils contaminated with crude oil by enrichment culture technique using an inoculum of 2,4-DNT. The best-growing bacterial strains were selected for identification. The partial 16S rDNA sequence of bacterial strains was determined, and a phylogenetic tree was constructed based on the fast minimum evolution tree analysis (Fig. 1). Comparative ribosomal DNA gene sequence analysis indicated that the bacterial strain that showed the highest growth was in the same group as *Arthrobacter*. The strain had 99.1 % sequence similarity with *Arthrobacter mysorens* LMG 16219T (GenBank accession no. AJ639831) and 99.1 % sequence similarity with *Arthrobacter arilaitensis* Re117T (GenBank accession no. FQ311875) (Nand and Rao 1972; Irlinger et al. 2005). However, further detailed analysis should be performed to more specifically determine the species of bacterial strains isolated. Hereafter, this species will be referred to as *Arthrobacter* sp. K1 in the study. The morphology of colonies on a plate was yellow-brown, convex, glistening, smooth, and wet. The Gram-staining procedure indicated that the bacterial strains were Gram-positive.

Effects of temperature and pH on biodegradation of 2,4-DNT

Batch biodegradation experiments were performed to determine the effects of temperature on biomass growth and 2,4-DNT degradation by *Arthrobacter* sp. K1 (Fig. 2a). Here, the measurements were taken on day 5 of incubation. Note that the best growth occurred at temperatures between 30 and 37 °C. Similarly, the extent of 2,4-DNT degradation reached a maximum at 30–37 °C, and decreased sharply at higher or lower temperatures. This indicates that *Arthrobacter* sp. K1 uses 2,4-DNT as a substrate for cell growth. The strong correlation between cell growth and 2,4-DNT consumption was also statistically confirmed using Kruskal–Wallis analysis ($P<0.05$).

The effect of solution pH on 2,4-DNT degradation and cell growth is shown in Fig. 2b. Note that the biomass growth reached a maximum at near-neutral pH 7, and decreased significantly towards more acidic or more alkaline conditions. Similarly, the highest DNT consumption occurred at pH ~7, which coincided well with the maximum cell growth (Fig. 2b), indicating that there is a significant correlation with cell growth and DNT degradation by *Arthrobacter* sp. K1.

The results of cell growth and 2,4-DNT degradation as a function of time at pH 7 and 30 °C are presented in Fig. 2c. While the cell growth increased with increasing incubation time, the highest cell growth rate was observed in the first 2 days of incubation. This time coincides well with the maximum DNT consumption rate. The decrease in cell growth rate at incubation times >2 days may be explained through the consumption of the substrate (2,4-DNT) in solution. Note that nearly 70 % of 2,4-DNT was consumed during the first 10 days of incubation.

Modeling of 2,4-DNT degradation

A simple rate law can be used to determine microbial 2,4-DNT degradation kinetics under variable environmental conditions (Wittbrodt and Palmer 1995; Dogan et al. 2011):

$$\frac{d[C]}{dt} = k[C]^a[X]^b[H^+]^c \quad (1)$$

Where k is the rate constant, and C, X, and H^+ represent 2,4-DNT, biomass, and proton concentrations, respectively. Similarly, a, b, and c are the reaction orders with respect to 2,4-DNT, biomass, and proton concentrations, respectively. If the biodegradation of 2,4-DNT at constant proton and biomass concentrations follows a pseudo-first-order model, the rate equation can then be simplified to:

$$\frac{d[C]}{dt} = k[C] \quad (2)$$

The integrated form of this rate law is

$$\ln[C] = \ln[C_o] - kt \quad (3)$$

where C is the model predicted concentration of 2,4-DNT (M), C_o is the initial 2,4-DNT concentration, and k is the first-order rate constant. Figure 3a shows 2,4-DNT degradation rates at different pH values. Note that the reaction kinetics follow a first-order pattern with reaction rates increasing with increasing solution pH. Reaction rate constants and model statistics are presented in Table 2. In all simulations, the

Fig. 1 Phylogenetic tree with bootstrap values based on 16S rDNA sequences, showing the position of strain K1 relative to the related strains

first-order model provided a good fit to the kinetic data with a regression coefficient $R^2 > 0.95$ (Table 2). The dependence of rate constants, k, on solution pH can be computed as follows:

$$k = k_1 [H^+]^c \quad (4)$$

Where c is the reaction order with respect to H^+, and k_1 is a pH-independent rate constant. A plot of log k versus log $[H^+]$ displays a trend with a slope equal to reaction order, c, for H^+ and an intercept of log k_1 (Fig. 3a). As seen in Fig. 3b, the reaction order, c, is 0.18 and the pH-dependent rate constant, log k_1, is

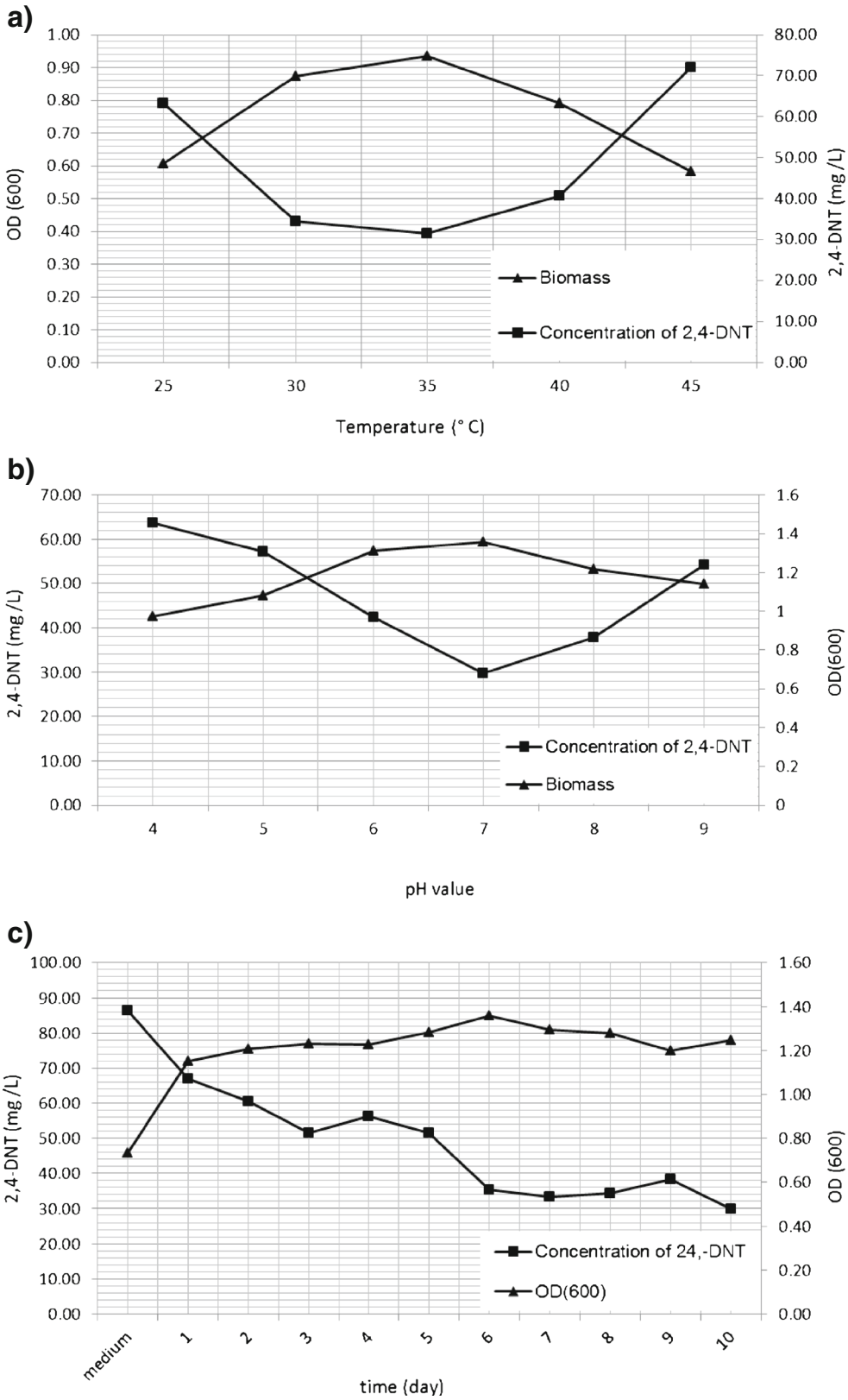

Fig. 2 The effects of pH and temperature on biomass growth and DNT degradation by *Arthrobacter* sp. K1 plotted in terms of **a** temperature versus OD(600) or 2,4-DNT concentration(mg/L); **b** pH versus biomass growth or 2,4-DNT concentration(mg/L), and **c** time versus cell growth or 2,4-DNT concentration (mg/L) at a fixed pH 7 and a temperature of 30 °C

−3.28. The non-integer value obtained for the reaction order (n) with respect to H^+ implies parallel reactions involved in different numbers of electron transfers (Wittbrodt and Palmer 1995).

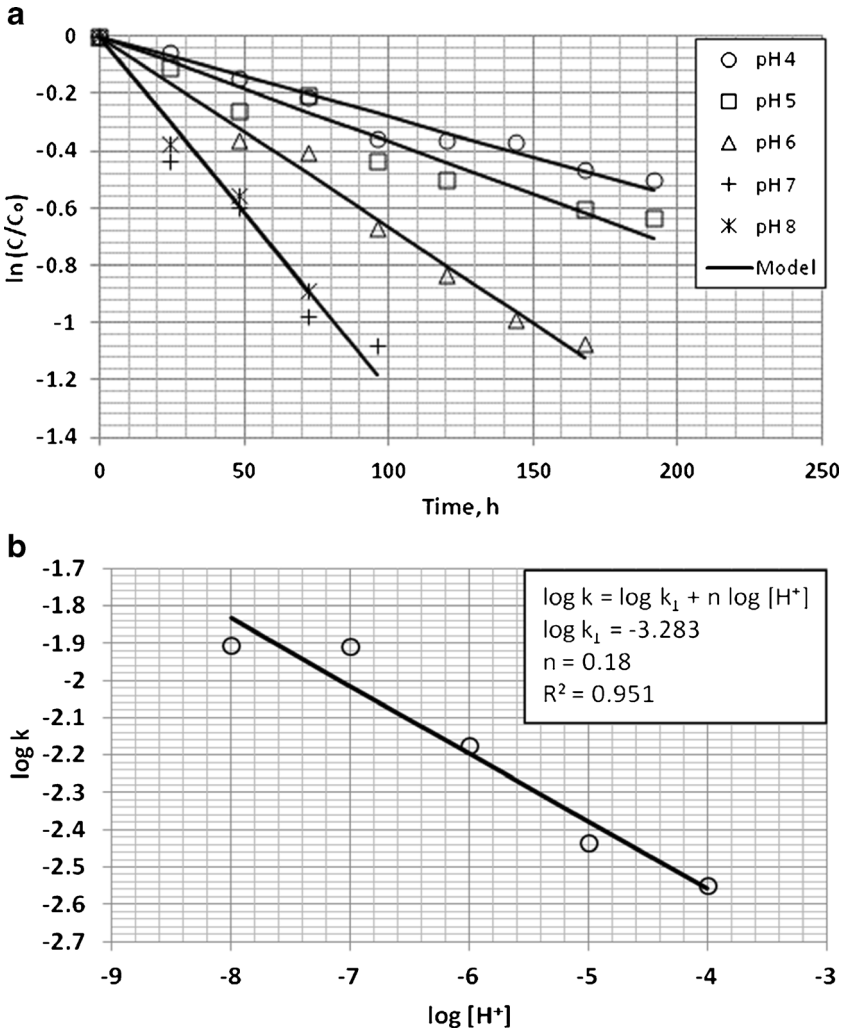

Fig. 3 The effects of solution pH on: **a** 2,4-DNT degradation by *Arthrobacter* sp. K1 at T=30 °C and an initial 2,4 DNT concentration of 100 mg/L), **b** reaction rate constants (logk). The *solid lines* show model fit to Eq. (3) in (**a**) and to Eq. (4) in (**b**)

Agarose gel electrophoresis

The results from the agarose gel electrophoresis analysis of *Arthrobacter* sp. K1 indicate a single plasmid (Lane 2) with a total size of ~8.1 kb which is named pArK1 (Fig. 4). In contrast, no band was observed after curing experiment (Lane 3).

Table 2 Biodegradation first-order reaction rate constants (k) and model statistics

2,4- DNT		Temperature		
pH	Conc. (gL)	(°C)	k (h^{-1})	R^2
4	0.1	30	−0.0027	0.99
5	0.1	30	−0.0037	0.95
6	0.1	30	−0.0067	0.99
7	0.1	30	−0.0123	0.95
8	0.1	30	−0.0124	0.98

Fluorescence in situ hybridization (FISH)

Figure 5 shows fluorescence images of *Arthrobacter* sp. K1. FITC (green)-stained cells indicating encoding of the *DntAa* gene. The hybridization signals were seen as bright green dots in these bacterial cells (Fig. 5a). The hybridization of the target genes resulted in very strong signals when the hybridization conditions were 30 % formamide in the hybridization buffer and at 46 °C hybridization temperature. Hybridization time was optimized as 3 h. The cells stained with DAPI were used to obtain the total cell count of the bacteria. Thus, DAPI-stained bacterial cells in the same microscopic areas were illustrated as controls in Fig. 5b. In addition, positive and negative hybridization controls have been performed with 5′ Cy3-EUB338 and NON338 probes (data not shown in Fig. 5).

Discussion

Crude oil-contaminated soil samples from a petroleum refinery in Mersin, Turkey, were used as source for 2,4 DNT

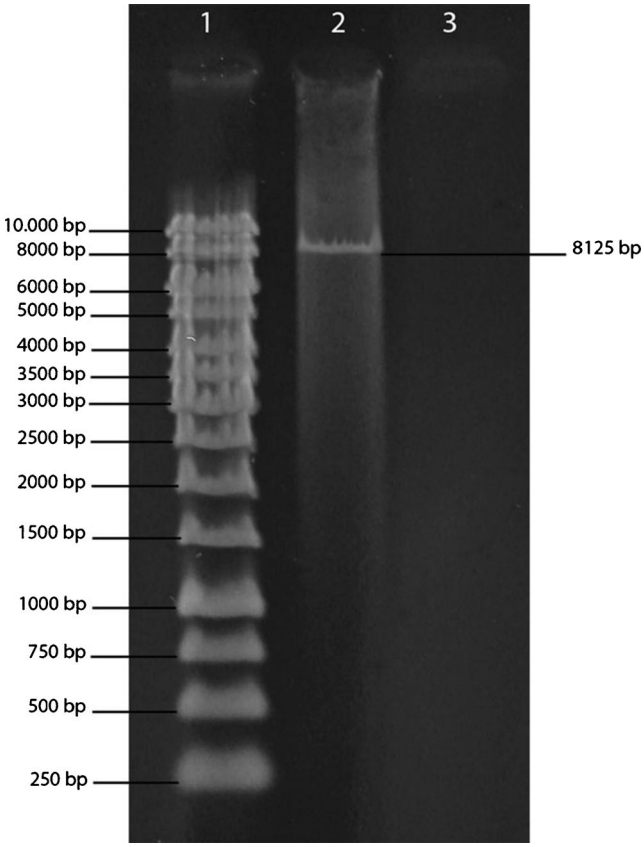

Fig. 4 Separation profile of plasmids from *Arthrobacter* sp. K1 by agarose gel electrophoresis. (*Lanes 1* DNA size marker, *2* plasmid DNA, *3* plasmid DNA after curing application)

degraders. FISH with *dntAa* probes encoding 2,4-DNT dioxgenase were used to detect 2,4-DNT-degrading bacteria. The direct FISH analysis of soil samples collected from the petroleum refinery showed very weak signals, indicating a low number of cells in the soil samples, most probably due to extensive crude oil soil contamination, which has an adverse effect on the size of the bacterial population. Thus, a selective enrichment culture technique using 2,4-DNT as the sole carbon source was used to isolate DNT-degrading bacterial cells. Following the culture enrichment procedure, the hybridization signals significantly improved. It has been reported that the metabolic pathway of 2,4-DNT in different species of bacteria was obtained with Southern hybridization using *dntAa* and *dntD* gene probes (Snellinx et al. 2003). This indicates that similar genes are involved in similar metabolic functions in different bacterial strains.

A phylogenetic tree was constructed based on the 16S rDNA sequence of the strains isolated. The results showed very high similarities (>99.1 %) to *Arthrobacter mysorens* LMG 16219T (GenBank accession no. AJ639831) and *A. arilaitensis* Re117T (GenBank accession no. FQ311875) (Nand and Rao 1972; Irlinger et al. 2005). However, further genetic and chemotaxonomic analysis was required for exact identification as stated above. Thus, this strain isolated was named as *Arthtobacter* sp. K1 in the study. Members of the genus *Arthrobacter* appear well adapted to soil habitat, as they are resistant to both starvation and desiccation, and are capable of utilizing a wide range of carbon sources. The nutritional versatility of this genus is also reflected in the fact that they have been found to degrade several pollutants (Westerberg et al. 2000).

The biodegradation of organic contaminants depends greatly on environmental factors including type of nutrients, soil pH, and temperature. Gupta and Bhaskaran (2004), for instance, show that nutrient addition to the medium stimulated the degradation of 2,4-DNT degradation by strains in poultry litter. In the present study, yeast extract was chosen as the additional nutrient element in medium for bacterial growth stimulation and effective degradation of 2,4-DNT by *Arthtobacter* sp. K1. Similarly, the optimum temperature for the highest growth and DNT degradation was in the range of 30–37 °C.

Our results indicate that the optimal pH was 7–8 for the biodegradation of 2,4-DNT (Fig. 2b). This observation is consistent with previous reports indicating that the optimum pH value lies in the range of 6.0–8.0 for strains belonging to the genus *Arthrobacter* for effective activity. Note that maximum DNT degradation obtained at pH ~7–8 can be explained through the maximum cell growth (Fig. 2b). This pH dependence of cell growth and organic substrate degradation was also observed in others (Johnson et al. 2002; Park et al. 2003;

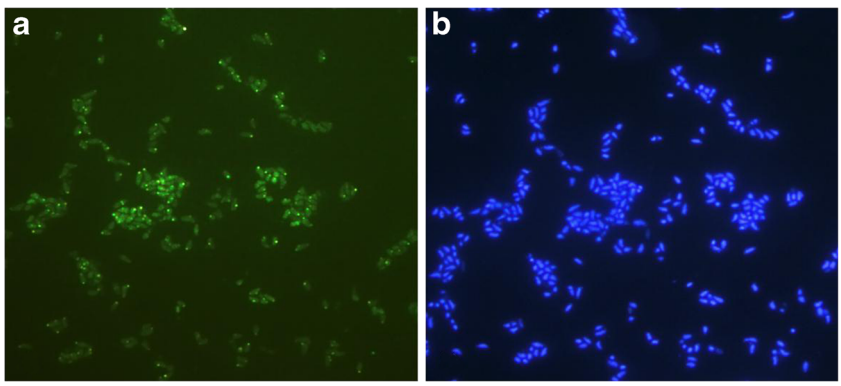

Fig. 5 FISH: **a** images of *Arthrobacter* sp. K1 and **b** control images of the same microscopic area. FITC-stained cells indicate encoding of the *DntAa* gene. The hybridization signals were seen as bright green dots in these bacterial cells

Shen et al. 2009; Dogan et al. 2011). For example, Singh et al. (2012) found that that the maximum p-nitrophenol biodegradation by *Arthrobacter chlorophenolicus* A6 occured at incubation temperature 30 °C and pH 7. Qui et al. (2009) observed a maximum p-nitrophenol removal by *Arthrobacter* sp. HY2 at 30 °C and under slightly alkaline pH conditions. As pointed out by Nishino et al. (2000), the bacterial pathway for degradation of 2,4-DNT involves multiple complex processes, first initiated by dioxygenation of 2,4-DNT, which results in the formation of 4-methyl-5-nitrocatechol (MNC) and the release of nitrite; monooxygenation of MNC then yields 2-hydroxy-5-methylquinone, which is subsequently reduced to 2,4,5-trihydroxytoluene prior to ring cleavage. The aromatic ring cleavage product hydrolases are serine hydrolases, which require the deprotonation (i.e., pH-dependence) of a serine to generate a nucleophilic residue in the active sites (Ollis et al. 1992; Nishino et al. 2000). As the pH increases, the active-site serine becomes deprotonated, which, in turn, increases the catalytic efficiency of the hydrolase.

Figure 3a shows that the 2,4-DNT degradation kinetics with *Arthrobacter* sp. K1 followed a first-order kinetic model with reaction kinetic constants given in Table 2. Note the reaction rates significantly increased with increasing solution pH, and reach a maximum value at pH~7. The degradation reaction rate constant at pH 7 and a temperature of 30 °C was found to be 0.0123 h^{-1}. This value coincides well with the maximum growth (OD_{600}=1.2) at an incubation time of 10 days (Fig. 2c.). However, at pH>7, the increase in reaction rates is not statistically significant. The reaction rate constants determined for DNT agree well with the rate constants obtained for NACs in the literature. For instance, the reaction rate constant of TNT degradation was highest at 30 °C (0.068 h^{-1}) (Park et al. 2003). The reaction rate constants for the TNT degradation increased from 0.073 to 0.079 and absorbance value increased from 1.32 to 1.96 when a carbon source was added to the media (Park et al. 2003). Gupta and Bhaskaran (2004) showed that the reaction rates of degradation of 2,4-DNT and 2,6-DNT by *Pseudomonas* sp. and *P. putida* were 0.15 and 0.05 h^{-1}, respectively, at an incubation time of 30 h.

Curing experiments demonstrate that cured strains were not able to grow on the nutrient agar plate containing 2,4-DNT. This indicates that the ability of *Arthrobacter* sp. K1 for degrading 2,4-DNT is plasmid-encoded. Electrophoretic separation profiles of plasmidic DNA isolated from non-cured cultures showed that *Arthrobacter* sp. K1 contained an approximately 8.1-kb-sized plasmid. But the size of this plasmid was much smaller than those reported previously. For instance, DNT dioxygenase gene for DNT degradation by *Pseudomonas* sp. strain is localized on a large (180 kb) plasmid (Suen and Spain 1993). Similarly, the degradation ability of *Arthrobacter* genus for wide variety of xenobiotic substances is also plasmid-originated (Hayatsu et al. 1999; Turnbull et al. 2001). Jerke et al. (2008) compared a total of 8 plasmids from 5 different strains of *Arthrobacter*, and found that the base lengths ranged from 49.633 to 408.000 kb. *Arthrobacter* strains using carbaryl as a carbon source contained three plasmids with sizes of 110, 120, and 130 kb. Degradation of carbaryl was achieved using two of the plasmids, but they did not achieve a complete degradation when they were used singly (Hayatsu et al. 1999).

FISH using rRNA-targeted oligonucleotide probes is a standard method for identification of microorganisms in environmental samples. In recent years, a modified FISH method, based on polynucleotide probes, has been used for the detection of chromosomal or plasmid-encoded genes in situ (Zwirglmaier et al. 2004; Pratscher et al. 2009). In the present study, FISH with *dntAa* polynucleotide probes encoding 2,4-DNT dioxygenase was used to detect 2,4-DNT-degrading bacteria. The direct FISH analysis of soil samples collected from a petroleum refinery showed very weak signals. Therefore, a selective enrichment culture technique using 2,4-DNT as the sole carbon source was then used to isolate DNT-degrading bacteria. Following the culture enrichment procedure, the intensity of hybridization signals significantly improved in the isolated bacterial strains. Zwirglmaier et al. (2004) indicated that signals are detected in bacterial cells even if the cells have low copy numbers of plasmids compared to plasmid-free bacterial cells. They also suggested that plasmid-targeted probes signal intensity was lower than rRNA-targeted polynucleotide probes. On the other hand, the decrease in the efficiency of the signal can be explained through the plasmids loss of the bacterial cells during lysozyme treatment which was used to increase the cell permeability. While the results from FISH show plasmid genes encoded this plasmid, curing results proved the metabolic instability of plasmid DNA in cytosol. However, our data also confirmed that increasing the cell permeability resulted in the loss of plasmid in the cells at the same time (Lechardeur et al. 1999).

This study suggests that the plasmid pArK1 could be involved in 2,4-DNT biodegradation, and might also play a significant role in the biodegradation other xenobiotics. The isolation of such bacteria harboring catabolic plasmids among indigenous bacteria from polluted soils is evidence of microbial adaptation to xenobiotics.

Acknowledgment This work has been supported by Mersin University Research Fund (Project Code: BAP-FBE BB (PK) 2008-8YL.

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