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TNT and nitroaromatic compounds are chemoattractants for *Burkholderia cepacia* R34 and *Burkholderia* sp. strain DNT

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Abstract Nitroaromatic compounds are toxic and potential carcinogens. In this study, a drop assay was used to detect chemotaxis toward nitroaromatic compounds for wild-type *Burkholderia cepacia* R34, wild-type *Burkholderia* sp. strain DNT, and a 2,4-dinitrotoluene (2,4-DNT) dioxygenase mutant strain (S5). The three strains are chemotactic toward 2,4,6-trinitrotoluene (TNT), 2,3-DNT, 2,4-DNT, 2,5-DNT, 2-nitrotoluene (NT), 4NT, and 4-methyl-5-nitrocatechol (4M5NC), but not toward 2,6-DNT. Of these, only 2,4-DNT is a carbon and energy source for *B. cepacia* R34 and *Burkholderia* sp. strain DNT, and 4M5NC is an intermediate in the 2,4-DNT degradation pathway. It was determined that the 2,4-DNT dioxygenase genes are not required for the chemotaxis for these nitroaromatic compounds because the DNT DDO mutant S5 has a chemotactic response toward 2,4-DNT although 2,4-DNT is not metabolized by S5; hence, 2,4-DNT itself is the chemoattractant. This is the first report of chemotaxis toward TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2NT, 4NT, and 4M5NC.

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Introduction

The enzymes and genetic pathways required for bacterial degradation of toxic organic compounds such as polychlorinated biphenyls, toluene, naphthalene, and nitroaromatic compounds have been determined (Grimm and Harwood 1997; Samanta et al. 2000; Parales and Harwood 2002). However, the role of chemotaxis in pollutant biodegradation and bioremediation has not been studied extensively (Samanta et al. 2000; Parales 2004). Bacterial chemotaxis, movement due to a chemical gradient (Pandey et al. 2002), enhances biodegradation by bringing cells close to the pollutant thereby reducing poor bioavailability, sequestration of the chemical on surfaces, and low solubility (Parales and Harwood 2002). For example, chemotaxis by *Pseudomonas putida* G7 was shown to enhance naphthalene degradation compared to nonchemotactic and nonmotile mutants, proving chemotaxis can overcome these mass transfer problems that may limit biodegradation rates (Marx and Aitken 2000; Law and Aitken 2003).

Most nitroaromatic compounds in the environment are man-made and are released from manufacturing pharmaceuticals, dyes, plasticizers, pesticides, and explosives (Spain 1995). Chemotaxis toward nitroaromatic compounds has been investigated previously as it has been reported that *Ralstonia* sp. SJ98 is chemotactic toward nitrocatechol, nitrophenols, nitrobenzoate, dinitrobenzenes, dinitrophenols, and dinitrobenzoates (Bhushan et al. 2000; Samanta et al. 2000; Pandey et al. 2002). *P. putida* PRS2000 was also found to be chemotactic toward nitrobenzoates and aminobenzoates (Parales 2004).

In this study, we focus on chemotaxis toward the nitroaromatic compounds 2,4,6-trinitrotoluene (TNT), 2,3-dinitrotoluene (2,3-DNT), 2,4-DNT, 2,5-DNT, 2,6-DNT, 2-nitrotoluene (NT), 4NT, and 4-methyl-5-nitrocatechol (4M5NC). 2,4-DNT and 2,6-DNT are found in soil and groundwater as contaminants from the manufacture of TNT, the most common explosive (Nishino et al. 2000). 2,4-DNT and 2,6-DNT are also intermediates in the production of

polyurethane foams (Nishino et al. 1999) and are listed as priority pollutants by the U.S. Environmental Protection Agency (Nishino et al. 1999). Various nitroaromatic compounds have been shown to be toxic and carcinogenic. For example, 2,3-DNT and 2,5-DNT are suspected human carcinogens (Anonymous 1994). Dinitrotoluenes also cause systemic intoxication (Gosselin et al. 1976).

Burkholderia sp. strain DNT was isolated from water samples from Waconda Bay near the Volunteer Army Ammunition Plant in Chattanooga, TN (Spanggord et al. 1991), and *Burkholderia cepacia* R34 was isolated from surface waters at the Radford Army Ammunition Plant in West Virginia (Nishino et al. 2000). These strains use 2,4-DNT as a sole carbon and energy source (Nishino et al. 2000; Johnson et al. 2002). Catabolism of 2,4-DNT is initiated both by 2,4-DNT dioxygenase from *Burkholderia* sp. strain DNT (DNT DDO) and *B. cepacia* R34 (R34 DDO) (Suen and Spain 1993; Johnson et al. 2002). The 2,4-DNT oxidation pathway was previously determined (Suen and Spain 1993; Johnson et al. 2002), and the first intermediate is 4M5NC. Both DNT DDO and R34 DDO oxidize 2,6-DNT to 3M4NC (Spanggord et al. 1991; Nishino et al. 2000), DNT DDO oxidizes 4NT to 4-methylcatechol and 4-nitrobenzyl alcohol (Parales et al. 1998; Leungsakul et al. 2005), R34 DDO oxidizes 4NT to 4-nitrobenzyl alcohol (Leungsakul et al. 2005), and alpha subunit variants I204L and I204Y of DNT DDO were constructed that oxidize 2,3-DNT to 4M3NC and 2,5-DNT to 4M5NC (Leungsakul et al. 2005). These results indicate that 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, and 4NT may be degraded by wild-type DNT DDO and its variants, so chemotaxis toward these pollutants would be beneficial for stimulating their biodegradation.

In addition to determining the chemotactic response of *Burkholderia* sp. strain DNT and *B. cepacia* R34 toward nitroaromatic compounds, we wished to determine whether the chemotaxis system of *Burkholderia* sp. strain DNT for these compounds is related to the 2,4-DNT degradation pathway. This is the first report indicating that TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2NT, 4NT, and 4M5NC are chemoattractants. Moreover, it was determined that the 2,4-DNT dioxygenase genes are not required for the chemotaxis of these nitroaromatic compounds. These results are significant because the *Burkholderia* sp. strain DNT and *B. cepacia* R34 appear to be able to detect these nitroaromatic compounds which may enhance their biodegradation.

Materials and methods

Bacterial growth conditions and chemicals

B. cepacia R34, *Burkholderia* sp. strain DNT, and DNT DDO deletion mutant S5 (Suen and Spain 1993) were grown from single colonies at 30°C and 250 rpm in 250-ml Erlenmeyer flasks containing 25 ml of Luria–Bertani (LB) medium (Sambrook et al. 1989), 25 ml of MSB medium (Spain and Nishino 1987) supplemented with 20 mM of sodium succinate and with 0.3 mM of 2,4-DNT (to induce 2,4-DNT degradation of *B. cepacia* R34 (Johnson et al. 2002) and *Burkholderia* sp. strain DNT), or MSB medium supplemented with 20 mM of sodium succinate and 0.03 mM of 4M5NC (to induce the 2,4-DNT degradation pathway of the mutant S5). After overnight growth, the three strains were grown in 100 ml of the same respective medium to an optical density at 600 nm (OD) of 1.0–1.5 in 250-ml Erlenmeyer flasks. These cells are all motile under the growth conditions used.

2,5-DNT was obtained from Accustandards Inc. (New Haven, CT), TNT was obtained from Chem Service (West Chester, PA), *N*,*N*-dimethylformamide (DMF) was obtained from Fisher Scientific (Fairlawn, NJ), and 4M5NC was provided by Dr. Glenn R. Johnson of Tyndall Air Force Base. 2,3-DNT, 2,4-DNT, 2,6-DNT, 2NT, and 4NT were obtained from Aldrich Chemical (Milwaukee, WI).

Chemotaxis assay

The drop assay used in this study was modified slightly from previous studies (Grimm and Harwood 1997; Samanta et al. 2000) by making an altered drop assay medium. Cells in the exponential phase of growth or heat-killed cells (negative control) were washed and resuspended in drop assay medium (MSB containing 0.2% bactoagar and 10 mM succinate as an energy source) and poured in Petri plates. Crystals of the nitroaromatic compounds (except TNT and 2NT), 10 μl of 50 mM of TNT in DMF, or 10 μl of 2NT were placed in the center of each plate, and 10 μl of a 20% casamino acids solution was used as a positive control (Grimm and Harwood 1997; Parales et al. 2000). Heat-killed cells for negative controls were prepared by autoclaving at 121°C for 30 min (control for nonchemotactic aggregation). No substrate negative controls were also used.

A chemotactic response of cells was observed after 1–3 days. All experiments were done at least two times. To photograph the drop assay plates, the plates were placed on the bottom of an upside down glass tray positioned one inch from a black surface to provide adequate contrast.

Results

The drop assay was used in this study to determine the chemotactic responses toward TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2,6-DNT, 2NT, 4NT, and 4M5NC for *B. cepacia* R34, *Burkholderia* sp. strain DNT, and the DNT dioxygenase mutant of *Burkholderia* sp. strain DNT (S5). Casamino acids were used as a positive control to verify the cells had sufficient motility to move toward the chemicals (Grimm and Harwood 1997; Parales et al. 2000). It was found that all three of these strains had a strong chemotactic response toward casamino acids (Fig. 1j). The negative controls showed that there is no chemotactic response toward the chemotaxis medium, and there is no movement of cells to the chemical when they are dead (no abiotic aggregation) (Fig. 1k). There was no chemotactic response toward DMF, which is the negative control for TNT che-

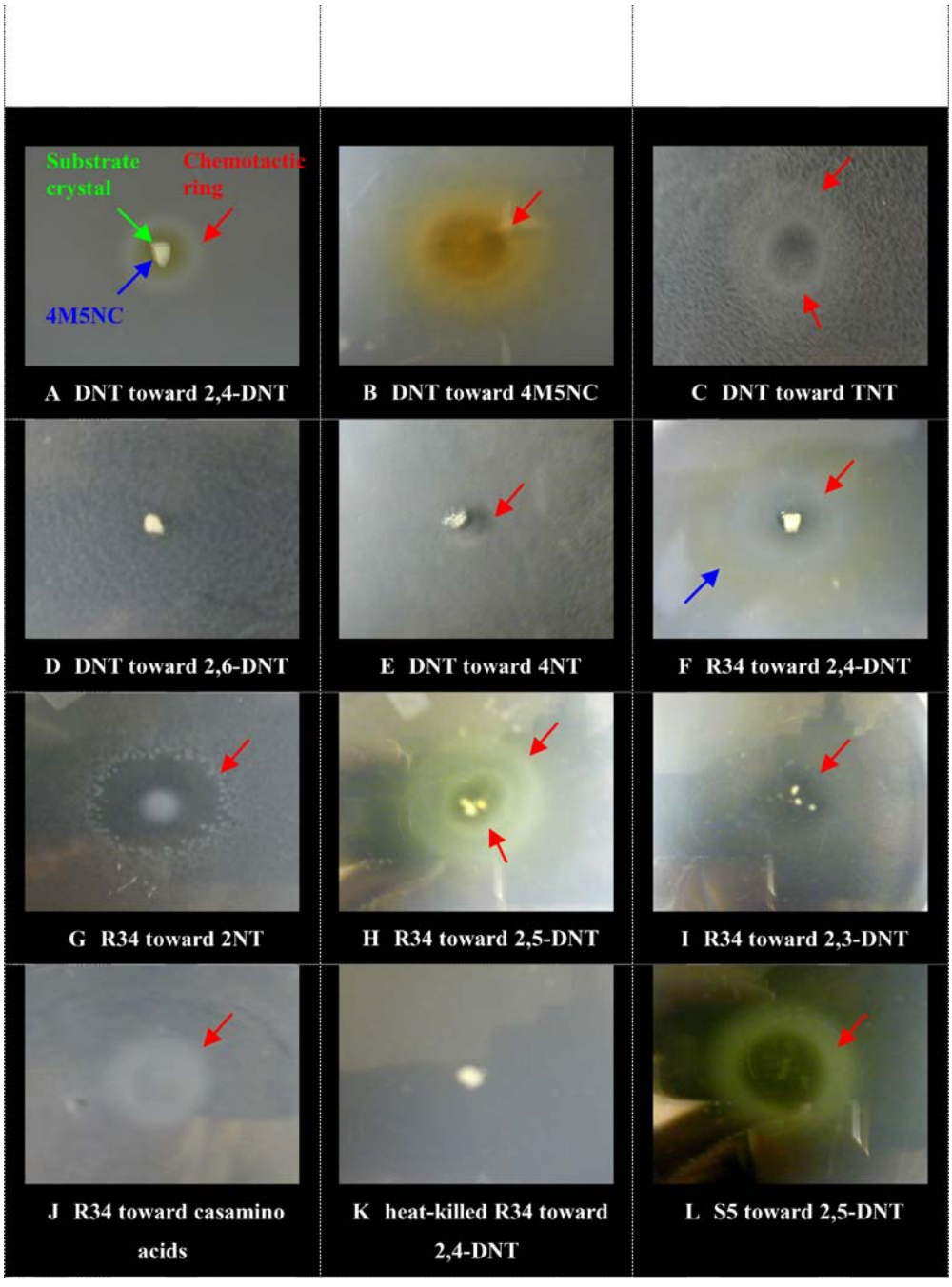

Fig. 1 Representative chemotactic responses toward the nitroaromatic compounds. Chemotaxis of *Burkholderia* sp. strain DNT toward 2,4-DNT (**a**), 4M5NC (**b**), TNT (**c**), 2,6-DNT (**d**), and 4NT (**e**). Chemotaxis of *B. cepacia* R34 toward 2,4-DNT (**f**), 2NT (**g**), 2,5-DNT (**h**), 2,3-DNT (**i**), and casamino acids (**j**). Chemotaxis of heat-killed *B. cepacia* R34 toward 2,4-DNT (**k**). Chemotaxis of mutant S5 toward 2,5-DNT (**l**). *Red arrows* indicate the chemotactic rings. *Green arrow* indicates the substrate crystal. *Blue arrows* indicate 4M5NC (*yellow color*) in panels **a** and **f**

motaxis (TNT was applied in DMF). The strength of the bacterial chemotactic response was quantified by measuring the diameter of the chemotactic ring (1–20 mm) in order to judge its significance for degradation (Table 1). Chemotactic rings with diameters greater than 5 mm indicate a stronger response (++); rings with diameters less than 5 mm indicate a weaker response (+). A stronger response indicates that more cells accumulated near the attractants, and that they formed a much denser ring.

Chemotaxis of *B. cepacia* R34 and *Burkholderia* sp. strain DNT toward nitroaromatic compounds

Chemotaxis of *B. cepacia* R34 and *Burkholderia* sp. strain DNT was observed in the form of a ring of cells that accumulated around, but did not touch, TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2NT, 4NT, and 4M5NC (Fig. 1a–c, e–i). Note that the chemotactic ring for 4NT was very weak. For casamino acids, a ring of cells formed and touched the

Table 1 Chemotaxis of *Burkholderia* sp. strain DNT, *B. cepacia* R34, and the dioxygenase deletion mutant S5 toward nitroaromatic compounds

Strains	Compound									
	TNT	2,3-DNT	2,4-DNT	2,5-DNT	2,6-DNT	2NT	4NT	4M5NC	Casamino acids	None
Burkholderia sp. strain DNT	+	+	+	++	−	+	+	++	++	−
Burkholderia sp. DNT mutant S5	+	+	+	++	−	+	+	+	++	−
B. cepacia R34	+	+	+	++	−	+	+	+	++	−

The width of the chemotactic ring (diameter in mm) is indicated by: ++ strong response (5–20 mm), + weak response (1–5 mm), and − no response. No chemotaxis-like aggregation was found for the heat-killed strains DNT, R34, and mutant S5 with these substrates

chemical (Fig. 1j). These results may indicate that TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2NT, 4NT, and 4M5NC are too toxic to cells at high concentrations; therefore, cells did not touch these chemicals (diffusion of the chemicals created a concentration gradient in the cell suspension). Interestingly, there were reproducible double chemotactic rings (two circular regions with concentrated cells) for both *Burkholderia* sp. strain DNT and *B. cepacia* R34 toward both TNT (Fig. 1c for *Burkholderia* sp. strain DNT) and 2,5-DNT (Fig. 1h for *B. cepacia* R34).

There was no chemotactic response toward 2,6-DNT for both strains (Fig 1d). In addition, cells grown in LB did not show any difference in their chemotaxis toward TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2NT, 4NT, and 4M5NC compared to cells grown in MSB containing 2,4-DNT (data not shown).

Chemotaxis of *Burkholderia* sp. DNT DDO mutant S5 toward nitroaromatic compounds

The chemotactic response of the S5 mutant was the same as *Burkholderia* sp. strain DNT and *B. cepacia* R34 for all the attractants, indicating that dioxygenase genes are not required for chemotaxis (Fig. 1l). Note that S5 did not produce nitrite from 2,4-DNT, as expected (data not shown). S5 grown in LB did not show any difference in chemotaxis compared to cells grown in MSB containing 4M5NC (4M5NC is metabolized by mutant S5) (data not shown).

Discussion

These results clearly show that TNT, 2,3-DNT, 2,4-DNT, 2,5-DNT, 2NT, 4NT, and 4M5NC are chemoattractants for *B. cepacia* R34, *Burkholderia* sp. strain DNT, and the DNT DDO-deficient mutant S5. The 2,4-DNT dioxygenase genes are not required for chemotaxis toward these nitroaromatic compounds.

B. cepacia R34 and *Burkholderia* sp. strain DNT express 2,4-DNT dioxygenases that are very similar to the naphthalene dioxygenase of *Pseudomonas* sp. strain NCIB 9816-4 and *Ralstonia* sp. strain U2 (Johnson et al. 2002). Previously, it was found that *nahY* of *P. putida* G7 is a naphthalene chemoreceptor, that it is cotranscribed with the degradation genes in the NAH7 catabolic plasmid, and that it is required for naphthalene chemotaxis (Grimm and Harwood 1999); however, *nahY* does not share significant similarity with the putative receptor encoded by ORF11 in the 2,4-DNT degradation pathway of *B. cepacia* R34 (Johnson et al. 2002). Additionally, it is not clear that naphthalene or a naphthalene degradation product is the chemoattractant because there is no naphthalene chemotaxis in a *P. putida* G7 strain that lacks the naphthalene degradation genes (Grimm and Harwood 1999). In contrast, our results show that 2,4-DNT is the chemoattractant for *B. cepacia* R34 and *Burkholderia* sp. strain DNT because the DNT DDO mutant S5 has a chemotactic response toward 2,4-DNT although 2,4-DNT is not metabolized by S5. Similarly, *P. putida* F1 that lack toluene dioxygenase activity are attracted to toluene, which indicates that toluene is the chemoattractant for *P. putida* F1 (not a metabolite) (Parales et al. 2000).

Although 2,4-DNT is the sole carbon and nitrogen source of *B. cepacia* R34 and *Burkholderia* sp. strain DNT, chemotaxis of these strains toward 2,4-DNT is not as strong as that toward 2,5-DNT, which is not a carbon and nitrogen source for these strains. Also, TNT, 2,3-DNT, 2,5-DNT, 2NT, and 4NT are chemoattractants but not carbon and energy sources for *B. cepacia* R34 and *Burkholderia* sp. strain DNT, which means that the chemotactic systems of these strains are active for more nitroaromatic compounds than just 2,4-DNT or the intermediate 4M5NC in the 2,4-DNT degradation pathway. These results also corroborate that the 2,4-DNT degradation genes are not necessary for chemotaxis.

Chemotaxis has been categorized into two groups based on whether the signal transduction strategies are metabolism-independent or metabolism-dependent (Pandey and Jain 2002). There are three criteria for metabolism-independent chemotaxis: (1) nonmetabolizable analogs of metabolizable attractants are attractants, (2) chemotaxis is not affected by mutations affecting the metabolism of a chemical attractant, and (3) bacteria are chemotactic even in the presence of more favorable metabolizable compounds (Pandey and Jain 2002). Our results fit these three criteria because TNT, 2,3-DNT, 2,5-DNT, 2NT, and 4NT are chemoattractants but are not metabolized; mutant S5 has a chemotactic response toward 2,4-DNT although S5 lacks 2,4-DNT dioxygenase; and TNT, 2,3-DNT, 2,5-DNT, 2NT, and 4NT were chemoattractants even in the presence of succinate, a carbon and energy source for these strains.

Previously, it was shown that *Ralstonia* sp. SJ98 degrades *p*-nitrophenol, 4-nitrocatechol, *o*-nitrobenzoic acid,

and *p*-nitrobenzoic acid, and these compounds are also chemoattractants (Samanta et al. 2000). *P. stutzeri* OX1 degrades chlorinated ethenes, which are also chemoattractants for this strain (Vardar et al. 2005). Similarly, in this work, we have demonstrated the chemotaxis of *Burkholderia* sp. strain DNT toward 4NT, which is degraded by this strain although it is not metabolized.

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