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ORIGINAL PAPER

Aerobic biotransformation of 2,4-dinitroanisole in soil and soil *Bacillus* sp.

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Abstract 2,4-Dinitroanisole (DNAN) is a low sensitive melt-cast chemical being tested by the Military Industry as a replacement for 2,4,6-trinitrotoluene (TNT) in explosive formulations. Little is known about the fate of DNAN and its transformation products in the natural environment. Here we report aerobic biotransformation of DNAN in artificially contaminated soil microcosms. DNAN was completely transformed in 8 days in soil slurries supplemented with carbon and nitrogen sources. DNAN was completely transformed in 34 days in slurries supplemented with carbons alone and persisted in unamended microcosms. A strain of *Bacillus* (named 13G) that transformed DNAN by co-metabolism was isolated from the soil. HPLC and LC–MS analyses of cell-free and resting cell assays of *Bacillus* 13G with DNAN showed the formation of 2-amino-4-nitroanisole as the major end-product via the intermediary formation of the arylnitroso (ArNO) and arylhydroxylamino (ArNHOH) derivatives, indicating regioselective reduction of the *ortho*-nitro group. A series of secondary reactions involving ArNO and ArNHOH gave the corresponding azoxy- and azodimers. Acetylated and demethylated products were identified. Overall, this paper provides the evidence of fast DNAN transformation by the indigenous microbial populations of an amended soil with no history of contamination with explosives and a first insight into the aerobic metabolism of DNAN by the soil isolate *Bacillus* 13G.

Keywords DNAN · Nitroaromatic · Explosive · Biodegradation · Nitroreduction

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Introduction

Defense industries in the US, Canada and Australia are currently developing new insensitive munitions compounds for use in various explosive formulations. As a consequence, new highly energetic chemicals will make their way into natural environments in the coming years. For one, the nitroaromatic 2,4-dinitroanisole (DNAN) is considered as a promising substitute for 2,4,6-trinitrotoluene (TNT); it is a less sensitive melt-cast medium than TNT, requiring a higher temperature for detonation, making it safer to manufacture, transport and store (Davies and Provatas 2006). The environmental risk associated with the use of DNAN at large scale is still unclear. A study showed that DNAN is metabolized to 2,4-dinitrophenol (2,4-DNP) in the body, a chemical with high acute and

chronic toxicity (U.S. HSS 1995); its transformation by natural microbial communities remained to be characterized. Recent papers on DNAN transformation in anaerobic bioreactors reported the reduction of DNAN to diaminoanisole (Arnett et al. 2009; Platten III et al. 2010). Clone libraries for three bioreactors revealed the dominance of a single clone of *Chloroflexi* (*Levilinea* sp.), suggesting that the organism played an important role in the anaerobic reduction of DNAN (Arnett et al. 2009). Aerobic biodegradation of DNAN has not been reported yet.

Biodegradation of nitroaromatic compounds is of general interest as they represent one of the largest groups of industrial chemicals produced today. Bacterial species of *Pseudomonas*, *Burkholderia*, *Comamonas*, *Arthrobacter*, *Acidovorax* and *Rhodococcus* have been reported to grow on several nitroaromatic compounds including nitrobenzenes, nitrotoluenes and nitrophenols as the sole nitrogen, carbon and energy sources (Jain et al. 1994; Nishino and Spain 1995; He and Spain 1999; Nishino et al. 2000; Ju and Parales 2010). *Rhodococcus* strains AS2 and AS3 were shown to use 4-nitroanisole as the sole source of carbon and energy (Schäfer et al. 1996). In these strains, the degradation was initiated by demethylation of 4-nitroanisole to 4-nitrophenol, which was hydroxylated to 4-nitrocatechol prior to denitration and ring cleavage leading to mineralization (Schäfer et al. 1996). However, under both aerobic and anaerobic conditions, the electron-withdrawing nature of the $–NO_2$ groups on the aromatic ring favours reduction of the compounds to arylamines without cleaving the aromatic ring (Spain 1995).

The present study was undertaken to first determine the intrinsic capability of a natural microbial community to transform DNAN in soil microcosms, secondly, to isolate a bacterial strain capable of degrading DNAN, and thirdly, to identify key intermediates and end-products of DNAN transformation by the isolate to gain insights into the aerobic transformation pathway of DNAN.

Materials and methods

Chemicals and culture media

2,4-Dinitroanisole (purity 98.4%) was provided by Defense Research and Development Canada (DRDC), Valcartier, Canada. Standard 2-amino-4-nitroanisole (2-A-4-NAN, purity 99.8%) was obtained from MP Biomedicals and standards 4-amino-2-nitroanisole (4-A-2-NAN), 2-amino-4-nitrophenol (2-A-4-NP), 4-amino-2-nitrophenol, 2,4-DNP and 2,4-dinitrotoluene (2,4-DNT) were obtained from Sigma-Aldrich. The mineral salts medium (MSM) (pH 7.0) consisted of (per liter of distilled water): 0.38 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.05 g $FeCl_3{\cdot}6H_2O$. R2A and Luria-Bertani (LB) were purchased from Difco and tryptone soya broth (TSB) was purchased from Oxoid.

Soil description and preparation

Topsoil was collected from an uncontaminated site at the DRDC-Valcartier (Quebec, Canada), stored at 4°C, and used within 3 months of collection. The soil had the following properties: sandy soil, pH 6.0, 2% total organic carbon and 7.5% moisture content. It was sieved (2 mm) prior to being used in microcosms. The soil samples to be used as killed controls were sterilized by autoclaving (121°C, 20 min) three consecutive days.

DNAN biotransformation in soil microcosms

The soil microcosms consisted of 10 g of soil and 20 ml of MSM in 100-ml sterile glass serum bottles. DNAN was added as an aqueous stock solution to a final concentration of 40 mg l^{-1} (202 μM). Microcosms were prepared with soil slurries either (i) unamended; (ii) amended with 10 mM glucose and 5 mM succinate; or (iii) amended with glucose, succinate and 5 mM NH_4Cl as a nitrogen source. Killed controls and controls without DNAN were prepared for the three treatments. Each treatment was prepared in 3–5 replicates and the microcosm bottles were incubated at room temperature ($\sim$20 to 22°C) away from light on a rotary shaker at 150 rpm. At periodic intervals, aliquots were collected from the aqueous phases for chemical analysis.

Isolation, identification and growth of a DNAN-reducing *Bacillus* strain

Serial dilutions of soil slurries from microcosms that underwent two cycles of complete DNAN transformation were plated on R2A, LB and 1/5 strength TSB solidified with 1.5% (w/v) agar. A number of colonies with diverse morphology, size and pigmentation were

isolated. Single colonies were tested for their ability to degrade DNAN in MSM and LB medium. A DNAN-transforming bacterium was purified by repeated streaking on LB agar plates. The genomic DNA of the pure culture was isolated with the QIAGEN Blood & Cell Culture DNA kit. A 700-bp fragment of the 16S rRNA gene was amplified by PCR and sequenced on both strands at the McGill University Genome Quebec Innovation Centre (Montreal, QC). The 16S rRNA gene sequence was submitted for comparison to the GenBank databases using the Blastn algorithm. The strain was identified as a *Bacillus*; the 16S rRNA gene sequence of the isolate, named *Bacillus* strain 13G, was deposited at GenBank under accession number JF912193. The phylogenetic identity was corroborated with microscopic visualization of Gram-strained cells. Growth with DNAN as the sole N and/or C source was tested in MSM supplemented with 40 mg l^{-1} of DNAN. In other bottles, glucose (10 mM) and succinate (5 mM) were used as the C sources and NH_4Cl (20 mM) as the N source when needed. Each flask was inoculated from a bacterial suspension at a starting optical density at 600 nm (OD_{600}) of 0.1. The OD_{600} values and DNAN concentrations were measured every day for a week.

Biotransformation assays

Growing cells of strain 13G

A time course assay with strain 13G was performed in 50 ml LB with 49 mg l^{-1} DNAN. Cultures were inoculated using mid-log phase cells also grown in LB with 49 mg l^{-1} DNAN (OD_{600} of 4.8). Cultures were grown aerobically at 25°C, away from light, in 500-ml baffled culture flasks shaken at 250 rpm. Periodically, 1 ml was sampled from each culture to monitor growth spectrophotometrically at 600 nm (Thermo Spectronic UV1) and then centrifuged at 16,000×*g* for 10 min using an Eppendorf Centrifuge 5415D. Supernatants were then used for chemical analysis. Controls were prepared with cells in the absence of DNAN. The results presented are the mean of triplicate assays.

Resting cells of strain 13G

A time course experiment was conducted with 13G cells grown aerobically in LB medium supplemented with 40 mg l^{-1} DNAN. Cells were harvested at late log phase, with an OD_{600} between 9.0 and 11.4, at 15,000×*g* at 4°C for 15 min using a Sorvall RC6 Plus 110 centrifuge (Thermo Electron Corporation, Milford, MA). Cells were washed and resuspended in sterile MilliQ water, then incubated with 40 mg l^{-1} DNAN, with a final OD_{600} of ~3.2. In some cases, cells were incubated with 175 mg l^{-1} DNAN for LC–MS analysis. The assays were conducted in 120-ml serum bottles and were incubated at 25°C, away from light, at 150 rpm. At selected times, aliquots of the cell suspensions were collected, centrifuged at 16,000×*g* for 10 min and the supernatants used for chemical analysis. Controls were prepared with cells in the absence of DNAN, as well as DNAN in the absence of cells. The results are presented as the mean of triplicate assays. To understand DNAN transformation, additional assays were performed with potential DNAN products (2-A-4-NAN, 4-A-2-NAN, 4-amino-2-nitrophenol, 2,4-DNP, 2-A-4-NP) or 2,4-DNT as sole substrate. Each of the above substrate was tested under the same conditions as described above, aside from an initial concentration of 20 mg l^{-1}.

Cell-free extract of strain 13G

Cells were grown at 25°C in LB medium with 50 mg l^{-1} DNAN. At mid-log phase, the cells were harvested by centrifugation, washed twice with 50 mM phosphate buffer pH 7.0 and resuspended in the same buffer at an OD_{600} of 10. The cells were broken by freeze/thaw followed by five cycles of sonication on ice (1 min on; 30 s off) with a 130-w sonicator set at 80% of maximum power. The cell lysate was centrifuged at 20,000×*g* for 30 min. Proteins were quantified with the Pierce BCA kit using albumin bovine as a standard. The reaction mixture (1 ml) contained DNAN at 100 μM, NADH or NADPH at 300 μM, and 2 mg of cell-free extract in 50 mM phosphate buffer pH 7.0. The reaction was performed at 25°C and stopped by filtration through 30-kDa cut-off spin columns (Microcon YM-30, Amicon).

Analytical methods

DNAN, 2-A-4-NAN, 4-A-2-NAN, 2-hydroxylamino-4-nitroanisole (2-HA-4-NAN) and 2,4-DNT were

analyzed by reversed-phase high-performance liquid chromatography (HPLC)–UV with a system that consisted of a W600 pump (Waters), a 717 plus autosampler and a 2996 Photodiode-Array Detector. 50-µl injection volumes were separated with a Discovery C18 column (25 cm × 4.6 mm × 5 µm) (Supelco, Oakville, Canada) at 35°C. The mobile phase (50% aqueous methanol) ran isocratically at 1 ml min^{-1} for 15 min. The detector was set to scan from 192 to 450 nm. Detection of DNAN was performed at 298 nm and its limit of detection at this wavelength was 0.010 mg l^{-1}; 2,4-DNT was detected at 230 nm with a detection limit of 0.005 mg l^{-1}. Liquid chromatography–mass spectrometry (LC–MS) analyses were performed using a mass spectrometer (MS, Bruker MicroTOFQ mass analyzer) attached to an HPLC system (Hewlett Packard 1200 Series) equipped with a DAD detector. Aliquots (10 µl) were injected into a 3.5 µm-pore size Zorbax SB-C18 column (2.1 mm ID × 150 mm; Agilent, Mississauga, Canada) at 25°C. The solvent system was composed of a CH_3OH/H_2O gradient (40–90% v/v) at a flow rate of 0.15 ml min^{-1}. For mass analysis, negative electrospray ionization (ES−) was used to produce deprotonated molecules $(M–H)^-$ and characteristic mass ion fragments. Mass range was scanned from 40 to 1000 Da. We determined HCHO by HPLC (Bhatt et al. 2006) and NO_2^- and NO_3^- by ion chromatography (Balakrishnan et al. 2004). Measurement of methanol was made on an Agilent 6890 gas chromatograph equipped with a flame ionization detector (FID). One hundred microliter of liquid sample was transferred in 20-ml headspace vial crimped with a Teflon coated septum. Before injection, the vial was heated at 80°C for 2 min. Headspace gas (1 ml) was automatically injected onto a DB-ACL2 capillary column (30 m × 530 mm × 2 µm) from J&W using a Combipal autosampler from CTC Analytics. The column was held at 40°C for 10 min. Helium was the carrier gas with a head pressure of 5 psi. The injector under a split ratio of 5:1 and the detector were maintained at 200 and 250°C, respectively. Measurements of 2,4-DNP and 4-amino-2-nitrophenol were made on an Agilent HPLC consisting of a quaternary pump model G1311A and an autosampler model G1329A equipped with a DAD detector model G1315A. Twenty-five microliter was injected on a reverse phase HPLC column Gemini-NX C18 3 µm from Phenomenex of 4.6 × 150 mm maintained at 40°C. An isocratic run using 45% methanol and 55% H_3PO_4 0.027 N was performed at a flow rate of 0.45 ml min^{-1}. An eluent switch was applied at 22 min to fasten the removal of unknown late peaks from the HPLC column. Compounds were quantified at 235 nm.

Results and discussion

Biotransformation of DNAN in soil

The soil indigenous microbial community readily transformed DNAN in aerobic microcosms supplemented with a nitrogen (NH_4Cl) and carbon (glucose and succinate) sources (Fig. 1). DNAN disappeared at the rate of 1.7 ± 0.2 nmol h^{-1} g^{-1} and the initial 4 µmol were completely transformed in 8 days. We observed disappearance of 88% of DNAN in only 28 h after fresh culture medium was supplied to actively transforming microcosms (data not shown). This suggested that the DNAN-degrading microbes became more efficient following adaptation to the prevailing conditions or that their number increased. In soil microcosms supplemented with the carbon sources alone, DNAN transformation began slowly during at least the first 8 days and was completed

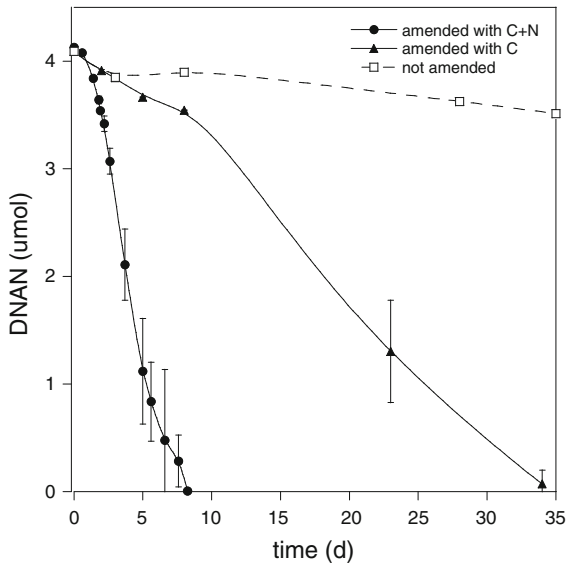

Fig. 1 Aerobic transformation of DNAN in soil microcosms. *Error bars* represent the standard deviation of the mean

after 34 days (Fig. 1). DNAN only slowly disappeared in unamended microcosms with the loss of 0.58 µmol after 35 days (Fig. 1) and 0.65 µmol after 5 months of aerobic incubation (data not shown). This suggests that DNAN may persist under natural conditions at least in soils with no history of contamination with DNAN. No significant loss of DNAN occurred in the sterile controls. HPLC analysis identified a peak corresponding to 2-amino-4-nitroanisole (2-A-4-NAN) in all DNAN-transforming microcosms. The molar fraction of 2-A-4-NAN increased with time, reaching 0.505 after complete DNAN disappearance. Formaldehyde (HCHO) was detected at a molar fraction of 0.041 before its concentration declined. A number of peaks were observed using LC–MS but interferences from the soil slurries prevented good identification of the corresponding products.

Isolation of *Bacillus* strain 13G

A screening of microbial isolates obtained from the soil slurries identified one strain able to transform DNAN in liquid medium. Microscopic examination of the bacterium showed gram-positive endospore-forming rods. 16S rRNA gene sequence analysis identified the bacterium as a *Bacillus* with 100% gene identity with strains of *B. cereus*, including the phenol-degrading sp. MRA1 (GenBank accession number JF729306). The *Bacillus* strain, named 13G, did not grow in MSM with DNAN as the sole N or C source. The strain only grew in MSM when supplemented with both N and C sources, in which the OD_{600} reached 1.6 (data not shown).

Biotransformation of DNAN with *Bacillus* 13G-products distribution

A series of degradation assays with strain 13G were performed to identify key intermediates and end-products of DNAN catabolism. Figure 2a is a typical LC–UV chromatogram of products (**2**–**7**) formed during DNAN incubation with resting cells of *Bacillus* 13G. Figure 2b represents a LC–MS chromatogram of DNAN incubated with cell-free extract of 13G showing the presumed nitroso product of DNAN (**1**). LC–MS analysis of product **2** showed a deprotonated molecular mass $[M–H]^-$ at *m*/*z* 183 Da, matching an empirical formula of $C_7H_8N_2O_4$, that was tentatively identified as 2-HA-4-NAN (Fig. 2c). We were unable to detect the nitroso derivative with resting cells, but when we incubated DNAN with cell-free extract we detected a trace amount of a compound (**1**) with $[M–H–CH_2]^-$ at *m*/*z* 167 Da and [M–H–NO] at *m*/*z* 151 Da, matching a molecular formula of $C_7H_6N_2O_4$ that we tentatively identified as 2-nitroso-4-nitroanisole (2-NO-4-NAN) (**1**; Fig. 2b, c). Product **3** was identified as 2-amino-4-nitroanisole by comparison with a reference standard.

We also detected a product (**4**) with a $[M–H]^-$ at *m*/*z* 347 Da, matching an empirical formula $C_{14}H_{12}N_4O_7$. The product was tentatively identified as 2,2′-azoxy-4,4′-nitroanisole (Fig. 2a, c). Hydroxylamino compounds are known to react with the corresponding nitroso derivative to produce azoxy-derivatives under aerobic/abiotic conditions (Haidour and Ramos 1996; Wang et al. 2000; Vasilyeva et al. 2000). Also we detected a peak with a $[M–H]^-$ at *m*/*z* 331 Da, matching an empirical formula of $C_{14}H_{12}N_4O_6$ and tentatively identified as 2,2′-azo-4,4′-nitroanisole (**5**; Fig. 2a).

Another peak was detected and identified as 2-acetamido-4-nitroanisole (2-AcA-4-NAN) (**6**; Fig. 2a) by comparison with a reference standard prepared by reacting 2-A-4-NAN with acetic anhydride. Peak **7** (Fig. 2a) was identified as acetamido-nitrophenol (**7**), a derivative of 2-A-4-NP. The same compound (**7**) was detected in a control containing resting cells of 13G and 2,4-DNP (loss of 11% mol/mol after 2.5 h of incubation).

Time profile of products detected during DNAN transformation with cells of *Bacillus* 13G-transformation pathway

During growth of 13G in LB, complete transformation of the initial 12 µmol DNAN was achieved in 6 h. DNAN disappearance was accompanied by the production of 2-A-4-NAN, which was formed via the intermediary formation of 2-HA-4-NAN (Fig. 3a). In resting cells, DNAN was transformed at the rate of 14.2 µmol min^{-1} g^{-1} protein and 2-A-4-NAN was formed at a molar fraction of 0.37. DNAN disappearance was accompanied with the formation of 2-HA-4-NAN and 2-A-4-NAN, as well as HCHO and small amounts of MeOH (Fig. 3b). Neither nitrite nor nitrate was detected which was supported by the absence of any DNAN denitrated products. Also we

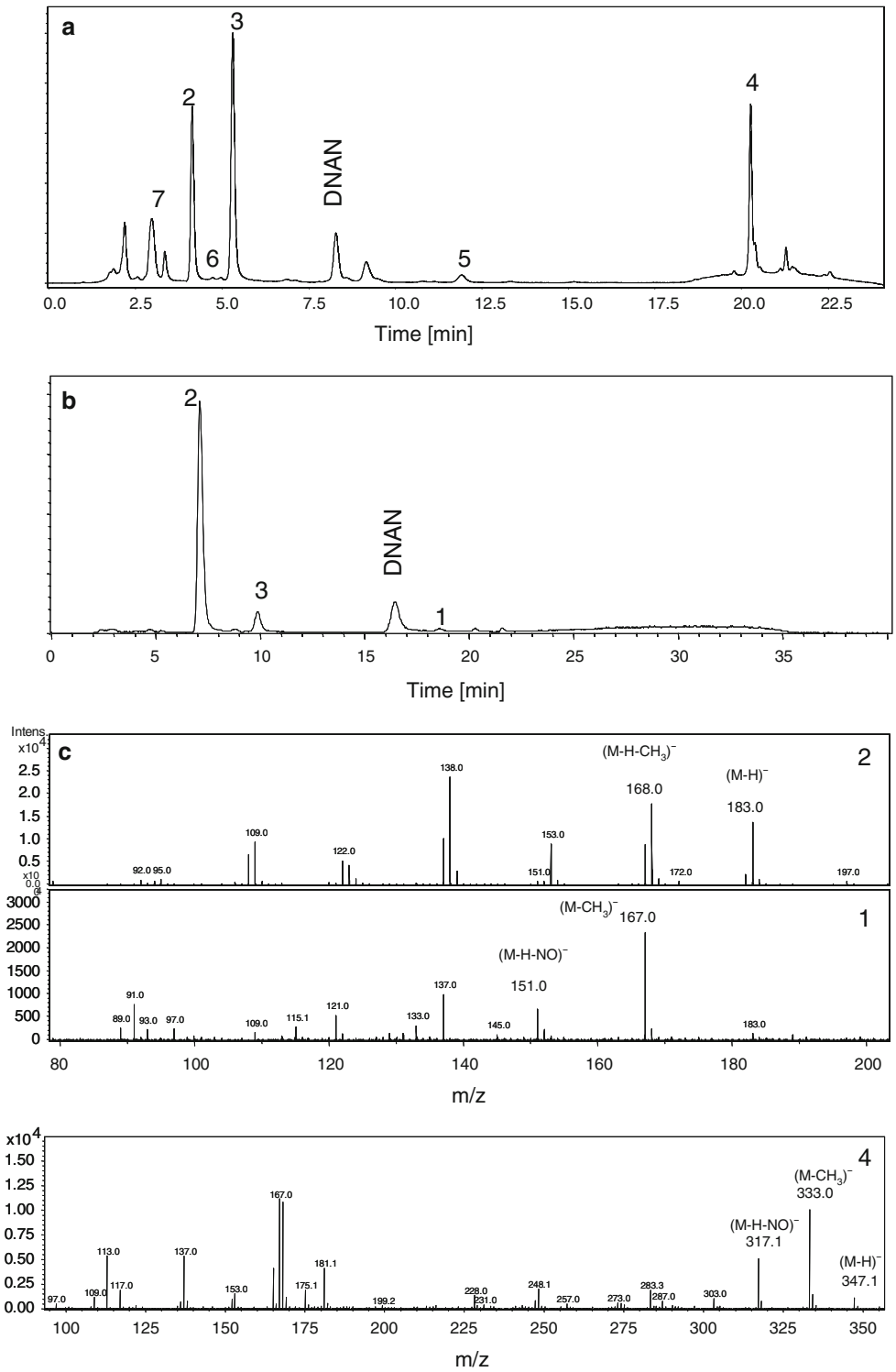

Fig. 2 **a** LC–UV (at 300 nm) chromatogram of DNAN and its metabolites produced by resting cells of *Bacillus* 13G after 46 h of incubation; **b** LC–MS chromatogram of DNAN incubated with cell-free extract of 13G after 5 h; **c** MS spectra of compounds **1**, **2** and **3**

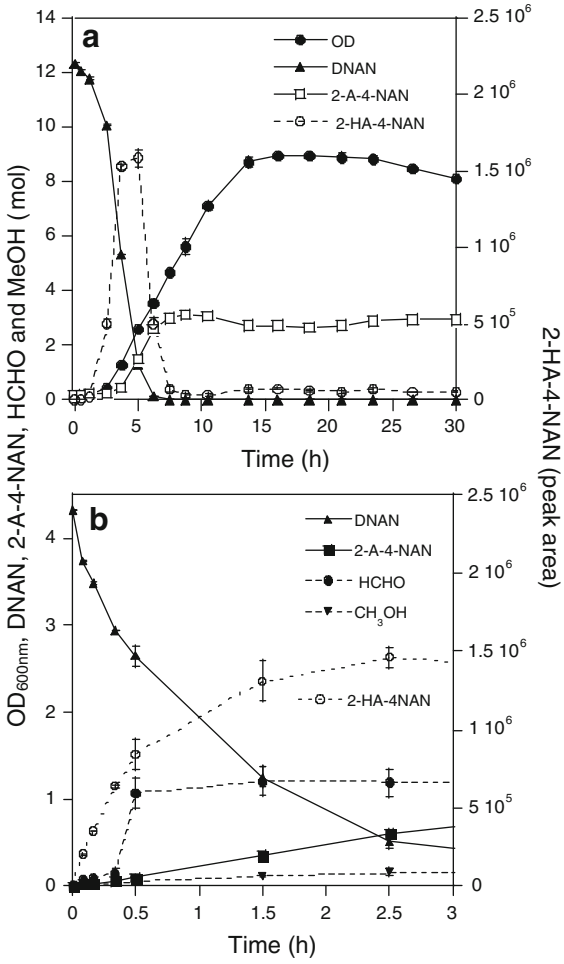

Fig. 3 DNAN disappearance and products by *Bacillus* 13G. **a** Growing cells in LB medium; **b** resting cells. *Error bars* represent the standard deviation of the mean

were unable to detect any products that might arise from the reduction of the $-NO_2$ group at the *para* position. Finally, diaminoanisole, an end product of DNAN anaerobic biotransformation (Arnett et al. 2009; Platten III et al. 2010), was not formed aerobically by 13G.

Using products distribution and time course study (Figs. 2, 3), we constructed a pathway for aerobic DNAN biotransformation by *Bacillus* 13G as shown in Fig. 4. The pathway consisted of a primary route involving a regioselective reduction of the *ortho*-NO_2 functional group to eventually produce the corresponding amine derivative (2-A-4-NAN) (**3**) via the intermediary formation of 2-NO-4-NAN (**1**) and 2-HA-4-NAN (**2**) (Fig. 4, path A).

Secondary routes consisted of a cascade of reactions involving the initially formed products 2-NO-4-NAN and 2-HA-4-NAN. One secondary route involved condensation between the nitroso and the hydroxylamino metabolites to give the azoxy (**4**) and the azo (**5**) derivatives (Fig. 4, path B). Another secondary route involved acetylation of 2-HA-4-NAN (**2**) to give the 2-AcA-4-NAN (**6**) (Fig. 4, path C) or demethylation of **2** followed by acetylation to give **7** (Fig. 4, path C). Indeed, as shown in Fig. 3b, HCHO was detected during incubation of DNAN with 13G. The formation of HCHO suggested demethylation of the O-Me group in DNAN. However, we are not sure at what stage in the degradation process HCHO was formed, but the absence of 2,4-DNP suggested that HCHO was formed later in the degradation process, i.e., after reducing $-NO_2$ to –NHOH. We presume that the acetylated products originated from either (1) demethylation of 2-HA-4-NAN (**2**) followed by acetylation or (2) demethylation of 2-AcA-4-NAN (**6**) (Fig. 4, path C). A control assay containing resting cells and 2-A-4-NAN did not degrade 2-A-4-NAN (**3**), confirming that the production of the 2-AcA-4-NAN did not originate from amino-nitroanisole.

As mentioned above, reduction of DNAN by 13G was regioselective favouring the reduction of the *ortho* $-NO_2$ to the $-NH_2$ group. In this respect, we conducted some preliminary experiments to degrade 2,4-DNT with 13G under the same conditions. We found that reduction of 2,4-DNT occurred principally at the *para* position leading to the formation of 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene in a 6:1 molar ratio (data not shown). Two other strains of *Bacillus* (99% 16S rRNA gene identity to *B. pumilus*), previously isolated by our team from explosives contaminated sediments, transformed 2,4-DNT by co-metabolism (Fournier and Hawari, unpublished data), however in these strains, it was the reduction of the *ortho*-nitro group that was favoured. Similarly and as opposed to 13G, nitroreductase I of *Klebsiella* sp. C1 was shown to catalyze the reduction of the nitro group of 2,4-DNT exclusively at the *ortho* position (Kim and Song 2005). The above experimental evidences gathered thus far indicate that regioselectivity of the initial steps involved in the reduction of dinitroaromatics may be determined by the type of the reducing system used.

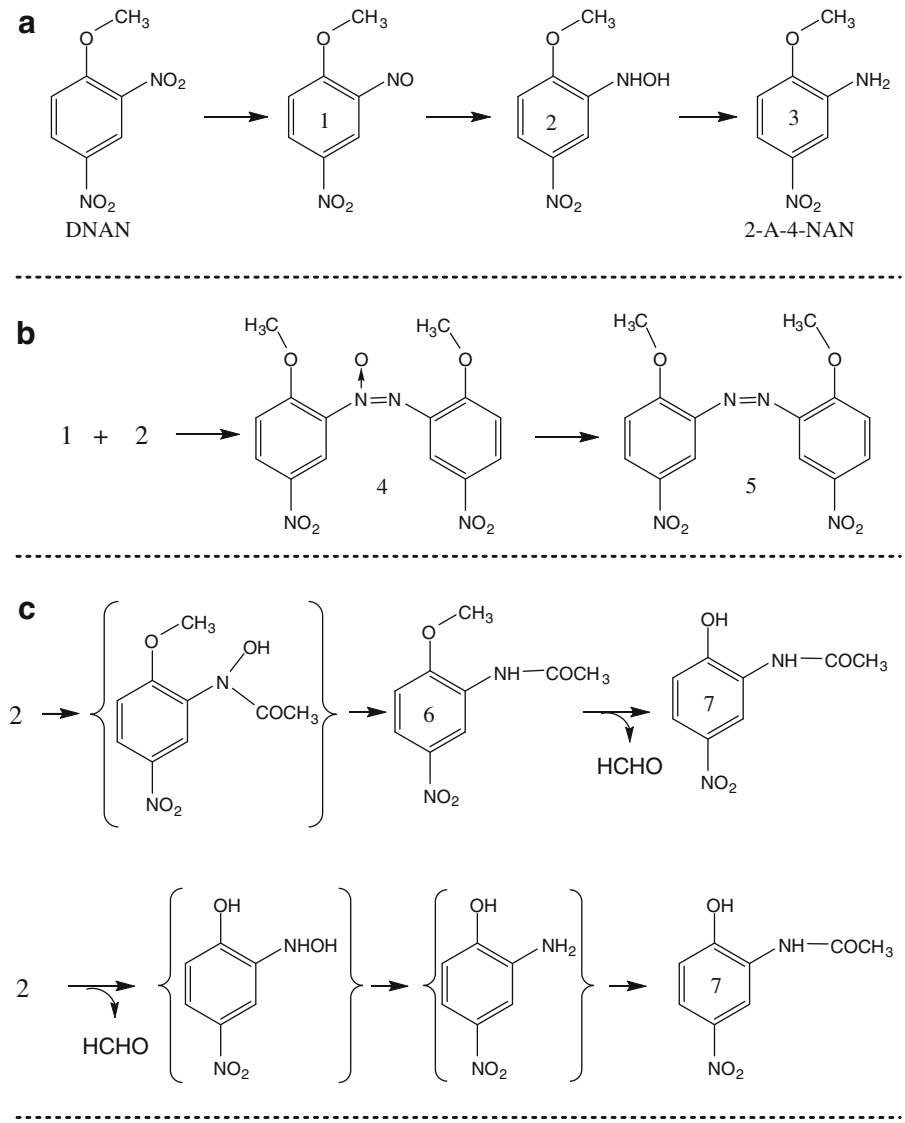

Fig. 4 Proposed degradation routes of 2,4-DNAN with *Bacillus* 13G. *Path A* Primary transformation routes of DNAN by *Bacillus* 13G; *path B* production of azoxy- and azo-compounds (**4** and **5**, respectively) from compound **1** and **2**; *path C* proposed secondary transformation routes of compound **2** to produce acetylated compounds (compounds in bracket were not detected)

Conclusion

This work describes a case of aerobic DNAN transformation in a soil with no history of contamination. The isolated *Bacillus* strain 13G did not grow or mineralize significant quantities of DNAN, suggesting cometabolism rather than a growth-linked process. Cometabolic reactions involving broad-specificity nitroreductases may represent an important mechanism of DNAN transformation in the environment, as it is for other nitroaromatic compounds. Additional DNAN biodegradation studies with other bacterial strains and natural environments will expand our understanding of DNAN degradation and its fate in situ.

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