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Publisher's version / Version de l'éditeur:

<https://doi.org/10.1021/es301047d>

Environmental Science & Technology, 46, 11, pp. 6035-6040, 2012-05-07

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Aerobic Mineralization of Nitroguanidine by *Variovorax* Strain VC1 Isolated from Soil

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ABSTRACT: Nitroguanidine (NQ) is an energetic material that is used as a key ingredient of triple-base propellants and is currently being considered as a TNT replacement in explosive formulations. NQ was efficiently degraded in aerobic microcosms when a carbon source was added. NQ persisted in unamended microcosms or under anaerobic conditions. An aerobic NQ-degrading bacterium, *Variovorax* strain VC1, was isolated from soil microcosms containing NQ as the sole nitrogen source. NQ degradation was inhibited in the presence of a more favorable source of nitrogen. Resting cells of VC1 degraded NQ effectively ($54 \mu\text{mol h}^{-1} \text{g}^{-1}$ protein) giving NH_3 (50.0%), nitrous oxide (N_2O) (48.5%) and CO_2 (100%). Disappearance of NQ was accompanied by the formation of a key intermediate product that we identified as nitrourea by comparison with a reference material. Nitrourea is unstable in water and suffered both biotic and abiotic decomposition to eventually give NH_3 , N_2O , and CO_2 . However, we were unable to detect urea. Based on products distribution and reaction stoichiometry, we suggested that degradation of NQ , $\text{O}_2\text{NN}=\text{C}(\text{NH}_2)_2$, might involve initial enzymatic hydroxylation of the imine, $-\text{C}=\text{N}-$ bond, leading first to the formation of the unstable α -hydroxynitroamine intermediate, $\text{O}_2\text{NNHC}(\text{OH})-(\text{NH}_2)_2$, whose decomposition in water should lead to the formation of NH_3 , N_2O , and CO_2 . NQ biodegradation was induced by nitroguanidine itself, L-arginine, and creatinine, all being iminic compounds containing a guanidine group. This first description of NQ mineralization by a bacterial isolate demonstrates the potential for efficient microbial remediation of NQ in soil.

INTRODUCTION

Nitroguanidine (NQ) is used by the defense industry as an oxidizer in triple-base propellants. It reduces flash and temperature during deflagration and it improves the firing power when compared to single and double-base propellants. It has been recently incorporated in a new formulation, IMX-101, that has been qualified as a TNT replacement for the production of new insensitive munitions. NQ also serves as an intermediate in the commercial synthesis of various organic chemicals and herbicides. It exists in two tautomeric forms: the nitrimine form $\text{O}_2\text{NN}=\text{C}(\text{NH}_2)_2$ predominates over the nitramino tautomer $\text{O}_2\text{NNHC}=(\text{NH})\text{NH}_2$, except under strongly alkaline conditions.¹ Like other explosive materials, NQ can be detected as a contaminant at military training ranges.² Although NQ itself is practically nontoxic (toxicity threshold of 2200 mg L^{-1}),³ its potential transformation products (e.g., nitrosoguanidine, guanidine, cyanoguanidine, melamine, urea, and nitrite) have varying degrees of toxicity. It has been reported that photolysis, the dominant transformation process of NQ in surface waters,⁴ can lead to the formation of hydroxyguanidine, guanidine, urea, cyanoguanidine, ammonia, and nitrosoguanidine.^{5,6} Others reported that NQ can be reduced by catalytic hydrogenation to nitrosoguanidine and aminoguanidine⁷ and by using zerovalent iron under anaerobic conditions (J. Hawari and R. Saad, personal communication).

On the other hand, very little information is available on the microbial transformation of NQ. Kaplan et al. studied NQ transformation in activated sludge and showed that it was transformed to nitrosoguanidine that further decomposed abiotically to cyanamide, cyanoguanidine, melamine, and guanidine under anaerobic conditions;⁸ conversely, aerobic NQ biotransformation in the activated sludge was negligible.⁸ NQ was biotransformed cometabolically to cyanamide in surface water samples under aerobic conditions.⁴ It has also been reported that NQ is poorly degraded in soils by indigenous bacteria and that degradation rates are related to soil organic carbon content.^{8–10}

So far, no report is available on the transformation or mineralization of NQ by a pure microbial isolate. Here we report aerobic mineralization of NQ by a *Variovorax* strain that we isolated from soil. Assays were performed to identify intermediate and final products of NQ catabolism and to propose a biodegradation pathway.

Received: March 16, 2012

Revised: May 2, 2012

Accepted: May 7, 2012

Published: May 7, 2012

MATERIALS AND METHODS

Chemicals and Culture Media. Nitroguanidine (NQ, contains 25% water) and acetohydroxamic acid (AHA, 98% purity) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Urea (electrophoresis grade) was obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada) and N-nitrourea was purchased from SelectLab Chemicals GmbH (Frankfurt, Germany). ^{18}O -labeled water (97 atom %) was from Isotec Inc. (Miamisburg, OH). The mineral salts medium (MSM; pH 7.0) consisted of (per liter of distilled water): 0.38 g of K_2HPO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.05 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. MSM supplemented with 10 mM glucose (MSMG) was used in the biodegradation assays with *Variovorax* (isolated as described below). The *Variovorax* isolate was routinely maintained on Luria–Bertani (LB) agar plates.

Soil Description and Preparation. Soil was collected from a non-contaminated area adjacent to a military live fire training range at the Defense Research and Development Canada-Valcartier (Quebec, Canada). The soil was stored at 4 °C and used within 2 months of collection. It consisted of sandy soil at pH 6.0, 2% total organic carbon, and 7.5% moisture content. The soil was sieved (2 mm) prior to being used in microcosms. Soil samples that were autoclaved (121 °C, 20 min) for 3 consecutive days were used as killed controls.

Nitroguanidine Degradation Assays in Soil Microcosms. The soil microcosms were prepared in 100-mL sterile serum bottles and contained 10 g of soil and 20 mL of MSM. NQ was added to a final concentration of 192 μM . The microcosms for the aerobic study were either unamended or amended with (i) 10 mM glucose and 5 mM succinate as C sources, or (ii) 10 mM glucose and 5 mM succinate plus 5 mM NH_4Cl as a nitrogen source. The anaerobic microcosms were prepared unamended and amended with 1% molasses. Anaerobic conditions were established by degassing with a vacuum pump for 20 min and introducing O_2 -free argon. The microcosms were then briefly degassed and filled with O_2 -free argon (4 times). Killed controls (autoclaved soil) were prepared in parallel for each condition tested. The microcosm bottles, in triplicate, were incubated in the dark at room temperature (~ 20 – 22 °C) on a rotary shaker at 150 rpm for aerobic conditions and statically for anaerobic conditions. Samples were collected at periodic intervals to monitor NQ disappearance.

Isolation of a Nitroguanidine-Degrading *Variovorax*. Serial dilutions of the aerobic soil slurries in MSM with 10 mM glucose, 5 mM succinate, and containing 385 μM NQ as the sole N source led to the isolation of one nitroguanidine-degrading bacterial strain. Genomic DNA was isolated from the strain using the QIAGEN Blood & Cell Culture DNA kit (QIAGEN, Mississauga, ON, Canada). The near full-length 16S rRNA gene (1392 bp) was amplified by PCR and the amplicon was sequenced on both strands at the McGill University Genome Quebec Innovation Centre (Montreal, QC, Canada). The sequence was submitted to the GenBank databases for phylogenetic comparison using the Blastn algorithm. The 16S rRNA gene sequence was deposited at GenBank under the name *Variovorax* strain VC1 and received the accession number JQ692086.

Biodegradation of Nitroguanidine by Growing Cells of Strain VC1. A time course assay with strain VC1 was conducted in 60 mL of MSMG supplemented with 834 μM NQ in 500-mL baffled culture flasks. Cultures were grown

aerobically at 25 °C, 180 rpm, and away from light. Periodically, samples were collected to monitor growth spectrophotometrically at 600 nm ($\text{OD}_{600\text{ nm}}$) and the supernatant was centrifuged and used for NQ analysis. The inocula used were cells grown in LB and washed three times with MSMG. The results presented are the mean of triplicates.

Biodegradation of Nitroguanidine and its Potential Transformation Products Urea and Nitrourea by Resting Cells of Strain VC1. Resting cells experiments were conducted using VC1 cells grown in MSMG with NQ, using the growth conditions mentioned above. Cells were harvested during midlog phase, washed and resuspended in sterile Milli-Q water, then incubated with either 488 μM NQ, 450 μM nitrourea, or 497 μM urea with a final $\text{OD}_{600\text{ nm}}$ of 0.7. Assays were performed in sealed 120-mL serum bottles and incubated at 25 °C, 180 rpm, aerobically and away from light. Samples of the liquid phase were removed at selected times for chemical analysis. The headspaces were sampled for gas phase analysis. Experiments conducted for the analysis of CO_2 had cells with a final $\text{OD}_{600\text{ nm}}$ of 1.8 incubated with 6 mM NQ, in 50 mM sodium phosphate buffer, pH 7.2, in sealed 20-mL headspace vials. CO_2 was analyzed 4 h after adding 250 μL of 2 M HCl to attain a pH of 1.4. Controls with substrates in the absence of cells and controls with cells in the absence of substrates were prepared. The results presented are the mean of triplicates.

Other resting cells assays were performed with NQ as substrate as described above but with VC1 cells grown with 10 mM glucose and 3 mM NH_4Cl , L-glutamic acid, L-arginine, L-histidine, cytosine, or creatinine (all purchased from Sigma Aldrich) to determine if they could induce NQ biodegradation.

Inhibition of Urease Activity by Acetohydroxamic Acid (AHA). For experiments with the urease inhibitor AHA, cells were allowed to incubate with 4 mM AHA for 30 min at 4 °C prior to exposure to NQ or urea. Once the AHA-treated cells were used in the assay, the final AHA concentration was 0.66 mM. Samples of the liquid phase were removed at selected times for chemical analysis and the headspaces were sampled for gas phase analysis. Controls with cells in the absence of any substrate and controls with substrates in the absence of cells were prepared. The results presented are the mean of triplicate assays.

Analytical Methods. NQ and nitrourea were analyzed using an HPLC system equipped with a 600 pump (Waters), a 717 plus autosampler and a 2996 Photodiode-Array Detector. Samples (50 μL) were separated with a Hypercab column (15 cm, 4.6 mm, 5 mm) (Cole-Parmer, Montreal, Canada) at 35 °C. The mobile phase, composed of 90% of 0.05% or 0.5% trifluoroacetic acid in water and 10% acetonitrile, ran isocratically at 1 mL min^{-1} . The detector was set to scan from 191 to 400 nm. The detection limit for NQ at 265 nm was estimated at 0.005 mg L^{-1} and for nitrourea the detection limit at 225 nm was estimated at 0.05 mg L^{-1} . The urea standards were measured using an HPLC system equipped with a 600 pump (Waters), a 717 plus autosampler, and an electrochemical detector (model 2465) with a gold cell in the pulse amperometric mode. Samples (25 μL) were injected on a cation exchange column IonPac CS14 (4 $\times$ 250 mm) (Dionex). The mobile phase was 5 mM methanesulfonic acid and ran isocratically at 1.25 mL min^{-1} at 35 °C. The detection limit for urea was estimated at 0.25 mg L^{-1} . Ammonium ion (NH_4^+) was analyzed by ion chromatography.¹¹ Nitrous oxide (N_2O) was determined by GC/ECD.¹² Measurement of CO_2 was made by gas chromatography using a thermal conductivity

detector on an Agilent 6890 gas chromatograph. Headspace of acidified samples (250 μL) were injected into a 3.5 m $\times$ 2 mm ID Chromosorb 102 packed column from SUPELCO. The column was heated at 50 $^{\circ}\text{C}$ and argon was used as the carrier gas. The injector and detector were maintained at 125 and 150 $^{\circ}\text{C}$, respectively. Degradation products were analyzed using a mass spectrometer (MS, Bruker MicroTOFQ mass analyzer) attached to an HPLC system (Hewlett-Packard 1200 Series) equipped with a DAD detector. Samples (10 μL) were injected into a 3- μm -pore size Gemini C18 column (3 mm ID $\times$ 150 mm; Phenomenex) at 25 $^{\circ}\text{C}$. The solvent system was composed of 50% CH_3CN and 50% HCOOH (0.05%) at a flow rate of 0.15 mL min^{-1} . For mass analysis, negative electrospray ionization was used to produce deprotonated ($\text{M} - \text{H}$) $^{-}$ molecular ions. Mass range was scanned from 40 to 500 Da.

RESULTS AND DISCUSSION

Biodegradation of NQ in Soil. To gain a broader view of NQ biotransformation in environmental samples and to isolate

Figure 1. Biotransformation of NQ in aerobic soil microcosms. C, carbon sources (glucose and succinate); N, nitrogen source (NH_4Cl). Error bars represent the standard deviation of the mean from triplicate experiments.

an efficient NQ degrader, we determined the fate of NQ in aerobic and anaerobic soil microcosms. Figure 1 shows the removal of 192 μM NQ in 6 days in carbon-amended (10 mM glucose and 5 mM succinate) aerobic microcosms. Transformation occurred quickly after a lag phase of ~ 4.5 days. NQ transformation in carbon-amended microcosms was slowed in the presence of ammonium chloride (NH_4Cl), which likely constituted a more favorable nitrogen source than NQ. NQ persisted in autoclaved controls as well as in aerobic microcosms not supplemented with carbons (Figure 1). There was no degradation even after 14 months (data not shown). Accordingly, previous studies showed that NQ biotransformation only occurs, or is greatly enhanced, in the presence of carbon supplements or nutrients.^{8,10} NQ did not transform in soil slurries, with or without nutrients (1% molasses), incubated anaerobically for 14 months (data not shown).

Isolation of the Nitroguanidine-Degrading *Variovorax* Strain VC1. A bacterial strain that grew aerobically with NQ as the sole N source was isolated from the soil slurries. The colonies had a yellow-pigmentation on LB agar plates and

Figure 2. NQ biodegradation by *Variovorax* strain VC1 (a) in growing cells, and (b) in resting cells with formation of NH_4^+ and N_2O as nitrogen end-products. The cell protein concentration was 0.7 mg mL^{-1} . Error bars represent the standard deviation of the mean.

microscopic examination revealed motile gram-negative rods. Analysis of the 16S rRNA gene sequence identified the isolate as a *Variovorax* with 100% identity, over the 1392-bp DNA sequence, to the β -proteobacterium *Variovorax paradoxus* EPS (GenBank accession CP002417) and 99.9% identity (one bp difference) with other *Variovorax* isolates such as *V. paradoxus* strain IHB B 1313 (GU186109). The strain was named *Variovorax* strain VC1. *Variovorax* species display multiple metabolic features including the biotransformation of the herbicides linuron¹³ and diuron¹⁴ and of synthetic (2,4-dinitrotoluene) and naturally occurring (3-nitrotyrosine) nitroaromatic compounds.^{15,16}

Growth of VC1 with NQ as the sole nitrogen source in the presence of 10 mM glucose is presented in Figure 2a. Strain VC1 degraded NQ at a maximum rate of $34.1 \mu\text{mol L}^{-1} \text{ h}^{-1} \pm 4.2$. NQ degradation was inhibited in the presence of a more

Figure 3. Percentage of NQ degraded by resting cells of *Variovorax* strain VC1 grown with NQ itself or with 3 mM creatinine, L-arginine, cytosine, L-glutamic acid, ammonium chloride, or L-histidine as the sole N source. The cell protein concentration was 1.3 mg mL^{-1} in 50 mM sodium phosphate buffer (pH 7.0) and incubated at 25°C for 24 h.

favorable source of nitrogen such as NH_4Cl or in LB medium (data not shown). VC1 resting cells degraded NQ at the maximum rate of $54.0 \pm 2.1 \mu\text{mol h}^{-1} \text{g}^{-1}$ protein (Figure 2b).

Induction of NQ Biodegradation. Because strain VC1 was isolated from a soil with no history of contamination, the enzymatic system responsible for NQ degradation is likely to be incidental to normal metabolism. We therefore performed resting cell assays using VC1 cells grown with the naturally occurring L-arginine and creatinine, both containing a guanidine group (imine), as the sole N source. Additional assays were performed with the amino-containing compounds L-glutamic acid, L-histidine, and cytosine, the latter being structurally similar to creatinine but without the guanidine group. As shown

Figure 5. Production of ammonium by resting cells of *Variovorax* strain VC1 during degradation of NQ and urea in the presence or absence of the urease inhibitor acetohydroxamic acid (AHA). Error bars represent the standard deviation of the mean.

in Figure 3, cells grown with NQ itself and creatinine degraded 100% of the initial $385 \mu\text{M}$ NQ in 24 h. Cells grown on arginine degraded 82.3% NQ. Only 3.6% NQ was degraded by cells grown on cytosine. Cells grown on L-glutamic acid, L-histidine, or ammonium chloride degraded less than 1.4% NQ after 24 h (Figure 3). The deimination product of creatinine, N-methylhydantoin,^{17,18} was detected by liquid chromatography–mass spectrometry (LC-MS), suggesting that the corresponding enzyme(s) (iminohydrolase) was also involved in NQ

Figure 4. (a) HPLC/UV (at 250 nm) chromatogram of NQ and product nitrourea (NU) from resting cells of VC1 incubated with $\sim 6 \text{ mM}$ NQ for 22 h; (b) mass spectrum of NU produced in H_2^{16}O ; and (c) mass spectrum of NU produced in H_2^{18}O .

Figure 6. Degradation of NQ and nitrourea (NU) and the concomitant formation of N_2O by resting cells of *Variovorax* strain VC1. Error bars represent the standard deviation of the mean.

degradation. Further works are required to elucidate the genes/enzymes involved in NQ biodegradation by VC1 and other bacterial strains.

NQ Transformation Products. Ammonia, detected as ammonium ions, and N_2O were found as final N-containing products (Figure 2b). The molar fraction of $\text{NH}_4^+:\text{NQ}$ was calculated at 2.00 ± 0.02 and the molar fraction of $\text{N}_2\text{O}:\text{NQ}$ was 0.97 ± 0.07 , representing a N-mass balance of 98.5%. None of the analyzed potential NQ degradation products (nitro-

soguanidine, aminoguanidine, hydroxyguanidine, guanidine, cyanamide, cyanoguanidine, hydrazine, urea, and nitrite) were detected. To detect some intermediates of NQ degradation, we performed a resting cell assay with ~10-times more NQ. Under these conditions, a peak was detected at 250 nm by HPLC and identified as nitrourea by comparison with a reference material (Figure 4a). Nitrourea was further confirmed by LC-MS, showing a deprotonated molecular mass $[\text{M} - \text{H}]^-$ at m/z 104 Da, representing an empirical formula $\text{C}_1\text{H}_3\text{N}_3\text{O}_3$ (Figure 4b). Nitrourea was previously detected as an abiotic degradation product of NQ.^{1,4} It decomposes spontaneously in water to nitroamide (NO_2NH_2) and cyanic acid (HNCO). In water, nitroamide decomposes spontaneously to N_2O while cyanic acid undergoes hydrolysis to NH_3 and CO_2 through carbamic acid.^{19,20}

Neither formaldehyde nor formate were observed, only CO_2 was found as C-containing degradation product. Carbon recovery as CO_2 was ~100% ($\text{CO}_2:\text{NQ}$ molar fraction of 1.05 ± 0.03).

Possible Routes for the Biodegradation of NQ by *Variovorax* strain VC1. We found that resting cells of strain VC1 degraded NQ ($\text{O}_2\text{NN}=\text{C}(\text{NH}_2)_2$) to quantitatively produce NH_3 (50.0%), N_2O (48.5%), and CO_2 (100%). The release of closely two moles of NH_3 and one mol of N_2O for each mol of NQ degraded pointed to the initial cleavage of a C–N bond rather than N–N bond in NQ to form either (i) urea (H_2NCONH_2) or (ii) nitrourea ($\text{H}_2\text{NCONHNO}_2$).

- (i) It has been shown previously that urea can be produced during photolysis of aqueous NQ.^{4,6} However, we were unable to detect urea among NQ degradation products. To test the possibility that urea actually formed but was degraded quickly after, we incubated urea with VC1 cells in the presence and absence of a urease inhibitor acetohydroxamic acid (AHA). Urea was degraded by

Figure 7. Proposed degradation route of nitroguanidine by *Variovorax* strain VC1 (compounds in brackets were not detected).

resting cells of VC1 grown with NQ (Figure 5). However, AHA inhibited the transformation of urea to NH_3 (catalyzed by urease) but did not significantly affect NQ degradation to NH_3 (thus urease is not involved) (Figure 5). This confirmed that urea was not an intermediate produced as a precursor of NH_3 during NQ degradation by VC1.

- (ii) As demonstrated above, the disappearance of NQ was accompanied by the formation of a key intermediate product that we eventually identified by HPLC/MS analysis and by comparison with a reference material as nitrourea. Abiotic controls containing nitrourea showed some decomposition, but in the presence of resting cells of VC1, nitrourea degraded with a rate ~ 5 times higher than the abiotic degradation rate (Figure 6), indicating that nitrourea was degraded biologically in addition to the spontaneous abiotic hydrolysis.

Based on products distribution and reaction stoichiometry, we suggest that degradation of NQ, $\text{O}_2\text{NN}=\text{C}(\text{NH}_2)_2$, involves initial enzymatic hydroxylation of the imine, 18,21 $\text{C}=\text{N}-$ bond, leading first to the formation of the unstable α -hydroxynitroamine intermediate, $\text{O}_2\text{NNHC}(\text{OH})(\text{NH}_2)_2$ (Figure 7). Although NQ exists as a pair of tautomer, both molecules upon hydration should lead to the same α -hydroxynitroamine intermediate. A proposed route for the aerobic biodegradation of NQ by VC1 is shown in Figure 7. NQ degradation was initiated by iminohydrolysis to eliminate ammonium, which formed the unstable nitrourea (Figure 7, path a). Participation of water was demonstrated by incorporation of ^{18}O in nitrourea when H_2^{18}O was used. An increase of 2 Da of the $[\text{M} - \text{H}]^-$ of nitrourea was detected by LC-MS, i.e., from m/z 104 Da to m/z 106 Da (Figure 4c). Nitrourea degraded to two unstable molecules, i.e. (i) cyanic acid (HNCO) that quickly hydrolyzed to carbamic acid (H_2NCOOH) and (ii) nitroamide (NH_2NO_2). Carbamic acid further degraded to CO_2 and NH_3 , while NH_2NO_2 gave N_2O . Experimental evidence indicates that NQ was not degraded via urea (Figure 7, path b).

This pathway has high environmental significance since, conversely to what was generally reported, NQ could be totally biodegraded to three harmless end-products by a soil isolate under aerobic conditions. NQ is a one carbon molecule and both the studies in soil microcosms and with VC1 suggested that, in order to achieve effective bioremediation of NQ-contaminated sites, organic carbons or nutrients should be available in sufficient amount or be added during treatment. *Variovorax* strain VC1 provides a much needed pure culture model for further studies on the metabolism of NQ by microorganisms.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by Defence Research Development Canada, Department of National Defence. We thank L. Paquet, A. Corriveau, and C. Beaulieu for the analytic support.

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