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Functional Characterization of Propane-Enhanced *N*-Nitrosodimethylamine Degradation by Two Actinomycetales

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ABSTRACT: Propane-induced cometabolic degradation of *n*-nitrosodimethylamine (NDMA) by two propanotrophs is characterized through kinetic, gene presence, and expression studies. After growth on propane, resting cells of *Rhodococcus sp*. RR1 possessed a maximum transformation rate ($v_{max,n}$) of 44 ± 5 μg NDMA (mg protein)$^{-1}$ h^{-1}; the rate for *Mycobacterium vaccae* (*austroafricanum*) JOB-5 was modestly lower with $v_{max,n}$ of 28 ± 3 μg NDMA (mg protein)$^{-1}$ h^{-1}. Both strains were capable of degrading environmentally relevant, trace quantities of NDMA to below the experimental limit of detection, calculated as 20 ng NDMA L^{-1}. However, a comparison of half saturation constants ($K_{s,n}$) and NDMA degradation in the presence of propane revealed pronounced differences between the strains. The $K_{s,n}$ for strain RR1 was 36 ± 10 μg NDMA L^{-1} while the propane concentration needed to inhibit NDMA rates by 50% (K_{inh}) occurred at 7,700 μg propane L^{-1} ($R^2 = 0.9669$). In contrast, strain JOB-5 had a markedly lower affinity for NDMA verses propane with a calculated $K_{s,n}$ of $2{,}200 \pm 1{,}000$ μg NDMA L^{-1} and K_{inh} of 120 μg propane L^{-1} ($R^2 = 0.9895$). Genomic and transcriptional investigations indicated that the functional enzymes involved in NDMA degradation and propane metabolism are different for each strain. For *Rhodococcus sp*. RR1, a putative propane monooxygenase (PrMO) was identified and implicated in NDMA oxidation. In contrast, JOB-5 was not found to possess a PrMO homologue and two functionally analogous alkane monooxygenases (AlkMOs) were not induced by growth on propane. Differences between the PrMO in this *Rhodococcus* and the unidentified enzyme(s) in the *Mycobacterium* may explain differences in NDMA degradation and inhibition kinetics between these strains.

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Introduction

N-nitrosodimethylamine (NDMA), a member of the mutagenic nitrosamines, is a confirmed animal carcinogen (IARC, 1987; Mitch et al., 2003). Environmental releases have been linked to industrial sources (Sedlak et al., 2005), aerospace facilities, (CalDHS, 2002; MacDonald, 2002) and to certain water-reuse scenarios (Njam and Trussell, 2001; OCWD, 2000). NDMA's persistence in groundwater aquifers has led to the closure of wells in contact with these waters, and state and provincial agencies in the US and Canada have set advisory levels at 10 and 9 ng/L in water, respectively (CalDHS, 2002; MOE, 2003).

NDMA's chemical properties render it mobile in subsurface aquifers and resistant to abiotic attenuation (Mirvish, 1975; Oliver, 1979; Thomas, 1982); however, microorganisms can play a role in the degradation of NDMA in undefined microcosms (Gunnison et al., 2000; Kaplan and Kaplan, 1985; Yang et al., 2005) and in pure strains (Fournier et al., 2006, 2009; Rowland and Grasso, 1975; Sharp et al., 2005, 2007; Yoshinari and Shafer, 1990). Though the potential for biological degradation exists, variability of NDMA removal in wastewater treatment systems (Sedlak et al., 2005) suggests a dependence upon unidentified conditions associated with the expression and activity of enzymes involved in NDMA biodegradation.

Environmental NDMA concentrations are typically orders of magnitude lower than most water contaminants

targeted for bioremediation. Analyses of municipal effluent have demonstrated that treated wastewater typically contains $<$1,000 ng NDMA L^{-1} (Gan et al., 2006; Sedlak et al., 2005) while groundwater associated with fuel spills can have concentrations 3–4 orders of magnitude higher (CalDHS, 2002; WateReuse, 2005). Even though investigations of the NDMA biodegradation pathway have revealed intermediates that could be used as a carbon or nitrogen source (i.e., methanol, formaldehyde, methylamine, and nitrate), strains and consortia have not been shown to grow on NDMA as a sole carbon and energy source (Fournier et al., 2006, 2009; Gunnison et al., 2000; Kaplan and Kaplan, 1985; Sharp et al., 2005, 2007; Tate and Alexander, 1975). Consequently, it is plausible that NDMA biotransformation may predominantly occur via cometabolic reactions with little direct benefit to the transforming cells. In this scenario, degradation could be controlled by the presence of inducing substrates that promote enzymes capable of oxidizing NDMA, while at the same time compete for active sites.

Bacteria possessing monooxygenases, including propane monooxygenase, have been shown to cometabolically degrade NDMA with rapid rates of biodegradation (Fournier et al., 2006, 2009; Sharp et al., 2005, 2007; Yoshinari and Shafer, 1990). Propane has previously been studied as an inducing substrate for the remediation of xenobiotics such as TCE (Wackett et al., 1989) and MTBE (Smith et al., 2003), and has demonstrated promise in field tests evaluating the enhanced remediation of these compounds (Steffan et al., 2000; Tovanabootr et al., 2001). Most reports on propanotroph isolates, which could be biased by ease of cultivability, tend to focus on Gram-positive, GC rich bacteria of the order Actinomycetales which include the *Corynebacterium*, *Nocardia*, *Mycobacterium*, and *Rhodococcus* genera (Ashraf et al., 1994).

In this report, we investigate two laboratory strains of the genus *Rhocococcus* and *Mycobacterium* that grow on propane and degrade NDMA (Sharp et al., 2005). A detailed kinetic analysis of NDMA degradation by *Rhodococcus sp.* RR1 and *Mycobacterium vaccae* (*austroafricanum*) JOB-5 is conducted to compare degradation rates and extent of NDMA degradation. Inhibition studies investigate the role of competition between propane and NDMA during cometabolism, while genomic and expression investigations are used to query for functional genes involved in NDMA degradation and propane metabolism.

Materials and Methods

Cellular Growth and Harvest

Unless noted, reagents and equipment were identical to those described in prior work (Sharp et al., 2005, 2007). The following bacteria were used: *Rhodococcus sp.* RR1 (GenBank # DQ889725); *Rhodococcus jostii* RHA1 (NCBI Taxonomy ID 101510); and *M. vaccae* JOB-5 (supplied by Daniel Arp and recently reclassified as *M. austroafricanum* JOB-5 ATCC 29678). Strains were grown in aerobic batch culture in sealed 250 or 1,000 mL glass flasks containing 30 or 150 mL of growth medium, respectively (150 rpm and 30°C). Culture growth was determined by measuring optical density at 600 nm (OD_{600}) using a Milton Roy Spectronic 20D+ visible light spectrophotometer. Basal salts medium (BSM) was amended with propane by injecting 30% (v/v headspace) of filter sterilized gas into the sealed liquid culture flasks; filter-sterilized pyruvate (20 mM) was added to BSM for comparative expression studies (Sharp et al., 2007). Tryptic soy broth (15 mg/L) was used to promote cellular growth while not inducing for a specific oxygenase enzyme.

Cells were harvested from culture medium late in the exponential phase of growth (OD_{600} ~0.6–2.0). Residual propane was removed by bubbling with nitrogen gas (300 mL min^{-1} for 2 min). A bacterial pellet was isolated by centrifugation at 15,000*g* for 5 min followed by suspension in 0.1 M phosphate buffer (pH 7). After two subsequent washing steps, cell density targeted optimal visualization of transformation rates (adjusted OD_{600} between 0.02 and 3.0). Subsamples were frozen for future protein analysis via the Pierce Coomassie Plus Protein Assay Kit (Rockford, IL). Cellular digestion was accomplished by NaOH addition with bead beating for 2 min followed by boiling for 15 min.

Biodegradation and Kinetic Assays

Experiments to evaluate the biodegradation kinetics of NDMA, including standards and controls, were conducted in 125 mL amber incubation bottles sealed with Teflon-lined Miniert valves (Altech, Deerfield, IL). Kinetic studies of propane degradation were conducted in 14 mL serum bottles capped with butyl rubber stoppers and aluminum crimp seals at neutral pressure (Wheaton Scientific, Millville, NJ). Aqueous phase concentrations of propane were calculated by using the dimensionless Henry's constant (air/water at 25°C) of $K_H = 26.6$ (Schwarzenbach et al., 1990). Where appropriate, a 10-min intermediate incubation with 5% (v/v headspace) acetylene gas was used for monooxygenase inactivation (Sharp et al., 2005).

Degradation rates were obtained by monitoring initial disappearance of propane or NDMA by bacterial assays and controls in phosphate buffer. Additional exogenous reductants were not added at this stage. Linear degradation curves contained 3–5 data points collected over the first 4 h of incubation when growth was negligible. Best fits to the Monod equation (Eq. 1), which relates the rate of removal to the concentration of the substrate at a constant cell density (Shuler and Kargi, 2002), were determined using nonlinear regression with 95% confidence intervals. The solution was generated using the iterative solver program embedded in Microsoft Excel where v_c is the reaction velocity (μg substrate (mg protein)$^{-1}$ h^{-1}) at substrate concentration C (μg substrate L^{-1}), v_{max} represents the maximum reaction velocity (μg substrate (mg protein)$^{-1}$ h^{-1}), and K_s the half-saturation constant (μg substrate L^{-1}). The resultant constants for propane and NDMA, respectively, are

$v_{max,p}$, $K_{s,p}$, $v_{max,n}$, and $K_{s,n}$.

$$v_c = \frac{v_{max} C}{K_s + C} \quad (1)$$

The inhibitory effect of propane on the NDMA degradation rate was quantified using Equation (2) with varying initial propane concentrations and a constant initial NDMA addition. A similar approach has been used to describe the effect of propane on MTBE degradation in strain JOB-5 (Smith et al., 2003).

$$\frac{v_{n,p}}{v_n} = \frac{C_p}{K_{inh} + C_p} \quad (2)$$

In Equation (2), $v_{n,p}$ is the reaction velocity (μg NDMA (mg protein)$^{-1}$ h-1) at aqueous propane concentration C_p (mg propane L^{-1}), v_n represents the reaction velocity in the absence of propane (μg NDMA (mg protein)$^{-1}$ h-1), and K_{inh} represents the propane concentration (mg propane L^{-1}) where NDMA degradation is slowed by 50%.

Analytical Methods

For experiments involving an initial NDMA concentration above 10 μg/L, extractions were performed by removing 2 mL of solution from incubations at each time point of interest. Samples and standards were extracted into methylene chloride as described previously (Sharp et al., 2005). Analysis of lower NDMA concentrations involved an adaptation of a batch solid phase extraction (Choi and Valentine, 2003; Mitch and Sedlak, 2002) where 100 mL subsamples were exposed to 0.01% sodium azide. The addition of 4 μL of a 2.5 ng/μL d_6-NDMA methanol stock (Cambridge Isotope Laboratories; Andover, MA) provided an internal standard for correction of extraction recoveries. Subsequently, 200 mg of Ambersorb 572 resin was equilibrated with active mixing for 3 h, separated by filtration, and then dried overnight. 550 μL of methylene chloride was added to the dried resin and the sample was mixed briefly. Following an equilibration of 30 min, 250 μL of the methylene chloride was removed for analysis by tandem mass spectroscopy.

Methylene chloride extracts containing NDMA and the deuterated standard were analyzed using previously described GC MS/MS methods (Mitch and Sedlak, 2002; Sharp et al., 2005). The detection limit for the liquid–liquid extraction was ~5 μg/L as determined by the standard curve (44 and 47 daughter ions). The limit of detection (LOD) for the resin/dichloromethane technique was 20 ng/L. This LOD was determined from replicate NDMA-free blanks and was calculated as the mean plus three standard deviation units (Anderson, 1989; Skoog and West, 1980).

Headspace measurements were analyzed by withdrawing 200 μL headspace samples from sealed bottles with a Hamilton gas-tight syringe (Reno, NV). The gas was then injected (250°C) into a Hewlett-Packard 5890 gas chromatograph with flame ionization detector (300°C) and a GS-GASPRO capillary column (30 m long with 0.32 mm I.D., Agilent JW Scientific, Santa Clara, CA). Gas chromatography temperature conditions were as follows: 35°C (hold time 1 min) ramping at 30°C/min to 180°C. The retention times for acetylene, propane, and acetaldehyde were 1.4, 1.6, and 5.8 min, respectively.

Degenerate Primer Design

In order to assess the presence of propane monooxygenase (PrMO) enzymes in bacterial strains, degenerate primers were designed to target homologues of the large (alpha) subunit gene, *prmA* based on the amino acid sequences of proteins from *Gordonia* TY-5, *Mycobacterium* TY-6, *Pseudonocardia* TY-7, *Mycobacterium smegmatis* MC2155, *R. jostii* RHA1, and *Methylibium petroleiphilum* PM1 (respective NCBI accession: BAD03956, BAF34294, BAF34308, ABK75704, ABG92277, and YP_001020147). The CODEHOP (Consensus Degenerate Hybride Oligonculeotide Primers) program was used (Rose et al., 1998) with the input of a multiple sequence alignment (MSA) of the six amino acid sequences produced from CLUSTAL X Version 2.0 (Larkin et al., 2007), to generate a pair of *prmA* degenerate primers, deg.prmA.F and deg.prmA.R (Table I). Amplification of degenerate primer

Table I. Molecular primers used for gene amplification and comparative expression.

Purpose	Primer	Sequence	Source
Degenerate primer (target: *prmA*)	deg.prmA.F	GCTCCTACTTCCCGATGGArsargaraarg	This study
	deg.prmA.R	GCTGGGCGATCAGGgtyttnccrtc	This study
Degenerate primer (target: *alkB*)	Rhose2	ACGGSCCAYTTCTACRTCG	Lopes Ferreira et al. (2007)
	Roas1	CCGTARTGYTCGAGRTAG	Lopes Ferreira et al. (2007)
qPCR	qPCR.RR1.alkB.F	TGCGTAACGACGTCCTCAAC	This study
	qPCR.RR1.alkB.R	AGGATCAGGAACGGGATGATC	This study
qPCR	qPCR.RR1.prmA.F	AACCTCAAGAAGCTCTACATGAACAA	This study
	qPCR.RR1.prmA.R	GAAGCCCTCCCCGAACTG	This study
qPCR	qPCR.JOB5.alkB1.F	TCGCCACACCCGAGGAT	This study
	qPCR.JOB5.alkB1.R	AACTGGAACGTGTAAATGCTCTCA	This study
qPCR	qPCR.JOB5.alkB2.F	TGCTCTACGGCGCATTGA	This study
	qPCR.JOB5.alkB2.R	GAGATCAGGATGTACGGGATCAG	This study

polymerase chain reaction (PCR) products from RHA1 genomic DNA (gDNA) was visualized on 1% agarose electrosphoresis gels and on HP Agilent's Bioanalyzer (Santa Clara, CA).

Alkane monooxygenase (*alkB*) degenerate primers, Rhose2 and Rhoas1 (Table I), were developed in a previous study (Lopes Ferreira et al., 2007).

Gene Expression and Sequence Analysis

Analysis of gene expression was determined by quantitative reverse transcriptase PCR (qRT-PCR). Real-time PCR primers for the *prmA* and *alkB* genes (Table I) were designed according to the sequence of the gene fragment produced by the degenerate primers using PerlPrimer (Marshall, 2004). Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) was used for quantification of genes and of complimentary DNA (cDNA) produced from transcripts. Differential expression ratios were calculated based on a standard curve method where cDNA copy numbers were normalized to gDNA copy numbers for each condition (Pfaffl, 2001).

Total RNA for analysis of gene expression was extracted and purified from RR1 and JOB-5 under propane- and pyruvate-growth conditions, in quadruplicate, using Qiagen's RNeasy Mini Kit (Valencia, CA). Each RNA sample was treated with Ambion's DNA-free Kit (Applied Biosystems) to remove any contaminating gDNA, until the C_T value was >35. This RNA was used to synthesize cDNA using Roche's Reverse Transcriptase (Mannheim, Germany). gDNA was purified using MO-BIO's UltraClean Microbial DNA Isolation Kit (Carlsbad, CA).

DNA sequencing was carried out at the University of California Berkeley DNA Sequencing Facility using an Applied Biosystems 96 capillary 3730xI DNA Analyzer. PCR amplification products from the degenerate primers were ligated into *Sma*I digested and phosporylated pUC19 linearized plasmids (Fermentas, Burlington, ON, Canada) and cloned into electrocompetent *Escherichia coli* cells (New England Biolabs, Ipswich, MA) for sequencing.

Results

Kinetics of NDMA Removal

Propane-grown cells of strain RR1 and JOB-5 were independently harvested, washed, and suspended in phosphate buffer. The cleaned cells were then exposed to varying concentrations of NDMA to assess biodegradation rates. Abiotic and azide-killed controls revealed no measurable loss of NDMA. Substrate toxicity as determined by agreement with Monod $\nu_{max,n}$ was not observed for either strain RR1 or JOB-5 during the transformation of up to 10,000 μg/L NDMA.

Resulting Monod constants (Eq. 1) associated with NDMA degradation for the two bacteria as well as prior literature values for a related *Rhodococcus* species are listed in Table II. Model fits employed no fewer than 10 rates measured over a range of kinetically relevant concentrations. Best-fit analysis revealed a threefold difference in maximum transformation rates ($\nu_{max,n}$) between studied strains ranging from 15 to 49 μg NDMA (mg protein)$^{-1}$ h^{-1}, while the half saturation constants ($K_{s,n}$) for the two *Rhodococci* were statistically similar with an average value of 28 μg NDMA L^{-1}. Propane grown cells of JOB-5 exhibited a two-orders of magnitude higher half-saturation constant for NDMA ($2{,}200 \pm 1{,}000$ μg NDMA L^{-1}).

Although RR1 can constitutively degrade NDMA after growth on the complex medium soy broth, the maximum transformation rate of 0.14 ± 0.01 μg NDMA (mg protein)$^{-1}$ h^{-1} attained by these cells was <1% of the rate achieved by the same cells when grown on propane. However, a calculated half saturation constant of 45 ± 10 μg NDMA L^{-1} for NDMA degradation by soy broth grown cells overlaps with the $K_{s,n}$ derived for propane grown cells (Table II). In contrast, no measurable NDMA degradation occurred when JOB-5 was grown on soy broth (Sharp et al., 2005).

Biodegradation of Trace Quantities of NDMA

The potential to degrade trace quantities of NDMA with lower concentrations of cells was assessed. Propane-grown

Table II. Monod parameters associated with NDMA and propane biodegradation by propane-grown cells.

Species	Substrate	$\nu_{max,s}$ [μg substrate (mg protein)$^{-1}$ h^{-1}]	K_s [μg substrate L^{-1}]
Rhodococcus sp. RR1	NDMA	44 ± 5	36 ± 10
	Propane	190 ± 65	$6{,}200 \pm 400$
Rhodococcus jostii RHA1[a]	NDMA	18 ± 3	20 ± 17
	Propane	NA	NA
Mycobacterium vaccae JOB-5	NDMA	28 ± 3	$2{,}200 \pm 1{,}000$
	Propane	33 ± 4	790 ± 400

NA, not available.
Monod constants were calculated by fitting data to Equation (1) using nonlinear regression with 95% confidence intervals.
The concentration of propane in the dissolved solution was adjusted using Henry's constant to account for liquid phase partitioning.
[a]Monod constants for NDMA degradation by RHA1 are from Sharp et al. (2007).

RR1 cells (2 mg protein L^{-1}) removed 100 ng NDMA L^{-1} to below the LOD (20 ng/L). An approximate rate of 0.2 μg NDMA (mg protein)$^{-1}$ h^{-1} was calculated for this removal, which closely matches the predicted rate of 0.14 μg NDMA (mg protein)$^{-1}$ h^{-1} derived from the calculated Monod kinetic constants (Table II). Complete NDMA removal was also observed for strain RR1 after growth on soy broth (110 mg protein L^{-1}). In contrast, cells deactivated with 0.01% sodium azide exhibited minimal NDMA removal (92 ± 7 ng NDMA L^{-1} remaining). Similarly, propane-grown JOB-5 (17 mg protein L^{-1}) degraded 150 ng NDMA L^{-1} to below detection limits. Since acetylene has been shown to be an effective inhibitor of monooxygenase activity in JOB-5, in contrast with strain RR1 (Sharp et al., 2005), it was used as an enzymatic inhibitor for NDMA-oxidizing activity of JOB-5. This control exhibited no significant degradation (140 ± 17 ng NDMA L^{-1}).

Competition Between Propane and NDMA

In order to investigate whether the propane-induced enzyme involved in NDMA degradation is inhibited by the presence of propane, the kinetics of propane degradation by these bacterial isolates was first characterized. Specifically, propane degradation rates by propane-grown cells of RR1 and JOB-5 that were degassed and washed to remove residual propane and subsequently exposed to defined concentrations of propane were quantified and fit to Equation (1) (Table II). Significant differences in both $K_{s,p}$ and $v_{max,p}$ values between the two strains were apparent. In particular, RR1 exhibited kinetic constants that were an order of magnitude higher than those for JOB-5.

Next, the effects of propane on NDMA degradation rates were measured over a range of propane concentrations. Although propane inhibited NDMA degradation (100–500 μg NDMA L^{-1}) in proportion to concentration (0–20,000 μg propane L^{-1}) for both strains, graphical interpretation using the double-reciprocal (Lineweaver–Burke) approach revealed complex inhibition kinetics that do not correspond with classic enzymatic models (data not shown). Alternatively, an approach that has previously been applied to quantify the inhibition of propane on MTBE degradation in JOB-5 (Smith et al., 2003) yielded a quantifiable comparison between the inhibition patterns of the two strains. When the NDMA degradation rate in the presence of a given propane concentration ($v_{n,p}$) is normalized by the NDMA degradation rate in the absence of propane (v_n), an inhibitory constant can be calculated (Eq. 2). As shown in Figure 1, the degradation of 200 μg NDMA L^{-1} by strain RR1 was inhibited by 50% (K_{inh}) at 7,700 μg propane L^{-1} ($R^2 = 0.9669$) while JOB-5 required approximately 1/30th of that value (120 μg propane L^{-1}; $R^2 = 0.9895$) for 50% inhibition. Consistent with these values, the presence of 1,400 μg propane L^{-1}, or 5% (v/v) in the headspace, had little effect on NDMA degradation by RR1 while it exerted an ~80% decrease for JOB-5.

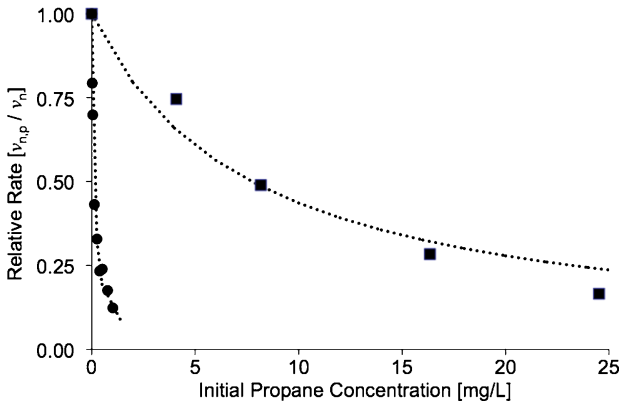

Figure 1. Inhibitory effect of propane on NDMA degradation. Initial relative rates for the degradation of 200 μg/L NDMA was normalized as degradation rate ($v_{n,p}$) in the presence of a given propane concentration divided by the rate (v_n) in the absence of propane. The concentration of propane in the dissolved solution was adjusted by the Henry's constant to account for liquid phase partitioning. ■ = relative rate of NDMA degradation for strain *Rhodococcus sp.* RR1; ● = relative rate of NDMA degradation for strain *Mycobacterium vaccae* JOB-5. Data were fit as dashed lines to Equation (2) using nonlinear regression.

Presence of Propane Monooxygenase

At the time of this study, only six amino acid sequences existed in the NCBI Entrez Protein database (see Materials and Methods Section) with the annotation of PrMO large (alpha) subunit, *prmA*. These sequences were used to develop the *prmA* degenerate primer set, deg.prmA.F and deg.prmA.R (Table I). Based upon the nucleotide sequence of the *prmA* gene in *R. jostii* RHA1 (accession NC_008268), these primers were calculated to produce a PCR amplification product of 1,333 bp or 90.1% of the total gene length. The efficacy of the PCR primers was verified by producing a 1,333 bp fragment derived from RHA1 gDNA that was found to be identical to the *prmA* gene deposited in NCBI GenBank for this strain.

These *prmA* degenerate primers were then applied to gDNA derived from strain RR1 and JOB-5. Of these two propanotrophs, only RR1 produced a PrMO homologue with the primers (Fig. 2). The nucleotide sequence of the partial *prmA* gene fragment from RR1 (1,318 bp) was found to be 91% identical to the *prmA* fragment of RHA1, while the corresponding amino acid sequences were 96% identical (NCBI's blastx-2 sequences tool). The partial nucleotide sequence for the RR1 *prmA* gene has been deposited as GenBank accession HM209445.

Presence of Alkane Monooxygenases

Previously developed *alkB* degenerate primers (Table I) were used to test for the presence of an alkane monoxygenase (AlkMO) in both RR1 and JOB-5. PCR amplification using the *alkB* degenerate primers produced an expected 343 bp fragment from both strains; sequence analyses revealed that RR1 possessed one *alkB* homologue while JOB-5 contained

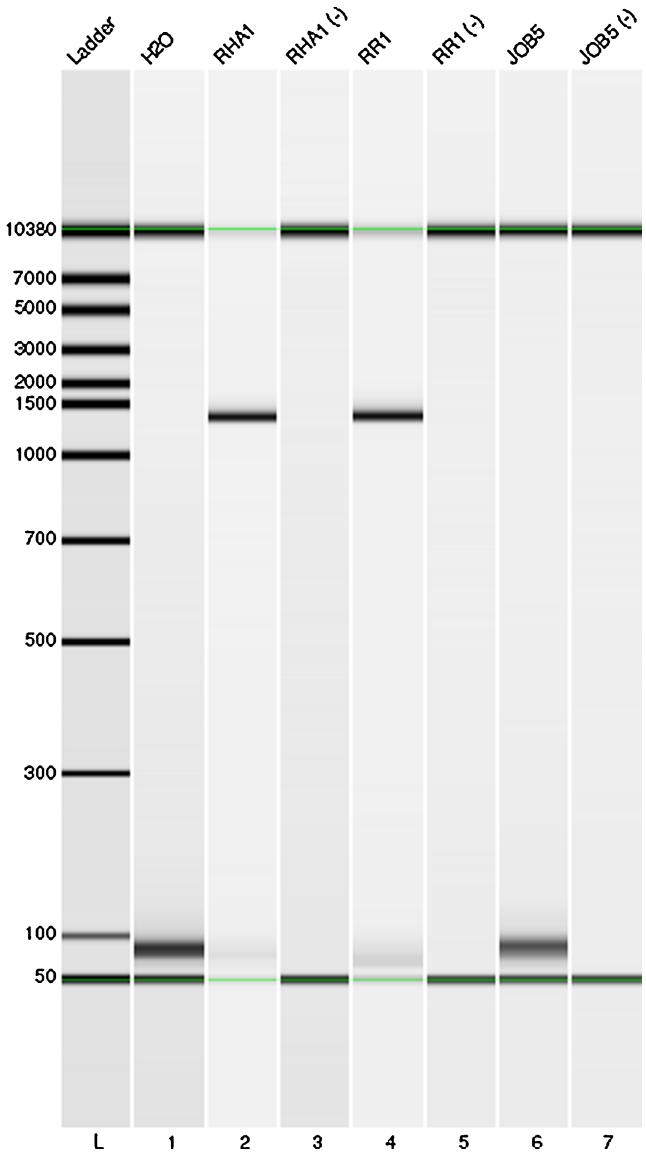

Figure 2. Identification of propane monooxygenase genes. PCR products of primer set deg.prmA.F and deg.prmA.R on genomic DNA from *R. jostii* RHA1 "RHA1," *Rhodococcus* RR1 "RR1," and *Mycobacterium vaccae* JOB5 "JOB5." Negative control wells "RHA1 (−)," "RR1 (−)," and "JOB5 (−)" contain template genomic DNA without the degenerate primers. "H_2O" lane is a control containing no template DNA. Samples were run on Agilent Technologies 2100 Bioanalyzer using DNA 7500 Assay Kit and imaged through Biosizing Software. Numbers to the left of the "Ladder" represent nucleotide length.

at least two distinct copies of an *alkB* gene, which share ~60% nucleotide identity.

Monooxygenase Expression in Pyruvate- Versus Propane-Grown Cells

Analysis by RT-qPCR confirmed that the PrMO gene in RR1 was induced in the presence of propane. The mRNA levels of *prmA* were 70-fold higher (P-value = 0.00055) when grown on propane versus pyruvate as the sole carbon source (Fig. 3). In contrast, *alkB* transcription levels were not significantly higher after growth on propane (<5-fold). Similarly, analysis of expression of *alkB* genes found in JOB-5 indicated that neither was differentially up-regulated (<5-fold).

Discussion

This study characterizes kinetic parameters associated with the cometabolic degradation of NDMA by two different strains of bacteria capable of growth on propane. It also investigates differences in candidate enzymes between the strains to help interpret kinetic variability. The *Rhodococcus* and *Mycobacterium* tested in this study are common soil bacteria of the order *Actinomycetales* that have previously been shown to metabolize propane (Ashraf et al., 1994 and references within). Increases in xenobiotic degradation rates after growth on propane suggests the involvement of a propane monooxygenase (Fournier et al., 2006, 2009; Sharp et al., 2005, 2007; Smith et al., 2003); however, our results demonstrate that the propane-induced monooxygenase differs significantly between the strains with one found to lack homology to other annotated PrMOs.

Kinetic Analysis

The potential for NDMA transformation following growth on complex, common organics is supported by the observations of others (Gunnison et al., 2000; Sedlak et al., 2005; Yang et al., 2005). However, orders of magnitude differences in transformation rates between constitutive and promoted conditions reported here and in prior studies (Fournier et al., 2009; Sharp et al., 2007), suggest that the addition of propane as an inducing substrate could substantially enhance the bioremediation of NDMA in contaminated soils. Furthermore,

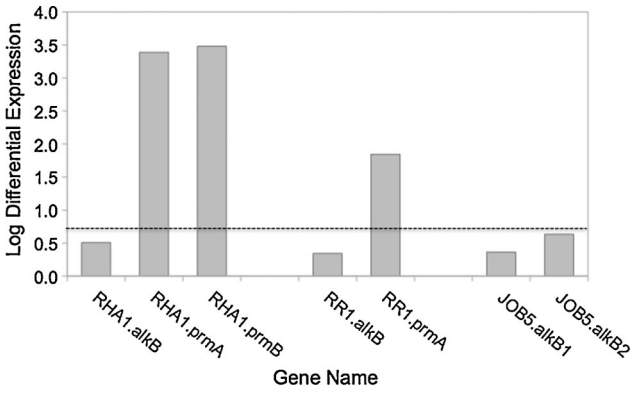

Figure 3. Difference in expression for candidate propane oxidizing genes when contrasting propane and pyruvate grown cultures. The dashed line represents a fivefold differential expression threshold. Statistical P-values from left to right: 0.00640, 1.63E-9, 5.76E-14; 0.00040, 0.00055; 0.00040, 0.00005. Expression data for *R. jostii* RHA1 is from Sharp et al. (2007).

measurable constitutive activity is not a characteristic of all propanotrophs, suggesting that propane amendment could be necessary to achieve effective biodegradation rates.

A comparison of half-saturation constants associated with NDMA degradation by axenic cultures indicates considerable variability between species (Table II). Although the maximum rate constants for RR1 and JOB-5 differs by <50%, RR1 exhibited dramatically higher enzymatic affinity for NDMA as reflected by $K_{s,n}$ (36 μg/L vs. 2,200 μg/L). Kinetic variables derived for strain RR1 after growth on propane were similar to those reported for *R. jostii* RHA1 with a statistically identical $K_{s,n}$ (Table II). When characterizing NDMA transformation by the methanotroph *M. trichosporium* OB3b, Yoshinari and Shafer (1990) generated a rate-to-concentration ratio that was constant from 2,200 to 740,000 μg/L and corresponded to linear increases in rates from 0.02 to 7 μg/mg/h. While they were unable to generate specific maximum rate constants, this result corresponds to a $K_{s,n}$ above 740,000 μg/L for that species, which is approximately 400- and 20,000-times the half saturation value for JOB-5 and RR1, respectively. Thus, propanotrophs may have a significantly higher affinity for NDMA than methanotrophs. Given that groundwater contamination levels are commonly <1 μg/L NDMA (OCWD, 2000; Sedlak et al., 2005; WateReuse, 2006), it is plausible that strains with a lower $K_{s,n}$, such as these propanotrophs, will play a more prominent role in environmental NDMA degradation. Collectively, the orders of magnitude variability in Monod constants exhibited by these three bacteria indicates that NDMA degradation kinetics in environmental communities will be a function of bacterial composition and induction conditions.

Impact of NDMA Concentration on Biodegradation

Advisory levels of ~10 ng NDMA L^{-1} (CalDHS, 2002; MOE, 2003) are nearly a thousand-fold lower than regulations set for more traditional water contaminants targeted for bioremediation. Therefore, an understanding of NDMA biodegradation at concentrations in the nanograms-per-liter range is crucial for predicting the fate of environmentally relevant concentrations and their potential for attenuation. In this study, both of the tested propanotrophs demonstrated the ability to transform parts-per-trillion levels of NDMA to concentrations below the experimental level of detection. Others have also reported that biological processes can remove NDMA at similar concentrations in pure culture (Fournier et al., 2009) and wastewater treatment systems (Sedlak et al., 2005), suggesting that different organisms show promise for participation in bioremediation strategies that approach the low NDMA threshold for acceptable water quality.

At the high end of the concentration spectrum, the presence of concentrations up to 10 mg NDMA L^{-1} appears to have little toxic effect on the tested strains. Since NDMA is rarely found at or above this concentration in contaminated environments (CalDHS, 2002), it seems that substrate toxicity will not play a prominent role in inhibiting NDMA biodegradation in the field. Similar effects have been observed by others where toxic effects on NDMA degradation rates were not observed for concentrations as high as 740 mg NDMA L^{-1} with a methanotroph (Yoshinari and Shafer, 1990) and for concentrations as high as 10,000 mg NDMA L^{-1} with undefined consortia (Kaplan and Kaplan, 1985).

Inhibition of NDMA Degradation

The inhibition of NDMA degradation by JOB-5 after exposure to acetylene is consistent with mechanistic inactivation of certain monooxygenases. This phenomenon has been documented for enzymes including soluble methane and toluene monooxygenases but is not absolute for all monooxygenases relevant to bioremediation applications (Prior and Dalton, 1985; Sharp et al., 2005; Smith et al., 2003). In this study, the propane-induced PrMO in RR1 is not irreversibly inhibited by acetylene. Differences in prosthetic groups and active hydroxylase units associated with short-chain alkane monooxygenases have been cited as potential causes for the different inhibition patterns (Ortiz de Montellano and Reich, 1986; Shanklin et al., 1997). The presence of a propane-induced PrMO in RR1 and the absence of an identifiable PrMO in JOB-5 (Figs. 2 and 3) may explain the differences in inhibition characteristics.

It appears that propane, which serves as an enzyme inducer in both of the studied strains, competes with NDMA for the active enzyme (Fig. 1). The K_{inh} values, which mark a propane concentration that results in 50% inhibition of the NDMA degradation rate, are the same order of magnitude as the respective $K_{s,p}$ values for both strains (Table II). This suggests that the same enzyme is involved in both processes. The K_{inh} value for JOB-5 observed in this study is similar to one previously reported for propane inhibition (244–325 μg propane L^{-1}) of MTBE degradation by JOB-5 (Smith et al., 2003). While our results did not identify the responsible enzyme in JOB-5, they demonstrate that AlkMOs in JOB-5 are not involved in either NDMA transformation or propane metabolism. The presence of distinct AlkMO's in strain JOB-5 is supported by functional assays that implicate two separate AlkMO's during growth on C_5–C_{14} *n*-alkanes (House and Hyman, 2010).

An interspecies comparison of $K_{s,n}$ and $K_{s,p}$ values indicates pronounced differences in the level of inhibition between these two strains (Table II). Specifically, while the kinetic and affinity constants exhibited by JOB-5 were similar for both propane and NDMA, RR1 exhibited a strong enzymatic preference for NDMA over propane (i.e., $K_{s,n} \ll K_{s,p}$) despite the requirement for induction by propane. This enzymatic preference for NDMA is consistent with the finding that additions of 5% (v/v) propane exerted no significant inhibition on NDMA degradation by RR1 while significantly inhibiting strain JOB-5. These results suggest that the extent of inhibition will be a function of the

characteristics of the active organisms, and specifically the enzymes involved.

Gene Presence and Expression

The promotion of *prmA* gene expression in *Rhodococcus sp.* RR1 after growth on propane is consistent with the behavior observed for *R. jostii* RHA1 (Sharp et al., 2007), except that differential expression of propane-induced *prmA* in RR1 was only 70-fold verses >2,000-fold for RHA1 (Fig. 3). This discrepancy may be explained both by biological variability and by differences in transcriptional measurement methods involving both primers/probe combinations (SYBR-green vs. Taqman) and reference normalization methods (DNA copy number vs. housekeeping gene) between the study of RR1 and RHA1. Through a series of transcriptional, biodegradation, and gene knockout studies, the RHA1 report conclusively demonstrated that the PrMO was involved in both NDMA and propane degradation and that PrMO was constitutively expressed (Sharp et al., 2007). Similarities in phylogeny, PrMO composition, and kinetic behavior suggest highly homologous enzymes are involved in NDMA and propane degradation in both *Rhodococci*.

Environmental Implications

This report presents pertinent kinetic constants associated with the biodegradation of NDMA, and it collectively expands upon findings that rapid and nearly complete NDMA degradation can be achieved by different propanotrophs even at nanogram-per-liter concentrations (Fournier et al., 2009; Sharp et al., 2007). Different characteristics associated with competition between propane and NDMA in these species is presumably due to different enzymes. Our findings suggest that even after functional biostimulation (i.e., propane amendment), the type of propane-induced enzymes present in the microbial community could dictate the effectiveness of a remediation strategy. To this end, the degenerate primers designed in this study could be used as biomarkers to assess the presence and expression of PrMOs in environmental systems.

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