Biodegradation **14:** 19–29, 2003.

Microbial consortia that degrade 2,4-DNT by interspecies metabolism: isolation and characterisation

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Accepted: 17 January 2003

Key words: 2,4-DNT, nitroaromatic compounds, consortium, ecology

Abstract

Two consortia, isolated by selective enrichment from a soil sample of a nitroaromatic-contaminated site, degraded 2,4-DNT as their sole nitrogen source without accumulating one or more detectable intermediates. Though originating from the same sample, the optimised consortia had no common members, indicating that selective enrichment resulted in different end points. Consortium 1 and consortium 2 contained four and six bacterial species respectively, but both had two members that were able to collectively degrade 2,4-DNT. *Variovorax paradoxus* VM685 (consortium 1) and *Pseudomonas* sp. VM908 (consortium 2) initiate the catabolism of 2,4-DNT by an oxidation step, thereby releasing nitrite and forming 4-methyl-5-nitrocatechol (4M5NC). Both strains contained a gene similar to the *dntAa* gene encoding 2,4-DNT dioxygenase. They subsequently metabolised 4M5NC to 2-hydroxy-5-methylquinone (2H5MQ) and nitrite, indicative of DntB or 4M5NC monooxygenase activity. A second consortium member, *Pseudomonas marginalis* VM683 (consortium 1) and *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 or *P. viridiflava* VM907 (consortium 2), was found to be indispensable for efficient growth of the consortia on 2,4-DNT and for efficient metabolisation of the intermediates 4M5NC and 2H5MQ. Knowledge about the interactions in this step of the degradation pathway is rather limited. In addition, both consortia can use 2,4-DNT as sole nitrogen and carbon source. A gene similar to the *dntD* gene of *Burkholderia* sp. strain DNT that catalyses ring fission was demonstrated by DNA hybridisation in the second member strains. To our knowledge, this is the first time that consortia are shown to be necessary for 2,4-DNT degradation.

Introduction

The compound 2,4-dinitrotoluene (2,4-DNT), a precursor of toluene diisocyanate is an important industrial intermediate in the production of polyurethane foams. Furthermore, dinitrotoluenes, but in particular 2,4-DNT, are precursors of the explosive 2,4,6-trinitrotoluene (TNT). In the past, waste streams from these industrial processes were released in the environment. Because of the toxicity and carcinogenic properties of 2,4-DNT, the U.S. EPA lists it as a priority pollutant (Keith & Telliard 1979). Therefore, its removal is of significant environmental concern.

Burkholderia sp. strain DNT was the first 2,4-DNT mineralising strain isolated, and its aerobic mineralisation of this compound has been extensively studied biochemically and genetically (Spanggord et al. 1991). Up to now, all other isolates that grow on 2,4-DNT use the same degradation pathway as strain DNT (Nishino et al. 2000). The pathway starts with the dioxygenation of 2,4-DNT resulting in the formation of 4-methyl-5-nitrocatechol (4M5NC) and the release of nitrite (Figure 1). Then, monooxygenation of 4M5NC yields the second nitrite group

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and 2-hydroxy-5-methylquinone (2H5MC) that subsequently is reduced to 2,4,5-trihydroxytoluene (2,4,5-THT) prior to ring cleavage (Suen & Spain 1993). The *dntA*, *-B*, and *-D* genes, encoding DNT dioxygenase, 4M5NC monooxygenase, and 2,4,5-THT oxygenase respectively, were cloned and characterised (Suen et al. 1996; Haigler et al. 1996, 1999).

The objective of our research was to isolate, characterise, and identify bacterial strains under aerobic conditions able to degrade nitroaromatic compounds including 2,4-DNT. Soil and water samples from a former ammunition production site were used as source for 2,4-DNT degraders in selective enrichment studies. In contrast with other studies, which isolated single strains that grow on 2,4-DNT as the sole source of carbon and/or nitrogen, we isolated microbial consortia that degrade 2,4-DNT by interspecies metabolism.

Materials and methods

Chemicals

2,4-Dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 2-nitrotoluene (2-NT), 4-nitrotoluene (4-NT), 2-amino-4-nitrotoluene (2-A-4-NT), 2-amino-6-nitrotoluene (2-A-6-NT), 4-amino-2-nitrotoluene (4-A-2-NT) were purchased from Sigma-Aldrich (Germany), 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) and 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT) from Supelco (PA). The purity of the chemicals was 95% to 99%. 4-Methyl-5-nitrocatechol (4M5NC) and 2,4,5-trihydroxytoluene (2,4,5-THT) were kindly provided by S. F. Nishino, AFRL/MLQR, Tyndall AFB, FL. The 2,4,5-THT sample contained 2-hydroxy-5-methylquinone (2H5MQ) due to spontaneous conversion of the hydroxytoluene to the quinone. 2,4,6-Trinitrotoluene (TNT) was kindly provided by J.C. Libouton, Nobel Explosifs, Centre de Recherches, Châtelet, Belgium.

Culture media

The composition of the minimal medium used to isolate nitroaromatic-degrading microorganisms was described previously (Ramos et al. 1996). However, the phosphate/NaCl stock solution (10× concentrated, pH 6.8) was slightly modified: $Na_2HPO_4{\cdot}2H_2O$, 40 g/L; KH_2PO_4, 30 g/L; NaCl, 5 g/L. When 2,4-DNT was added as the sole source of nitrogen, different carbon sources were added simultaneously, i.e., fructose, gluconate, glucose, lactate, and succinate (3 mM of each). A selective minimal medium contained 275 μM (50 mg/L) 2,4-DNT either as a source of nitrogen and/or carbon. Rich medium 869 (pH 7.0) was prepared as described previously (Mergeay et al. 1985). Solid media were prepared with 15 g Difco agar per litre.

Enrichment of 2,4-DNT degrading cultures

Soil and water samples from an ammunition brownfield were inoculated separately into selective medium with 2,4-DNT as nitrogen source. A first enrichment experiment occurred with freshly collected samples, a second enrichment experiment with a soil sample that had been stored at −80 °C in a solution of glycerol (15%) and NaCl (0.85%) at a ratio of 1.5 ml solution per g soil. After centrifuging and removing the supernatant, the sample was used as inoculum for enrichment. Table 1 shows the nitroaromatic contents of the samples used as inoculum. One gram soil samples or 10 ml water samples were used as inoculum into 100 ml selective minimal medium in which 2,4-DNT was present as the sole source of nitrogen. Samples were incubated at 28 °C while shaken at 150 rpm. Cultures were monitored for growth, and transferred to fresh medium at appropriate intervals ($OD_{660} \geq 0.25$). Initially, the dilution-factor was 10, but was gradually increased to 50 within 8 transfers.

Isolation and identification of bacteria

Individual strains were isolated by morphology from the adapted consortia by plating on solid 1/10 869 medium. After purifying the isolates, REP-PCR was performed as described by de Bruyn (1992) to identify similar strains, using the Goldstar Red DNA Polymerase kit (Eurogentec, Belgium). Strains were phenotypically characterised by basic bacteriological tests (Gram-straining, FAME-analysis, Biolog GN and API20NE) and by 16S rRNA gene sequence analysis on genomic DNA. 16S rRNA gene sequences were determined using the Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit (Amersham Pharmacia, UK). As primers 685R or 279F were used. The gene sequences obtained were compared with published sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/blast).

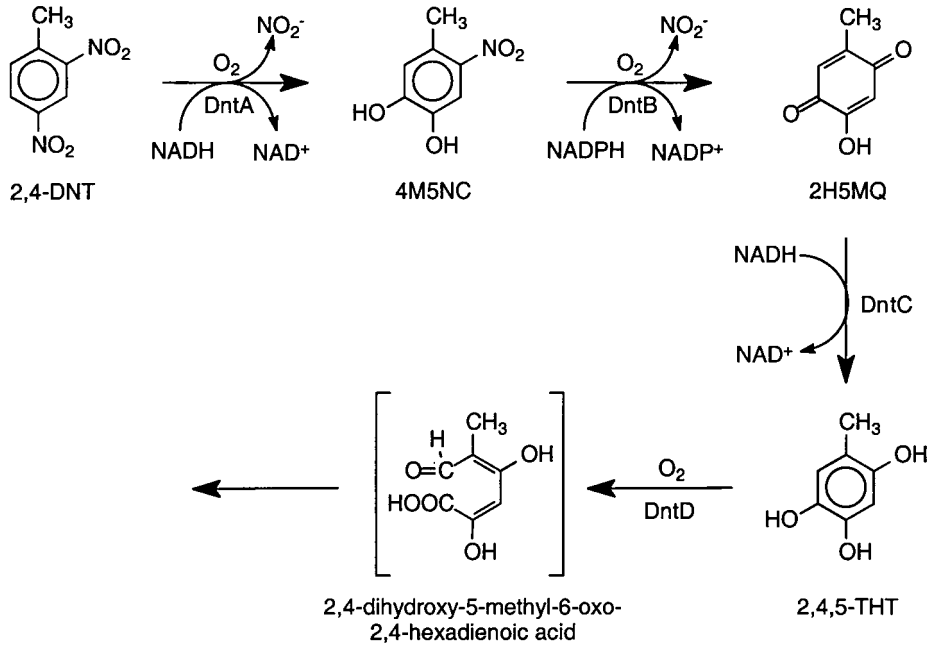

Figure 1. Proposed pathway for the degradation of 2,4-DNT by *Burkholderia* sp. Strain DNT. DntA, 2,4-DNT dioxygenase; DntB, 4M5NC monooxygenase; DntC, 2H5MQ reductase; DntD, THT oxygenase; NO_2^-, nitrite (based on Suen & Spain 1993).

Reconstitution and combinations of consortium constituents

A consortium was reconstituted by adding 0.5 ml of pure cultures of the consortium constituents into 100 ml selective medium. The pure cultures were obtained after overnight growth on 869 liquid medium, washed twice, and brought to an optical density of 1.0 in $MgSO_4$ (10 mM). A reconstituted consortium was transferred twice (1/50 dilution) to fresh selective medium before use. Furthermore, for both consortia, all possible permutations of two and more consortium members were made.

Enzyme activity

Microplate assays for detecting DNT dioxygenase, and 4M5NC monooxygenase, based on concomitant colour changes in the medium, were performed as described by Suen and Spain (1993). Enzyme activity was evaluated after 24 hours with the naked eye and judged as positive or negative. 2,4,5-THT oxygenase activity was detected by suspending cells in microplate wells containing 100 μl phosphate buffer (pH 6.8) with 100 μM 4-methylcatechol, a structural analogue for 2,4,5-THT (Suen & Spain 1993). The conversion of the medium from colourless to yellow was indicative for 2,4,5-THT oxygenase activity. This alternative was chosen, as simply dissolving 2,4,5-THT from the received sample imparted a pink coloration to the buffer, indicating oxidation from 2,4,5-THT to 2H5MQ. *Burkholderia* sp. strain DNT was used as a positive control in the enzyme assay.

Analytical methods

Nitrite release was measured as described by Daniels et al. (1994). For High Performance Liquid Chromatography (HPLC), soil samples and water samples were extracted and prepared for analyses as described by EPA method 8330A (1998). To follow the breakdown of nitroaromatic compounds in liquid cultures, 500 μL liquid culture samples were supplemented with 500 μL methanol, mixed thoroughly, and filtered through a 0.45 μm PTFE filter. HPLC analyses were performed on an Alltima C_{18} reverse-phase column, 250 mm $\times$ 4.6 mm, 5 μm particle size (Alltech, IL). The mobile phase was 55:45 methanol-water and detection occurred at 254 nm with an UV-VIS detector (Model L-4250, Hitachi, Japan). The flow rate was 1 ml/min and the injections loop volumes 20 μL. Products were identified by comparison with authentic standards. Under these conditions, retention times of 2,4-DNT, 4-A-2NT, 2-A-4NT and 4M5NC were 16.99, 9.42, 8.44 and 6.53 minutes respectively. Retention times of 2-A-

Table 1. Nitroaromatic concentrations in the soil and water samples used as inoculum for selective enrichment under aerobic conditions

Sample*	TNT	4-A-2,6-DNT	2-A-4,6-DNT	2,4-DNT	2,6-DNT
S1 (1)	3046.3	11.2	28.7	51.6	43.4
W1 (1)	9.8	0.5	1.9	ND	ND
W2 (2)	10.0	0.6	2.2	ND	ND
S17-2 (3)	173.7	4.6	7.9	ND	ND
S72 (4)	ND	ND	ND	ND	ND
S163 (5)	27130.2	131.8	286.5	ND	ND

S, soil sample, concentration in mg/kg dry matter; W, water sample, concentration in mg/L; ND, not detected (<0.01 mg/L). The water samples were taken in former non-covered pits where the groundwater welled up.
*Sampling place: codes 1, 2, 3 were taken at the front site of the TNT manufacturing plant; code 1, distance < 5 m; code 2, distance 15 m; code 3, distance 22 m. Code 4, a remote spot at the production site; code 5, at a TNT storage place.

4,6-DNT, 4-A-2,6-DNT and TNT were 15.66, 14.55 and 12.98 minutes respectively.

To prepare the samples for Gas Chromatography–Mass Spectrometry (GC–MS), 20 ml aliquots of the liquid culture were extracted three times with 10 ml of diethylether. The extracts were pooled and dried by adding 3 g Na_2SO_4. After decanting, the organic phase was evaporated completely. Hydroxylated compounds were derivatised overnight at 80 °C with 100 μl BSTFA (containing 1% trimethylchlorosilane (TMCS)). Injection and identification occurred onto a GC-MS Voyager system (Interscience, Belgium) with a Zebron ZB-5 capillary column, 25 m × 0.25 mm × 0.25 μm Df (Bester, Netherlands). Products were identified by comparing the retention time and masses with standards. Important mass spectral data for 2H5MQ were 210 (2), 195 (100), 167 (72) and 151 (17). Mass spectral data to identify 2,4,5-THT were 356 (34), 268 (8), 253 (12) and 179 (6). Standards of the tetranitroazoxytoluene isomers were not available. Tentative identification was based on molecular weight and mass spectra obtained by Direct Insert Probe Mass Spectrometry (DIP-MS) and Electrospray Ionization Tandem Mass Spectrometry Collision-Induced Dissociation (ESI-MS/MS-CID). DIP-MS was carried out on a Finnigan TSQ-70, positive chemical ionization mode, mass range 90–400 and a scan rate of 2s. Reagens gas was isobutane at a pressure of 3000–4000 m Torr. The mass spectrum showed ions at *m/z* 407 ([MH^+], 100) and *m/z* 377 ([MH^+–NO], 55). ESI-MS/MS-CID was performed as described by Yinon et al. (1997), on a ThermoFinnigan LCQ. LC analyses showed four distinct peaks. All four peaks accumulated a negatively charged ion at *m/z* 405 ([M^-], 100) in the ESI-MS spectrum. This indicates the presence of four related metabolites. However, the CID-spectra of the negatively charged ions gave little additional information. To prepare the samples for both DIP-MS and ESI-MS/MS-CID, cell pellet of 25 ml bacterial culture was extracted with 5 ml acetonitrile.

DNA hybridisation

The genomic DNA of the test strains was digested using *Eco*RI as restriction enzyme (Life Technologies, CA). After Southern blotting, DNA hybridisation was applied using the probes *dntAa* and *dntD* from the 2,4-DNT degrading genes. The gene probes were obtained by PCR on the genomic DNA of *Burkholderia* sp. strain DNT using the primerset 646F (5′-AAC TGG TAG TAG AAC CCC TC-3′) and 1687R (5′-GGG GTT CAC TCA TGG CTT GG-3′) for probe *dntAa*, and 81F (5′-CCA TGT CCG TTG CAG ACA TT-3′) and 1040R (5′-GCT ACT GGG TTC AGT TGT GC-3′) for probe *dntD*. Probes were labelled using the Gene Images random prime labelling module RPN 3540 (Amersham Life Science, UK). Detection was performed using the *Gene Images* CDP-*Star* detection module RPN 3510 (Amersham Life Science, UK). The membrane was washed twice with a mixture of 2× SSC and 0.1% SDS, at room temperature for 15 minutes, and twice with a mixture of 0.5× SSC and 0.1% SDS, at 60 °C for 15 minutes. *Burkholderia* sp. strain DNT was used as a positive control in the hybridisation.

Polymerase Chain Reaction (PCR) amplification

A Platinum *Taq* DNA Polymerase kit (Life Technologies, CA) was used to perform PCR. The PCR thermal profile, conducted in a reaction volume of 100 μl (5 μl PCR Buffer minus Mg (10×), 1 μl dNTP (2.5 mM each), 1.5 μl $MgCl_2$ (50 mM), 1 μl of each primer (1 μg/μl), 0.5 μl Platinum *Taq* DNA Polymerase (5 U/μl), 89 μl autoclaved, distilled water, and 1 μl template DNA (100 ng/μl)), consisted of an initial denaturation step at 94 °C for 2 min, 35 cycles of 30 sec denaturation at 94 °C, primer annealing for 30 sec at 55 °C and extension for 1 min at 72 °C and finally an extension step of 8 min at 72 °C.

Denaturing Gradient Gel Electrophoresis (DGGE) profiling

PCR amplification of 16S rDNA fragments was carried out with the following eubacterial primer set: GC-63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (El Fantroussi et al. 1999). DGGE was performed on an Ingeny-phorU system (Ingeny, Netherlands) in an 8% (w/v) polyacrylamide gel (acrylamide/bisacrylamide 37,5:1) with a denaturing gradient ranging from 35% to 65%. The electrophoresis was run at 60 °C for 15 hours at 120 V. Afterwards, the gel was fixed in 0.5% acetic acid and stained in TAE buffer with 1% sybr green I solution (BioWhittaker Molecular Applications, ME).

Results

Isolation of 2,4-DNT degrading consortia

Four soil and two water samples (listed in Table 1) from a former ammunition production site were used to inoculate minimal medium with 2,4-DNT as the sole nitrogen source to enrich for 2,4-DNT degrading cultures. This resulted in the isolation of only one 2,4-DNT degrading culture. The inoculum originated from soil sample 1 (S1) and the optimised culture, which showed stable DGGE patterns and identical 2,4-DNT degrading properties, was designated as consortium 1. A second enrichment was started eight months later with sample S1, to establish a second consortium (consortium 2). REP-PCR and DGGE revealed initially similar strains to consortium 1, but later on its composition diverged (data not shown).

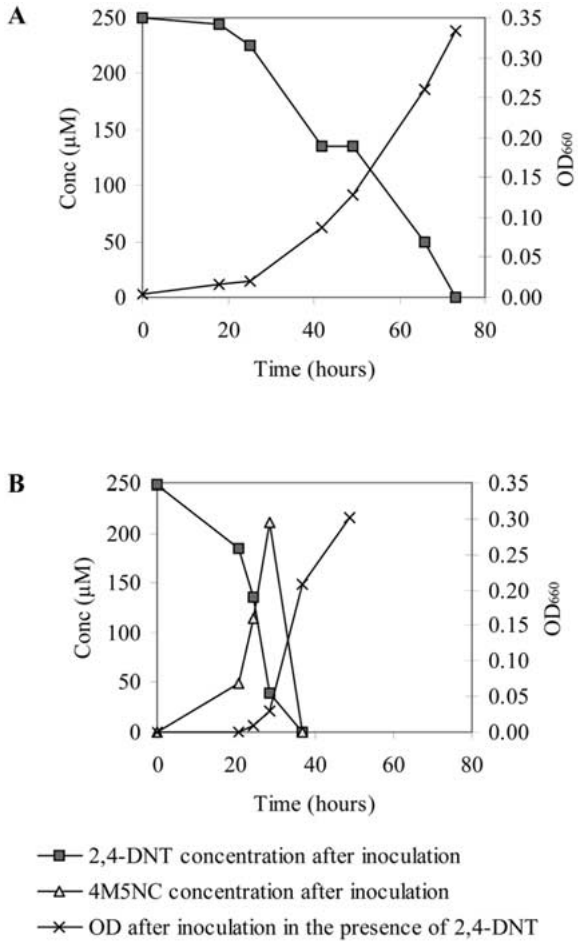

Figure 2. Growth and 2,4-DNT degradation characteristics under nitrogen-limiting conditions. A, consortium 1; B, consortium 2. The increase in optical density was negligible in the absence of 2,4-DNT, and the concentration of 2,4-DNT remained around 250 μM in the control samples (data not shown).

Figure 2 shows the growth and degradation characteristics of the two consortia on 2,4-DNT as sole nitrogen source. In consortium 1, growth was accompanied by gradual disappearance of 2,4-DNT, without the accumulation of metabolic end products detectable by the applied HPLC method (Figure 2A). After the log phase (from 18 hours) 2,4-DNT disappeared at 0.07 μM/min. However, within the first period of growth (from 18 to 48 hours), the culture medium turned pink. This colour faded away during the second period of growth (from 48 to 73 hours), indicating the transient accumulation of a metabolite. Using GC-MS, this intermediate was identified as 2-hydroxy-5-methylquinone (2H5MQ). Consortium 2 degraded 2,4-DNT faster than consortium 1 (at 0.19 μM/min

after the log phase (from 21 hours)) accompanied by a transient accumulation of an orange metabolite (Figure 2B). This compound was identified by HPLC as 4-methyl-5-nitrocatechol (4M5NC). During the first 29 hours of incubation, 84% of the initial 2,4-DNT concentration was degraded to 4M5NC. Subsequently, the accumulated 4M5NC was removed within 8 hours. Furthermore, the decline in 4M5NC concentration was accompanied by a strong increase in growth (starting at 29 hours). None of the consortia grew in nitrogen-free medium and no nitrite accumulated. Additional tests showed that the maximum optical density was linearly related to the initial concentration of 2,4-DNT (to a concentration of 80 mg/L) (data not shown). These findings demonstrated that both consortia used nitrite released from 2,4-DNT as their nitrogen source.

Characterisation of the 2,4-DNT-degrading consortia

Four distinct strains were isolated from consortium 1, and six from consortium 2. Table 2 shows their identification. It was remarkable that although both enrichments started from the same soil sample, they had no single common strain among their members.

The reconstituted consortia 1 and 2 showed degradation characteristics similar to the original consortia, indicating that the key strains in the degradation of 2,4-DNT had been isolated. In addition, DGGE separation patterns of the individual strains, the original consortia, and the reconstituted consortia were compared after growth on 2,4-DNT (Figure 3). Both consortium 1 and reconstituted consortium 1 showed a comparable pattern and *Pseudomonas marginalis* VM683 and *Variovorax paradoxus* VM685 were present in both. The absence of *Microbacterium* sp. VM684 and *Alcaligenes* sp. VM686 in the patterns of these consortia might reflect their low abundance in the consortia, fewer 16S rDNA copies, and/or preferential amplification during the PCR reaction. The two latter hypotheses are not unlikely as demonstrated in lane 7 that shows the result of PCR on a DNA-mixture of the participating strains, made by mixing equal amounts of equal DNA concentrations (100 ng/μl). However, in consortium 2, the presence of Pseudomonas aeruginosa VM903 and/or *P. viridiflava* VM907 and *Pseudomonas* sp. VM908 is clear in both consortium 2 and reconstituted consortium 2. A weak or no signal was observed for *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905, and *Serratia proteamaculans* VM906, which we can account for in the same ways as for *Microbacterium* sp. VM684 and *Alcaligenes* sp. VM686.

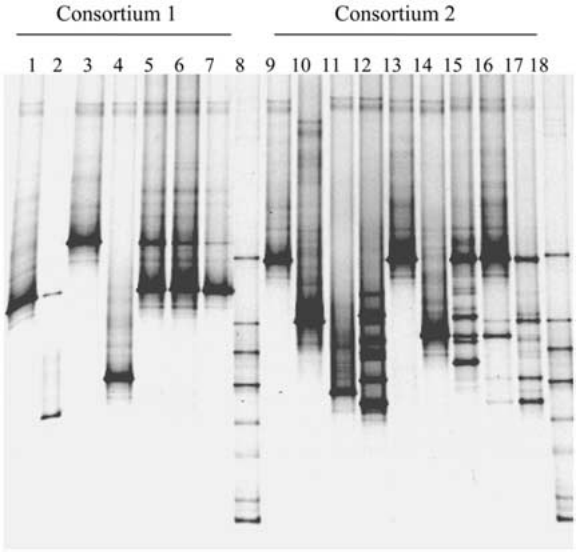

Figure 3. DGGE fingerprint characterising consortium 1 and 2. 1, *Pseudomonas marginalis* VM683; 2, *Microbacterium* sp. VM684; 3, *Variovorax paradoxus* VM683; 4, *Alcaligenes* sp. VM684; 5, consortium 1; 6, reconstituted consortium 1; 7, PCR on DNA-mix of participating strains; 8, marker; 9, *Pseudomonas aeruginosa* VM903; 10, *Sphingomonas* sp. VM904; 11, *Stenotrophomonas maltophilia* VM905; 12, *Serratia proteamaculans* VM906; 13, *Pseudomonas viridiflava* VM907; 14, *Pseudomonas* sp. VM908; 15, consortium 2; 16, reconstituted consortium 2; 17, PCR on DNA-mix of participating strains; 18, marker.

Co-operation of strains in breaking down 2,4-DNT

On selective medium with 2,4-DNT as the sole nitrogen-source, co-operation among the organisms of consortium 1 was observed. When the isolates were streaked separately on the same plate, *Variovorax paradoxus* VM685 generated a brown-pink metabolite, which diffused into the agar. Subsequently, *Pseudomonas marginalis* VM683 started to grow only in close proximity of *V. paradoxus* VM685, and this growth was always accompanied by disappearance of the brown-pink metabolite. In addition, the disappearance of the metabolite coincided with a stimulated growth of *V. paradoxus* VM685 in close proximity of *P. marginalis* VM683 (Figure 4). The results show that *V. paradoxus* VM685 partially degrades 2,4-DNT and in doing so, excretes one or more intermediates. The presence of *P. marginalis* VM683 is indispensable in the next step to continue the degradation and to support the growth of *V. paradoxus* VM685.

Table 2. Identification of the isolates from consortium 1 and consortium 2

Strain code (LMG)	Biochemical identification* (Blast)	Genetical identification
Consortium 1		
VM683	*Pseudomonas marginalis*	*Pseudomonas marginalis* (96%)
VM684	*Clavibacter michiganense*	*Microbacterium* sp. (96%)
VM685	Not identified	*Variovorax paradoxus* (95%)
VM686	*Alcaligenes* sp.	*Alcaligenes* sp. (98%)
Consortium 2		
VM903	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* (99%)
VM904	*Sphingomonas* sp.	*Sphingomonas* sp. (97%)
VM905	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia* (98%)
VM906	*Serratia* sp.	*Serratia proteamaculans* (98%)
VM907	*Pseudomonas fluorescens* group	*Pseudomonas viridiflava* (97%)
VM908	*Janthinobacterium lividum*	*Pseudomonas* sp. (98%)

*Biochemical identification included basic bacteriological tests: Gram-staining, FAME-analysis, Biolog GN, and API20NE. For genetical identification, the 16S rRNA gene was amplified, cloned, sequenced, and the sequence was compared against published sequences from GenBank.

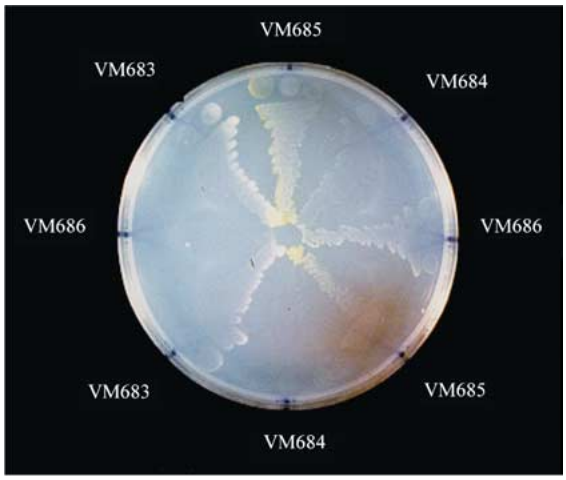

Figure 4. Individual strains, isolated from consortium 1, streaked out in combination on selective medium with 2,4-DNT as nitrogen source. List of strains: *Pseudomonas marginalis* VM683; *Microbacterium* sp. VM684; *Variovorax paradoxus* VM685; *Alcaligenes* sp. VM686.

The complementary action of strains in breaking down 2,4-DNT was further examined in liquid cultures. Accordingly, reconstitutions with all possible combinations of two or three members of the respective consortia were made. They showed that for both consortia, two main members were required to observe continued growth and degradation beyond the colour intermediates (4M5NC and 2H5MQ). In consortium 1, total degradation of 2,4-DNT could be described as an interaction between *V. paradoxus* VM685 and *P. marginalis* VM683. Thus, within four days, the supply of 2,4-DNT was depleted and the increase in growth slowed. Other combinations of members from consortium 1 partly transformed 2,4-DNT to its reduced intermediates (2-A-4-NT and 4-A-2-NT, which were detected using HPLC analysis), but no growth was observed. A three-member consortium was successful only when *V. paradoxus* VM685 and *P. marginalis* VM683 were present. These results indicate that the other isolates are not important for the consortium's major catabolic activity. In consortium 2, various efficient two-member combinations could degrade 2,4-DNT, but the presence of *Pseudomonas* sp. VM908 was essential. The best results were achieved when this species was in combination with *Sphingomonas* sp. VM904 or *P. viridiflava* VM907. After two days of incubation, neither 2,4-DNT nor any of its expected degradation products could be determined in its HPLC and GC-MS analyses. In combination with *P. aeruginosa* VM903 or *Stenotrophomonas maltophilia* VM905, complete degradation took three days. Degradation was inefficient with *Serratia proteamaculans* VM906; 63% of the initial 2,4-DNT concentration still remained in the medium after three days.

An important finding was that none of the individual strains from consortium 1 and 2 could grow alone on 2,4-DNT as the sole nitrogen source. However, transformations did occur: cultures with *V. paradoxus* VM685 from consortium 1 and *Pseudomonas*

sp. VM908 from consortium 2 accumulated the intermediate 4M5NC in the medium and nitrite was detected (though this compound was never observed in consortium 1), indicating that they initiate the attack on 2,4-DNT. The accumulation of 4M5NC was toxic and reduced the growth of *V. paradoxus* VM685 from consortium 1 and *Pseudomonas* sp. VM908 from consortium 2. In contrast, the other pure cultures exhibited reduction reactions leading to the formation of 4-A-2-NT and 2-A-4-NT. These results indicate that both consortium 1 and 2 use the same initial oxidative step in their 2,4-DNT degradation pathway. The identification of 4M5NC and 2H5MQ as degradation intermediates of 2,4-DNT in consortium 1 and 2 pointed to the presence of the same oxidative pathway for 2,4-DNT as in *Burkholderia* sp. strain DNT (Suen and Spain, 1993; Figure 1). To examine this hypothesis and also the role of the different members in 2,4-DNT degradation, enzyme-activity tests and DNA-DNA hybridisation were performed on the individual members. Only *V. paradoxus* VM685 and *Pseudomonas* sp. VM908 exhibited 2,4-DNT dioxygenase activity, as indicated by concomitant colour changes and the detection of nitrite. Furthermore, only these two strains hybridised with the *dntAa* probe (Figure 5A). We never succeeded in amplifying the *dntB* fragment in strain DNT to use further as a probe, despite choosing different primers as well as PCR conditions. However, 4M5NC monooxygenase activity was demonstrated in the presence of both *V. paradoxus* VM685 and Pseudomonas sp. VM908. 4M5NC was oxidised to 2H5MQ. We have no evidence about which strains further reduce 2H5MQ to 2,4,5-THT in the consortia. The enzyme activity test was negative, and as the 2H5MQ reductase gene (*dntC*) has not been sequenced, hybridisation could not be performed. The presence of 2,4,5-THT was demonstrated by GC-MS in the culture media of both consortium 1 and consortium 2. A DNA fragment containing the *dntD* gene, which is required for oxidising 2,4,5-THT into 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid, was used as probe to detect a homologous gene in our consortia (Figure 5B). *P. marginalis* VM683, *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 and *P. viridiflava* VM907 showed a strong hybridisation signal. This finding seems to coincide with our previous observations where strain VM683 in combination with strain VM685 and the strains VM903, VM904, VM905, or VM907 in combination with VM908 showed efficient break down of 2,4-DNT. *Pseudomonas* sp. VM908 and *Alcaligenes* sp. VM686 gave a weak hybridisation signal. No signals were obtained from *Serratia proteamaculans* VM906, *Microbacterium* sp. VM684 and *V. paradoxus* VM685. The possible presence of 2,4,5-THT oxygenase activity was further explored with 4-methylcatechol as the substrate. In addition to activity towards 2,4,5-THT the enzyme also shows activity towards 4-methylcatechol in *Burkholderia* sp. strain DNT (Haigler et al. 1999). However, our results were negative. To provide extra evidence for ring cleavage, the ability of consortium 1 and 2 to metabolise 2,4-DNT was assayed under carbon-limiting conditions. The compound was supplemented at 550 μM (100 mg/L). Both consortia completely degraded the 2,4-DNT within 7 days. Transformations to 4-A-2-NT and 2-A-4-NT occurred, but the sum of both aminonitrotoluenes was less than 1% of the initial 2,4-DNT concentration. Other metabolites were not detected and growth was observed throughout the exposure time (an OD660 at eight days of about 0.050 compared to an OD660 of 0.003 in the control without 2,4-DNT).

Metabolism of other nitroaromatic compounds under nitrogen-limiting conditions

Neither of the two 2,4-DNT-grown consortia transformed 2-NT, 4-NT, 2-A-4-NT, 2-A-6-NT, 4-A-2-NT, and 2,6-DNT. All substrates were provided at 50 mg/L. After 96 hours, consortium 1 transformed TNT partially (59%) into 2-A-4,6-DNT (2%), 4-A-2,6-DNT (5%) and several unidentified metabolites. During this exposure, the culture became progressively more red-brown. The causative compound(s) has not been identified. After 96 hours, consortium 2 transformed TNT completely into aminodinitrotoluenes (21%) and unidentified metabolites. Unlike consortium 1, this medium did not discoloured. DIP-MS and ESI-MS/MS-CID analyses gave strong indications for the presence of azoxy compounds (binuclear compounds, which arise from the condensation of partially reduced intermediates of TNT).

Discussion

Efforts to isolate 2,4-DNT degrading cultures were successful only when detectable 2,4-DNT contamination was present in the starting material, as was the case for sample S1 from which consortium 1 and consortium 2 were isolated (Table 1). Here, exposure must have favoured the selection of strains able

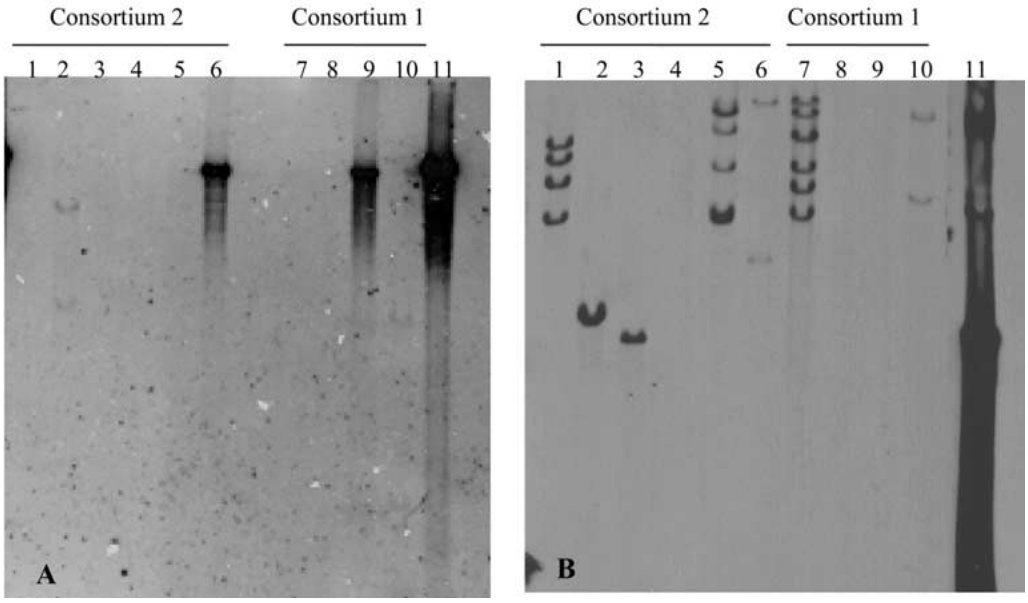

Figure 5. Result of hybridisation with probe dntAa (A) and probe dntD (B). 1, *Pseudomonas aeruginosa* VM903; 2, *Sphingomonas* sp. VM904; 3, *Stenotrophomonas maltophilia* VM905; 4, *Serratia proteamaculans* VM906; 5, *Pseudomonas viridiflava* VM907; 6, *Pseudomonas* sp. VM908; 7, *Pseudomonas marginalis* VM683; 8, *Microbacterium* sp. VM684; 9, *Variovorax paradoxus* VM683; 10, *Alcaligenes* sp. VM684; 11, *Burkholderia* sp. DNT.

to degrade the nitroaromatic compound. This observation is consistent with previous reports in which 2,4-DNT-mineralising strains could be isolated only from 2,4-DNT-contaminated sites (Spanggord et al. 1991; Johnson et al. 2000; Nishino et al. 2000). Similar findings were illustrated for some agriculture chemicals, such as the herbicides atrazine and linuron (De Souza et al. 1998; El Fantroussi 2000).

In contrast with other studies, which isolated single strains that grow on 2,4-DNT, we isolated 2,4-DNT-degrading consortia. This result suggests that, so far, the prevailing conditions at our site did not naturally select a single organism able to degrade the compound. However, the interaction between microorganisms with diverse degradative properties might be an important starting point in the evolution of novel catabolic strains.

The two isolated consortia had no common strain, despite the fact that they originated from the same homogenised soil sample. However, in an early stage of the second enrichment, which gave us consortium 2, two strains were identified that had the same REP-PCR patterns as observed for strains present in consortium 1. This result demonstrates that selective enrichment cannot always be reproduced. Complex biotic and abiotic interactions determine the adaptive responses, evolution, and final structure of a community. Competition among strains can change during enrichment by horizontal gene transfer, mutations, and small differences in selective pressure.

The fact that the intermediates 4M5NC, 2H5MQ and 2,4,5-THT were detected in the culture media of consortia 1 and 2 indicate that they use the same degradation pathway as *Burkholderia* sp. strain DNT. This finding is not unexpected as, to date, all strains that grow on 2,4-DNT appear to follow this same degradation pathway (Nishino et al. 2000). Analogous results were obtained from bacteria able to degrade the herbicide atrazine. All pure and mixed cultures of atrazine-mineralising bacteria were shown to contain genes homologous to *atzA*, *-B*, and *-C* from *Pseudomonas* sp. strain ADP. These results suggest a recent evolutionary origin and distribution of the degrading genes (De Souza et al. 1998). However, it might indicate as well that the particular pathway is the most advantageous and that it develops easily *de novo*.

The genetic and biochemical data demonstrate that *Variovorax paradoxus* VM685 (consortium 1) and *Pseudomonas* sp. VM908 (consortium 2) initiate the 2,4-DNT catabolism of the consortia by carrying out reactions similar to the first two metabolic steps observed for *Burkholderia* sp. DNT: 2,4-DNT is converted to 2H5MQ via 4M5NC, with release of both nitro groups as nitrite. However, the conversion of

4M5NC requires the interaction of other species from the consortia, as this compound accumulates in their absence. It was demonstrated that growth and activity of *V. paradoxus* VM685 and *Pseudomonas* sp. VM908 ceased in the presence of 2H5MQ. Elimination of 2H5MQ by the interacting bacteria pushes the equilibrium of the reaction towards the quinone. However, as nitrite was released, but growth was not observed for *V. paradoxus* VM685 or *Pseudomonas* sp. VM908 when they were individually inoculated under selective conditions, intervention of other strains also could also be at the point of detoxifying nitrite. 4M5NC was never detected when consortium 1 or its functional two-member derivative consortium was inoculated in selective conditions. This finding contrasts from that with consortium 2, where 4M5NC accumulated until 2,4-DNT was almost completely depleted, suggesting additional inhibition of 2,4-DNT on 4M5NC monooxygenase in consortium 2.

The results of *dntD* hybridisation, and the observation that both consortia grow on 2,4-DNT as carbon source, suggest that *Pseudomonas marginalis* VM683 (consortium 1), *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905 or *P. viridiflava* VM907 (consortium 2) are essential to continue the degradation of 2,4-DNT beyond 2,4,5-THT. However, the catabolic step resulting in ring fission could not be confirmed by an enzyme activity test using 4-methylcatechol as substrate, suggesting there is similarity in the ring-fission enzymes despite a difference in substrate specificity towards 4-methylcatechol.

In both consortium 1 and 2 the 2,4-DNT degradation occurred by specific two-member derivative consortia, though both consisted of more than two strains. Lappin et al. (1985) similarly selected a five-member mecoprop- (herbicide) degrading consortium in preference to several equally capable two-member derivative consortia, indicating the existence of metabolic cross-feeding. In addition, De Souza et al. (1998) examined an atrazine-catabolizing bacterial consortium containing four or more members, of which two members, *Clavibacter michiganese* ATZ1 and *Pseudomonas* sp. CN1, collectively mineralised atrazine. Timmis et al. (2001) concluded that the diversity of interacting species in a community along with the presence of redundant species favours stability and ensures effective utilisation of resources under changing environmental conditions. Interspecies metabolic interaction also can be proposed for our consortia. The nitrite released by *Variovorax paradoxus* VM685 or *Pseudomonas* sp. VM908 may allow nitrogen assimilation by the other consortia members. The surplus value to the consortium of the other strains (*Microbacterium* sp. VM684, *Alcaligenes* sp. VM686, *Serratia* sp. VM906) may be based on the transfer of micronutrients (e.g., vitamins, amino acids) or complexing agents for heavy metals. We did not investigate this hypothesis further.

Acknowledgements

We thank J. Spain and S.F. Nishino for their contribution to the work by providing *Burkholderia* sp. strain DNT, samples of 4M5NC and 2,4,5-THT, and their helpful suggestions. In addition we thank J.C. Libouton for donating a sample of TNT, J. Czech for GC-MS analyses, and D. Springael for useful discussions. This work was supported by a PhD grant from Vito to Z. Snellinx.

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