

Aerobic Biodegradation of N-Nitrosodimethylamine (NDMA) by Axenic Bacterial Strains

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Abstract: The water contaminant N-nitrosodimethylamine (NDMA) is a probable human carcinogen whose appearance in the environment is related to the release of rocket fuel and to chlorine-based disinfection of water and wastewater. Although this compound has been shown to be biodegradable, there is minimal information about the organisms capable of this degradation, and little is understood of the mechanisms or biochemistry involved. This study shows that bacteria expressing monooxygenase enzymes functionally similar to those demonstrated to degrade NDMA in eukaryotes have the capability to degrade NDMA. Specifically, induction of the soluble methane monooxygenase (sMMO) expressed by *Methylosinus trichosporium* OB3b, the propane monooxygenase (PMO) enzyme of *Mycobacterium vaccae* JOB-5, and the toluene 4-monooxygenases found in *Ralstonia pickettii* PKO1 and *Pseudomonas mendocina* KR1 resulted in NDMA degradation by these strains. In each of these cases, brief exposure to acetylene gas, a suicide substrate for certain monooxygenases, inhibited the degradation of NDMA. Further, *Escherichia coli* TG1/pBS(Kan) containing recombinant plasmids derived from the toluene monooxygenases found in strains PKO1 and KR1 mimicked the behavior of the parent strains. In contrast, *M. trichosporium* OB3b expressing the particulate form of MMO, *Burkholderia cepacia* G4 expressing the toluene 2-monooxygenase, and *Pseudomonas putida* mt-2 expressing the toluene sidechain monooxygenase were not capable of NDMA degradation. In addition, bacteria expressing aromatic dioxygenases were not capable of NDMA degradation. Finally, *Rhodococcus* sp. RR1 exhibited the ability to degrade NDMA by an unidentified, constitutively expressed enzyme that, unlike the confirmed monooxygenases, was not inhibited by acetylene exposure. © 2005 Wiley Periodicals, Inc.

Keywords: n-nitrosodimethylamine; NDMA; monooxygenase; biodegradation

INTRODUCTION

N-nitrosodimethylamine (NDMA) is considered a probable human carcinogen (IARC, 1987). This compound is regulated in United States waters with an Environment Protection Agency cleanup level of 0.7 ng/L and a interim California action level of 10 ng/L (Cal DHS, 2002; US EPA, 2001). Its presence in the environment has been linked to aerospace facilities through the decomposition of hydrazine-based rocket fuels (Cal DHS, 2002; MacDonald, 2002) and more generally to the discharge of water and wastewater disinfected with chlorine (Cal DHS, 2002; Mitch et al., 2003; Njam and Trussell, 2001; OCWD, 2000). In the latter case, it appears that secondary amines react with chloramine to form a hydrazine intermediate that is in turn oxidized to NDMA (Mitch and Sedlak, 2002). NDMA is highly water-soluble (Chemfinder, 2003), and although photolysis, and to a lesser extent volatilization (Henry's constant of 2.6×10^{-4} atm M⁻¹ at 20°C) (ATSDR, 1989; Mirvish et al., 1976), contribute to NDMA disappearance from surface waters, these processes are expected to have little mitigating effect in subsurface waters. Furthermore, NDMA does not significantly sorb to activated carbon or soils, regardless of organic carbon, humic acid, pH, clay, or cation exchange capacity (ATSDR, 1989; Dean-Raymond and Alexander, 1976; Gunnison et al., 2000; Kaplan and Kaplan, 1985; Oliver, 1979). It is therefore highly mobile in the subsurface. Its persistence in groundwater aquifers has been responsible for the closure of municipal drinking water wells and its listing as a priority pollutant (Cal DHS, 2002; Mitch et al., 2003; OCWD, 2000). Concern over this contaminant is likely to increase, particularly in water-limited regions where indirect and direct wastewater reuse is becoming more prevalent.

Studies conducted with undefined aerobic microbial consortia have demonstrated that NDMA is biodegradable in laboratory incubations (Gunnison et al., 2000; Kaplan and

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Kaplan, 1985; Tate and Alexander, 1975). In addition, biological activity was implicated for the disappearance of NDMA from groundwater at a field site in Colorado (Gunnison et al., 2000). It has been observed (Kaplan and Kaplan, 1985) that an activated carbon trickling filter acted as a growth matrix for NDMA-degrading organisms, while a sterilized column did not remove NDMA. Attempts to isolate NDMA-degrading bacterial strains from these environmental samples have failed. Previous studies conducted with axenic cultures to evaluate NDMA degradation have been limited to anaerobic experiments with bacteria common to the human gastrointestinal tract (Rowland and Grasso, 1975) and to aerobic experiments conducted with a methanotroph, *Methylosinus trichosporium* OB3b (Yoshinari and Shafer, 1990). Interestingly, both of these studies utilized ¹⁴C-labeled NDMA to measure biodegradation, and both reported less than 4% transformation of the added NDMA. Further, the studied NDMA concentrations were well in excess of those found in the environment and evidence for the involvement of specific enzymes in the degradation of NDMA was not presented.

Studies investigating the transformation of NDMA in higher organisms are more abundant. The microsomal fraction of tulip bulbs (Stiborova et al., 2000), spinach, and lettuce (Dean-Raymond and Alexander, 1976) can all metabolize NDMA. In addition, mammals have the ability to oxidize NDMA (Kroeger-Koepke et al., 1981; Tu and Yang, 1985). In both flora and fauna, it has been concluded that a cytochrome P-450 enzyme catalyzes the NADPH-dependent oxidation of NDMA. Since eukaryotic cytochrome P-450s are monooxygenase enzymes, it is reasonable to expect that functionally analogous enzymes could be responsible for NDMA metabolism in prokaryotes. Bacterial monooxygenases split molecular oxygen into two atoms, binding one onto an electron donor that is typically the inducer of the enzyme while consuming reducing equivalents in the form of NADH (Chang and Alvarez-Cohen, 1995). Interestingly, bacterial oxygenases that are active on donors such as methane, propane, ammonia, and toluene have also been shown to have the ability to oxidize xenobiotic compounds such as chlorinated aliphatic hydrocarbons (Arciero et al., 1989; Nelson et al., 1987; Tsien et al., 1989; Wackett et al., 1989) and MTBE (Deeb et al., 2001; Smith et al., 2003). In the case of trichloroethene, this oxidation reaction can be catalyzed by both monooxygenases (Leahy et al., 1996; Shields et al., 1989) and dioxygenases (Leahy et al., 1996; Nelson et al., 1988; Wackett and Gibson, 1988; Zylstra et al., 1989). The relaxed regiospecificity found in enzymes that are functionally analogous to those determined to degrade NDMA in higher organisms suggests that bacterial enzymes may have the capability to degrade NDMA.

The objective of this study was to induce expression of monooxygenase and dioxygenase enzymes in a variety of environmentally relevant strains in order to test the hypothesis that monooxygenase-expressing bacteria have the capability to degrade NDMA.

MATERIALS AND METHODS

Chemicals

N-nitrosodimethylamine (99+%) was purchased from Acros Organics (Geel, Belgium) and toluene (99.9%) was purchased from JT Baker (Phillipsburg, NJ). Sodium formate (99.2%), sodium azide, casamino acids, media salts, and agarose were purchased from Fisher Scientific (Fair Lawn, NJ). Becton Dickinson (Sparks, MD) Bacto LB agar was used for culture maintenance and to verify strain purity. DL-Lactic Acid, tetrazotized o-dianisidine, and tryptic soy broth were purchased from Sigma Chemical (St. Louis, MO). EM Science (Darmstadt, Germany) high-purity methylene chloride was used for liquid-liquid extraction samples. Naphthalene (99%) was purchased from Aldrich Chemical (Milwaukee, WI). Certified analyzed gases were used without further purification. Propane (99.5%) was purchased from Matheson (Twinsburg, OH), acetylene from Matheson (Newark, CA), and methane from Quadren Cryogenic (Robbins, CA). Deionized water, produced from a Barnstead Nanopure II water purifying system, was used for preparation of stock solutions, buffer, and medium.

Bacteria

Methylosinus trichosporium OB3b was purchased from the American type culture collection (ATCC #35070). Strain *Mycobacterium vaccae* JOB-5 was kindly supplied by Professor Daniel Arp at Oregon State University. The toluene-oxidizers *Pseudomonas mendocina* KR1, *Ralstonia pickettii* PKO1, *Burkholderia cepacia* G4, *Pseudomonas putida* mt-2, *Pseudomonas sp.* JS150, *Pseudomonas putida* F1, *Pseudomonas fluorescens* CFS215, and *Pseudomonas sp.* W31 were generously supplied by Professor Jerry Kukor at Rutgers University. *Rhodococcus sp.* RR1 was previously isolated from gasoline-contaminated soils (Deeb and Alvarez-Cohen, 1999). *Escherichia coli* pCR 2.1-TOPO was purchased from Invitrogen (Carlsbad, CA). Strains *E. coli* TG1/pBS(Kan)T4MO, *E. coli* TG1/pBS(Kan)T3MO, and *E. coli* TG1/pBS(Kan)TOM were designed to constitutively express toluene monooxygenases from strains KR1, PKO1, and G4, respectively (Tao et al., 2004). These recombinant strains will be referred to as TG1(T4MO), TG1(TpMO), and TG1(T2MO).

Culture Conditions

The variety of bacterial strains used in this study necessitated culture-specific modifications of growth and maintenance conditions; however, the conditions were kept as similar as possible to facilitate interspecies comparisons. The wildtype strains used in this study were grown on minimal basal salts medium (Deeb et al., 2002) containing 11.76 mM NaNO₃, 0.98 mM K₂SO₄, 0.15 mM MgSO₄*

7H₂O, 3.9 mM KH₂PO₄, 6.1 mM Na₂HPO₄, 0.05 mM CaSO₄*2H₂O, 0.08 mM FeSO₄*7H₂O, 0.1 mM H₂SO₄, 0.001 mM KI, 0.002 mM ZnSO₄*7H₂O, 0.002 mM MnSO₄*H₂O, 0.002 mM H₃BO₂, 0.004 mM CoMoO₄*H₂O and referred to hereafter as BSM. The copper fraction was excluded from this recipe when growing strain OB3b to induce the soluble (sMMO) rather than the particulate (pMMO) methane monooxygenase (Stanley et al., 1983). BSM used to grow strains PKO1 and JS150 was amended with 0.1% (wt/vol) casamino acids to enhance cellular growth without interfering with oxygenase expression (Kukor and Olsen, 1996). For toluene induction, toluene was supplied in the vapor phase by adding 30 µL toluene to a secondary container within the growth flask. Propane and methane were supplied by injecting 30% (vol/vol) of the gas into the sealed liquid culture flasks. Tryptic soy broth was used as a rich medium for experiments designed to promote cellular growth while not inducing a specific oxygenase enzyme. The *E. coli* strains containing the plasmids were grown in LB (Luria Bertani: Becton Dickinson) medium supplemented with kanamycin at 100 mg/L. These cells were given 1 mM IPTG ~2 h before harvesting the cells. All cells were incubated aerobically while shaking at 150 rpm and 30°C. Standard microbial aseptic techniques were used throughout. Cells were incubated in sealed 500 mL or 1000 mL clear flasks containing 100 or 200 mL BSM, respectively, and were harvested in the late exponential phase (OD₆₀₀ ~0.5–0.9) of growth while the cells containing the plasmids were harvested at OD₆₀₀ ~1.2 in accordance with methods described in Tao et al. (2004).

Between experiments, cells were maintained on solid medium consisting of either LB or BSM amended with 2% agar. *E. coli* plasmid strains were supplemented with 100 mg/L kanamycin to provide selective pressure to maintain the plasmid-containing population and discourage contamination. For the BSM agar, toluene was supplied in the vapor phase by saturating a piece of filter paper with 150 µL of toluene and sealing both the plate and filter paper in a large desiccator. Alternatively, propane was injected (30% vol/vol) into a vial containing BSM agar and cells. To ensure cellular purity, experiments were begun either from a single, large colony or from liquid medium containing an uncontaminated strain. Samples of liquid suspension were streaked onto LB plates to verify colony purity at the onset of experiments. Most results were performed in duplicate and all were independently repeated from the original cultures to ensure reproducibility.

Transformation of NDMA by Microorganisms

At the beginning of each experiment, cells were harvested by centrifugation at 15,000g for 5 min followed by suspension of the cellular pellet in fresh BSM. This process was repeated a total of three times to ensure that the experimental medium was devoid of unintended substrates. The concentration of the resultant suspension was adjusted

with BSM to a strain-specific target density (OD₆₀₀ between 0.7 and 1.8).

In studies that involved acetylene, a rapid intermediate incubation preceded the degradation study. For this intermediate step, freshly harvested cells were transferred into two 250-mL clear glass bottles capped with Teflon-lined Miniert valves (Altech, Deerfield, IL). Acetylene gas was added to the headspace of one bottle to achieve a final concentration of 6% (vol/vol). Both bottles were then incubated for 10 min at 30°C with shaking at 150 rpm. At the end of this time, both bottles were purged with nitrogen gas at a flow rate of 300 mL min⁻¹ for 2 min to remove any residual acetylene gas. Following this step, duplicate 30-mL samples were taken from both the acetylene-treated and untreated bottles and transferred into 100-mL incubation bottles for time-course incubations. NDMA (200 µg/L) was added to the bottles at the same time as the cellular addition. Strain OB3b was amended with 10 mM sodium formate at the onset of the experiments to eliminate the possibility of insufficient reducing equivalents hindering the cometabolic reaction (Alvarez-Cohen and McCarty, 1991); however, similar augmentation was not considered necessary for the remaining strains (Leahy et al., 1996; Smith et al., 2003). The addition of fixed concentrations of NDMA to BSM was used to generate both standards and abiotic controls. Time-course experiments were conducted by removing 2 mL of culture solution from incubations at each time point of interest. Unless otherwise stated, all incubations were performed at 30°C and 150 rpm to ensure viable cellular populations, enzyme activity, and phase partitioning.

Liquid-Liquid Extraction

A 2-mL sample of culture solution was removed from incubation bottles using a Gilson Pipetman (Middletown, WI) and combined with an equal volume of methylene chloride in a 20-mL scintillation vial. This mixture was shaken for 10 min in a cellular incubator (150 rpm and 30°C). Then, ~1 ml of the methylene chloride fraction was transferred to a crimp-sealed vial for analysis. Both active and control vials were extracted simultaneously.

Analytical Methods

Methylene chloride extracts containing NDMA were analyzed using the procedures of Mitch and Sedlak (2002). Analysis was performed on a Varian CP-3900 gas chromatograph coupled to a Saturn ion trap 2100T MS/MS containing an HP-5MS capillary column (30 m long by 0.25 mm I.D. by 1 µm thick, JW Scientific, Folsom, CA). An autosampler was employed for splitless injections of 2 µL with an injection port temperature of 200°C, a transfer line temperature of 260°C, and a trap temperature of 150°C. Gas chromatography temperature conditions were as follows: 35°C (hold time 1 min) ramping at 10°C/min to 70°C, then ramping at 2°C/min to 83°C, followed by

ramping at 10°C/min to 220°C and holding for 2.4 min. Helium carrier gas (99.999% purity) was maintained at 1.2 mL/min. Tandem mass spectrometry was performed by chemical ionization with methanol. The emission current was 60 µA, excitation amplitude 0.32 V, and multiplier offset was 100 V. NDMA was quantified by using the 44 and 47 daughter ions. The retention time was 7.1 min. The detection limit using the liquid-liquid extraction was ~ 5 µg/L.

Headspace measurements of toluene were performed as described previously (Deeb et al., 2002). Briefly, 200 µL headspace samples were removed from sealed bottles with a Hamilton constant rate gas-tight syringe (Reno, NV). The gas was then injected into a Hewlett-Packard 5880 gas chromatograph with flame ionization detector and a 0.75 mm × 30 m glass capillary column. Temperatures were fixed at 85°, 250°, and 300°C, respectively, for the oven, injector, and detector.

Naphthalene Oxidation Assay

A colorimetric assay for monooxygenase activity was adapted from Brusseau et al. (1990). A 5-mL aliquot of cellular suspension was incubated at 150 rpm and 30°C for 30 min in the presence of 1 mg naphthalene crystals to facilitate the potential oxidation to naphthol. A 200-µL aliquot of 0.2% (wt/vol) tetrazotized *o*-dianisidine was then added to the suspension. The resultant naphthol-diazo complex that formed was visualized as a purple liquid whose absorbance can be measured at 528 nm in order to quantify naphthalene oxidation as a surrogate for monooxygenase activity.

Protein Measurements

Cellular mass was quantified using a Pierce Coomassie Plus Protein Assay Kit (Rockford, IL). Cellular digestion was accomplished by bead beating the cells for 2 min followed by boiling for 15 min.

RESULTS

NDMA Degradation by Oxygenase-Expressing Cultures

To test for the biodegradation of NDMA, bacteria that express a variety of oxygenases induced by aliphatic and aromatic hydrocarbons were chosen (Table I) and cells were grown in the presence of inducing substrates. The results demonstrate that while five phylogenetically distinct monooxygenase-expressing bacteria can degrade NDMA, none of the dioxygenase-expressing cultures exhibited this capability. Not surprisingly, the oxygenase-deficient *E. coli* pCR 2.1-TOPO culture was also unable to biodegrade NDMA, confirming that cellular sorption of NDMA was negligible in these experiments.

Degradation of NDMA by Aliphatic Hydrocarbon-Induced Monooxygenases

The two aliphatic hydrocarbon-induced cultures were chosen due to their widely studied broad substrate specificities: *Methylosinus trichosporium* OB3b that expresses either the soluble (sMMO) or the particulate (pMMO) form of methane monooxygenase (Stanley et al., 1983), and *Mycobacterium vaccae* JOB5 that expresses a propane/butane monooxygenase (PMO) (Hamamura et al., 1997;

Table I. Bacterial strains tested for the ability to degrade NDMA.

Bacterial strain	Induced enzyme ^a	Growth substrate ^b	Detectable degradation ^c	Rate ^d [ng/mg/min]
<i>Methylosinus trichosporium</i> OB3b	soluble methane monooxygenase	methane	yes	3
<i>Methylosinus trichosporium</i> OB3b	particulate methane monooxygenase	methane	no	—
<i>Mycobacterium vaccae</i> JOB-5	propane monooxygenase	propane	yes	100
<i>Pseudomonas mendocina</i> KR1	tol 4-monooxygenase	toluene	yes	5
<i>Ralstonia pickettii</i> PKO1	tol 4-monooxygenase	toluene	partial	1
<i>Burkholderia cepacia</i> G4	tol 2-monooxygenase	toluene	no	—
<i>Pseudomonas putida</i> mt-2	tol side chain monooxygenase	toluene	no	—
<i>Pseudomonas sp.</i> W31	tol 2,3 dioxygenase	toluene	no	—
<i>Pseudomonas putida</i> F1	tol 2,3 dioxygenase	toluene	no	—
<i>Pseudomonas fluorescens</i> CFS215	tol 2,3 dioxygenase	toluene	no	—
<i>Pseudomonas sp.</i> JS150	tol 2,3 dioxygenase	toluene	no	—
<i>Rhodococcus sp.</i> RR1	unknown	soy broth	yes	13
<i>Escherichia coli</i> pCR 2.1-TOPO	no oxygenase	soy broth	no	—

^aThe list depicts the primary enzyme expected to be induced under the growth conditions. OB3b grown in the absence of copper induces sMMO while copper addition promotes pMMO. Secondary enzymes as identified by gene presence have been observed in the case of JS150 (tol 2 and 4-monooxygenase).

^bStrain RR1 degraded NDMA whether grown on soy broth or toluene. Strains PKO1 and JS150 were amended with casamino acids in addition to toluene to facilitate robust growth.

^cPartial degradation denotes ~40% NDMA disappearance over the course of 1 day. Undetectable degradation was identified as having no significant difference when comparing the degradation curve to incubated controls.

^dRates are reported as the average of at least 3 data points from the maximum slope of NDMA degradation curves and are expressed as a function of protein density. Controls demonstrated that headspace partitioning and sorption were not significant factors to consider when calculating rates of disappearance over the duration of these experiments.

Wackett et al., 1989). Resting cells of methane-induced OB3b that were grown in the absence of copper to specifically induce sMMO (Stanley et al., 1983) were capable of NDMA degradation (Fig. 1A) at a maximum rate of 3 ng/mg/min (Table I). Inhibition of NDMA degradation by these cells was achieved by briefly exposing them to acetylene gas (Table II), suggesting that sMMO was indeed responsible for the NDMA degradation by this culture. In contrast, when OB3b was grown in the presence of copper and methane to induce the pMMO rather than the sMMO enzyme, no NDMA degradation was observed (Fig. 1B).

Resting cells of propane-grown strain JOB5 rapidly degraded NDMA (Fig. 1C) at much higher rates than OB3b (Table I). In fact, degradation by JOB5 was the most rapid of all tested cultures, with degradation of 200 µg/L NDMA to below the detectable threshold in just over 1 h resulting in a maximum degradation rate of 100 ng/mg/min (Table I). Similar to OB3b, brief exposure to acetylene gas also inhibited NDMA degradation by this culture, providing evidence of the involvement of the propane monooxygenase in this degradation reaction.

In addition, growth of this strain on soy broth resulted in the loss of NDMA-degrading capabilities, supporting the involvement of propane as an inducing substrate for NDMA biodegradation.

Degradation of NDMA by Organisms Capable of Growth on Toluene

A variety of bacteria expressing toluene oxygenases were tested, including both mono- and dioxygenase-expressing cells (Table I). *Pseudomonas mendocina* KR1 expresses a toluene 4-monooxygenase (T4MO) (Yen et al., 1991); *Ralstonia pickettii* PKO1 that primarily expresses a T4MO similar to that of KR1 (Fishman et al., 2004); *Burkholderia cepacia* G4 expresses a toluene 2-monooxygenase (T2MO) (Shields et al., 1989); and *Pseudomonas putida* mt-2 expresses a xylene monooxygenase that is also referred to as a toluene sidechain monooxygenase (TMO) (Worsey and Williams, 1975). Four strains were tested that degrade toluene using 2,3 dioxygenase enzymes (TDO): *Pseudomonas* sp. W31 (Kukor and Olsen, 1996); *Pseudomonas putida* F1 (Gibson et al., 1970; Zylstra et al., 1989);

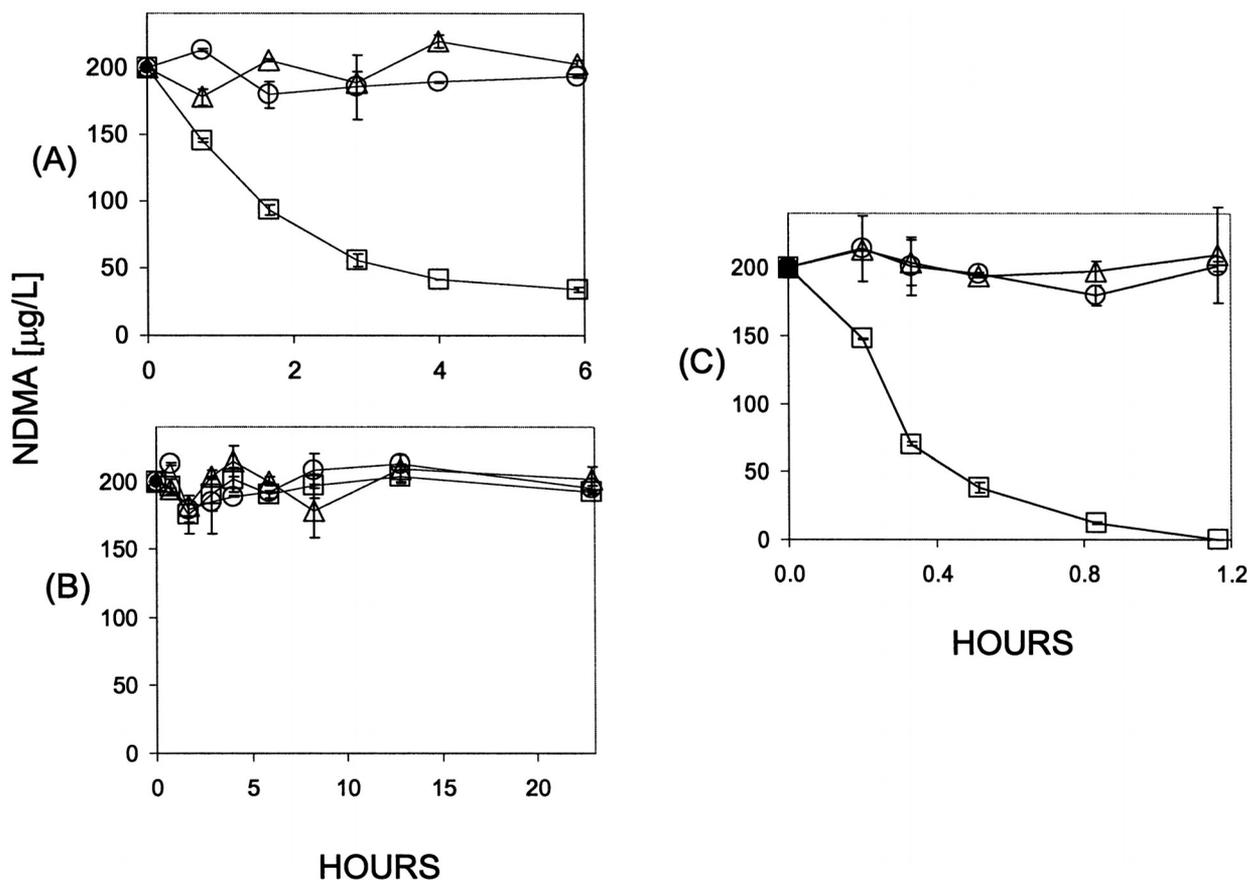


Figure 1. Degradation of NDMA by organisms induced with aliphatic hydrocarbons. **A:** *Methylosinus trichosporium* OB3b (270 mg protein/L) grown on methane in the absence of copper to induce sMMO. **B:** *Methylosinus trichosporium* OB3b (318 mg protein/L) grown on methane in the presence of copper to induce pMMO. **C:** *Mycobacterium vaccae* JOB-5 (52 mg protein/L) grown on propane to induce PMO. □ = active bacteria; Δ = active bacteria exposed briefly to acetylene gas; ○ = abiotic controls; ● = estimated initial addition of NDMA. Experiments were conducted in duplicate bottles with error bars depicting data range. In some cases the error bars are smaller than the symbols.

Table II. Effects of acetylene gas exposure on NDMA degradation, results of the naphthol assay for monooxygenase activity, and the effects of acetylene gas on the naphthol assay.

Bacterial strain (enzyme) ^a	Inhibition of NDMA degradation	Naphthol assay (no acetylene)	Inhibition of naphthol assay ^b
<i>Methylosinus trichosporium</i> OB3b (sMMO)	yes	yes	yes
<i>Methylosinus trichosporium</i> OB3b (pMMO)	—	no	—
<i>Mycobacterium vaccae</i> JOB-5	yes	yes	yes
<i>Pseudomonas mendocina</i> KR1	yes	yes	yes
<i>Ralstonia pickettii</i> PKO1	yes	yes	yes
<i>Burkholderia cepacia</i> G4	—	yes	no
<i>Pseudomonas putida</i> mt-2	—	no	—
<i>Pseudomonas</i> sp. W31	—	no	—
<i>Pseudomonas putida</i> F1	—	no	—
<i>Pseudomonas fluorescens</i> CFS215	—	no	—
<i>Pseudomonas</i> sp. JS150	—	no	—
<i>Rhodococcus</i> sp. RR1	no	no	—

^aThe enzyme form expressed by OB3b (sMMO or pMMO) was controlled by growth conditions (\pm copper).

^bAcetylene inhibition of the naphthol assay could not be measured for bacteria that did not test positive for that assay. Inhibition was observed as a significant decrease in the naphthol colorimetric signal when compared to cells that were not exposed to acetylene.

Pseudomonas fluorescens CFS215, and *Pseudomonas* sp. JS150. JS150 is unusual in that it appears to have a redundant system that encodes for both the aromatic monooxygenases T4MO and T2MO as well as the more readily expressed toluene dioxygenase; however, expression as determined by metabolic products was primarily demonstrated to be the dioxygenase (Haigler et al., 1992; Johnson and Olsen, 1995, 1997; Kahng et al., 2001). Figure 2 depicts the variety of initial toluene oxidation reactions catalyzed by the oxygenase-expressing bacteria used in this study (Leahy et al., 1996; Shields et al., 1989).

Of the four types of toluene-induced monooxygenase cultures tested, NDMA degradation was observed in only two. Strains KR1 and PKO1, cultures that express two distinct T4MOs, were both capable of degrading NDMA

(Fig. 3A,B), with PKO1 exhibiting only partial degradation and the lowest transformation rate (1 ng/mg/min) of all tested strains. As with the propane and methane oxidizers, brief exposure to acetylene inhibited NDMA degradation by these cultures, implicating monooxygenases in the reaction. In addition, these cultures lost their NDMA-degrading capabilities when toluene monooxygenase genes were not induced by growth of cells on soy broth.

In contrast, strains G4 and mt-2, the T2MO- and TMO-expressing cultures, respectively, did not exhibit NDMA degradation when incubated at comparable cellular densities (~ 100 mg/L protein). In addition, none of the four strains expressing toluene 2,3 dioxygenase enzymes exhibited NDMA-degrading capabilities under the tested conditions (Fig. 3C). The viability of these cultures and the level of dioxygenase activity were confirmed during the experiments by monitoring the oxidation of toluene by parallel incubations of the cultures.

In order to confirm the role of monooxygenases in the NDMA degradation, the degradation capabilities of three strains of *E. coli* TG1/pBS(Kan) with independent plasmid constructs coding for three toluene monooxygenases were tested. These bacteria have been demonstrated to constitutively express the T4MOs expressed by KR-1 (TG1(T4MO)) and PKO1 (TG1(TpMO)), and the T2MO expressed by G4 (TG1(T2MO)) (Canada et al., 2002; Tao et al., 2004). Expression of the specific enzymes in the same host eliminates the variability of intrastain comparisons. Strains TG1(T4MO) and TG1(para) were both capable of degrading NDMA, as were their parent strains. A 20-h incubation of these cells resulted in the degradation of $\sim 80\%$ and 60% of $200 \mu\text{g/L}$ NDMA by these respective strains, while acetylene exposure resulted in NDMA concentrations statistically indistinguishable from the

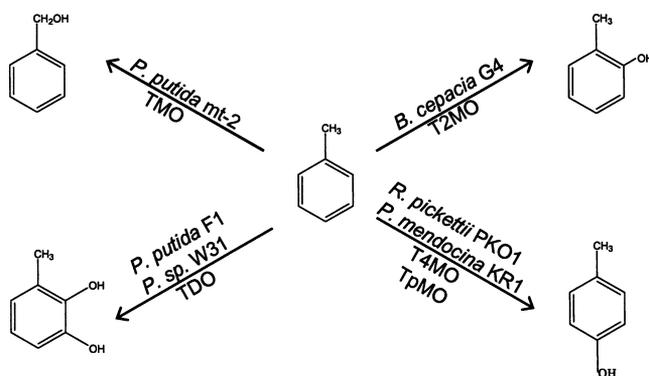


Figure 2. Defining toluene oxidation processes as they relate to the bacteria and oxygenases employed in this study (adapted from the work of Shields et al., 1989). A summary of the enzymes and their relationships to the bacteria can be found in Leahy et al. (1996).

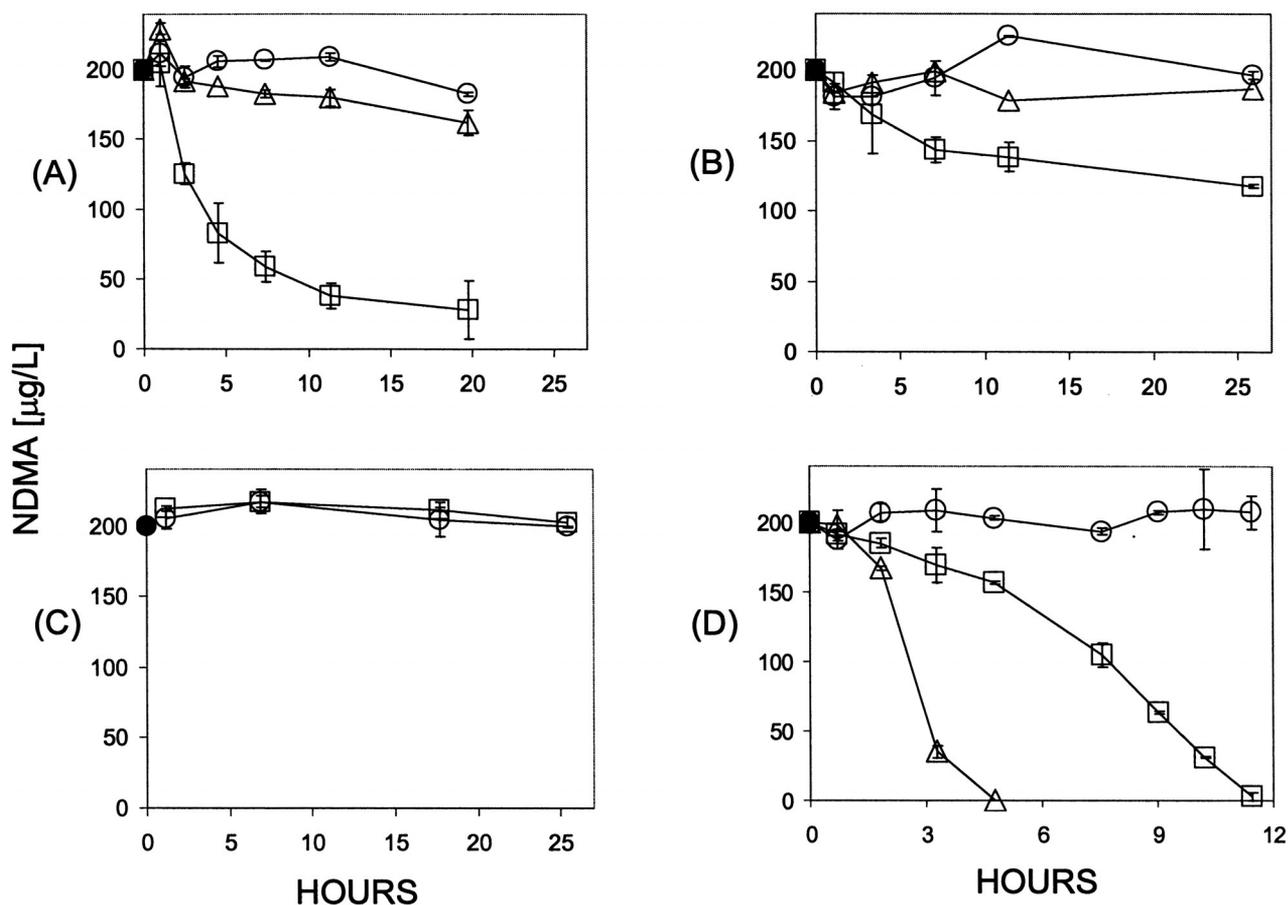


Figure 3. Degradation of NDMA by organisms capable of growth on toluene. **A:** *Pseudomonas mendocina* KR1 (90 mg protein/L). **B:** *Ralstonia pickettii* PKO1 (122 mg protein/L). **C:** *Pseudomonas sp.* JS150 (130 mg protein/L). **D:** *Rhodococcus sp.* RR1 (66 mg protein/L). Cells in A–C were grown with toluene as an inducing substrate, while **D** was grown on soy broth. □ = active bacteria; △ = active bacteria exposed briefly to acetylene gas; ○ = abiotic controls; ● = estimated initial addition of NDMA. Experiments were conducted in duplicate bottles with error bars depicting the data range, with the exception of the acetylene-exposed PKO1, which represents a single bottle. In some cases the error bars are smaller than the symbols.

abiotic control (Fig. 4). Conversely, strain TG1(T2MO) had no significant impact on NDMA concentrations, mimicking the behavior of its parent strain G4.

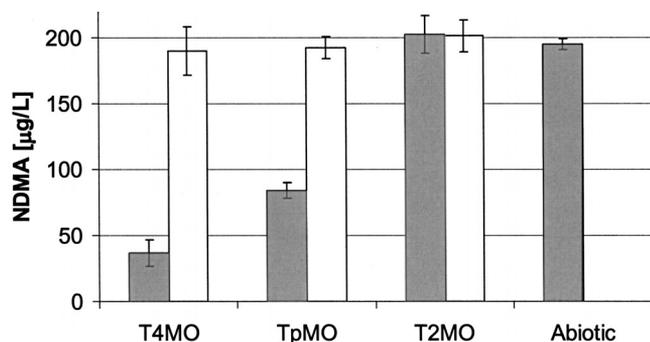


Figure 4. Final NDMA concentrations after a 20-h incubation for *E. coli* TG1(T4MO), TG1(TpMO), and TG1(T2MO) (420–455 mg protein/L). All experimental bottles were initially amended with 200 µg/L NDMA. ■ = active bacteria or abiotic control; □ = active bacteria exposed briefly to acetylene gas. Representative results of two independent experiments are shown with error bars depicting the mean deviation of multiple injections on the GC/MS/MS.

Rhodococcus sp. RR1, a toluene, benzene, xylene, and ethylbenzene-degrading strain with an undefined oxygenase enzyme (Deeb and Alvarez-Cohen, 1999; Deeb et al., 2002) degraded NDMA with a slight lag, followed by a degradation rate increase (Fig. 3D). Unexpectedly, acetylene did not inhibit NDMA degradation in this strain. In fact, brief exposure to acetylene actually increased NDMA degradation rates for cells harvested in the exponential phase, while it appeared to have little effect on cells harvested in the postexponential phase of growth. Since acetylene could not be used as a negative control for NDMA degradation by this strain, killed controls were produced by exposure to 0.01% sodium azide, resulting in near complete recovery of NDMA after 12 h (data not shown). Further, RR1 exhibited similar NDMA degradation behavior regardless of whether it was grown on rich substrate (soy broth) or BSM with toluene, suggesting that toluene is not responsible for induction of the NDMA-degrading enzyme in this strain. Conversely, cells grown on soy broth did not have the ability to immediately metabolize toluene (data not shown). This behavior contrasted with the other tested wildtype strains identified as NDMA degraders, as

they were unable to biodegrade NDMA after growth on soy broth.

Naphthol Assay as a Visual Indicator of Monooxygenase Activity

A colorimetric assay developed as a rapid method to detect monooxygenase activity (Brusseau et al., 1990; Ensley et al., 1983) was tested on the strains used in this study. This assay is based on the ability of certain monooxygenases to convert naphthalene to the dead-end products 1-naphthol and 2-naphthol. Addition of a diazo dye after naphthol production results in a dark purple naphthol-azo complex that can be visualized with the naked eye. It was hypothesized that this assay would correlate positively with NDMA degradation activity by monooxygenases, providing a rapid screening tool to detect this activity in cultures.

All but one of the strains that had the ability to degrade NDMA also tested positive with the colorimetric assay (Table II). Specifically, strains PKO1, KR1, JOB5, and OB3b (sMMO) produced naphthol end-products when incubated with naphthalene, while RR1 did not. Brief acetylene exposure inhibited naphthol production by the former strains, as expected. Interestingly, although strain G4 did not demonstrate NDMA degradation capabilities in our experiments, it did test positive for the naphthol assay. Unlike the other naphthol-producing incubations, the addition of acetylene gas did not inhibit this bacterium's production of naphthol. The null effect of acetylene on monooxygenase activity on strain G4 has been documented previously (Yeager et al., 1999). Conversely, strain mt-2 tested negative for both NDMA degradation and the naphthol assay, highlighting the unusual nature of the sidechain TMO when compared to the other toluene monooxygenases. None of the dioxygenase-expressing bacteria produced the dark purple color characteristic of the naphthol assay (Table II).

DISCUSSION

Our results suggest that a variety of monooxygenase-expressing bacterial strains are capable of degrading NDMA. Specifically, strains expressing three of the known bacterial monooxygenases: sMMO, PMO, and T4MO, exhibited the ability to degrade NDMA, while strains expressing pMMO, T2MO, and sidechain TMO did not. Moreover, protein-normalized NDMA degradation rates varied over two orders of magnitude for strains exhibiting NDMA degradation. There are many lines of evidence that the monooxygenases are responsible for the degradation of NDMA in these whole-cell assays. First, NDMA degradation was observed when the cells were grown under conditions that have been demonstrated to result in expression of the target monooxygenases, and enzyme activity in each strain was independently verified by the strains' abilities to convert naphthalene into naphthol. Growth of

these cells on soy broth to avoid monooxygenase gene expression also hindered the biodegradation of NDMA. Further, growth of the methane oxidizer OB3b under conditions that favored induction of the soluble form of MMO over the particulate form was necessary for NDMA degradation. Additionally, brief exposure to acetylene gas, which serves as a mechanism-based inhibitor of monooxygenases such as T4MO (McClay et al., 1996), sMMO (Prior and Dalton, 1985), and PMO (Hamamura et al., 1999), led to the cessation of NDMA degradation, lending strong evidence to the role of monooxygenases in catalyzing NDMA degradation. Finally, recombinant *E. coli* strains containing cloned toluene monooxygenases derived from strains KR1 and PKO1 were capable of NDMA degradation and were inactivated by brief exposure to acetylene, mimicking the behavior of their parent strains.

Since monooxygenase enzymes catalyze NDMA degradation in eukaryotic systems, it seems logical that bacterial NDMA degradation may occur by a biochemical degradation pathway that is similar to the one identified for the eukaryotic systems. In mammals, cytochrome P-450 2E1 monooxygenases react with NDMA to yield a reactive intermediate (Koop, 1992; Yamazaki et al., 1992). The first step of this reaction is reported to involve hydroxylation of one of the methyl carbons by the P-450 enzyme (Kroeger-Koepke et al., 1981). The resultant alkyl nitrosamine is unstable and undergoes a fragmentation reaction to form formaldehyde and a diazonium ion, which then decays to nitrogen gas (Carey, 1992). In addition to this hydroxylation pathway (Kroeger-Koepke et al., 1981), studies with rats have shown that P-450 enzymes (Tu and Yang, 1985) can mediate a denitrosation reaction. It is possible that both of these pathways share the same initial enzymatic step followed by exergonic noncatalytic reactions. The detection of formaldehyde and methylamine during microbial transformation of NDMA in the Kaplan and Kaplan study (1985) supports this analogy between bacterial and mammalian biochemical pathways.

Although cells expressing certain monooxygenase enzymes were capable of NDMA degradation, this capability did not extend to the tested cells expressing particulate MMO, T2MO, and sidechain TMO. A possible explanation for the lack of activity observed for the pMMO and the sidechain TMO is the membrane-spanning nature of these enzymes (Shaw and Harayama, 1995; Stanley et al., 1983), which may lead to capabilities that differ from those of the suspended monooxygenases (Newman and Wackett, 1995; Pikus et al., 1996). The inability of G4 expressing T2MO to degrade NDMA is more surprising, as this enzyme's structure and function is quite similar to the T4MOs. However, this unexpected result was confirmed by the observed lack of NDMA degradation in recombinant strain TG1(T2MO), which was designed to express the T2MO found in strain G4. Since *E. coli* expressing T4MO clearly degrades NDMA, there is a true enzymatic difference rather than merely a difference in transport.

Functional differences have previously been observed between T2MO and T4MO. For instance, the monooxygenase activity in strain G4 has been shown to be unaffected by exposure to acetylene gas and phenylacetylene, while KR1 and PKO1 experienced near complete inhibition of toluene monooxygenase activity or growth after brief incubations with these respective substrates (Keener et al., 2001; Yeager et al., 1999). Of course, the most obvious difference between these enzymes is that they hydroxylate different regions of the target toluene molecule (Fig. 2). With regard to differences in substrate affinity, past studies have shown KR1 and its T4MO to be unusual and particularly robust when compared to other toluene-induced oxygenases (Leahy et al., 1996; McClay et al., 2000), as was the case in this study.

The observation that strain RR1 had the capability to degrade NDMA when grown on soy broth, a rich and undefined medium, was entirely unexpected and in marked contrast with the results for other strains. Unlike simple substrates such as pyruvate and citrate, rich substrates involved in the main cycle of carbohydrate metabolism such as glucose have been shown to inhibit toluene oxygenase expression at the level of catabolic operons and promoters (Holtel et al., 1994). Hence, the expression by RR1 of an NDMA-degrading enzyme after growth on soy broth suggests that aromatic induction did not play an important role in NDMA degradation by this bacterium. Furthermore, since brief acetylene exposure did not inhibit NDMA degradation by RR1 as it did for the other strains, it is possible that the constitutive enzyme responsible is not a monooxygenase.

The naphthol-diazo colorimetric assay for monooxygenase activity has previously been used to assess the monooxygenase activity of KR1 (Keener et al., 2001) and OB3b (Brusseau et al., 1990) and clearly applies to a number of related monooxygenase enzymes. In this study, the naphthol assay demonstrated an imperfect correlation between naphthol production and NDMA oxidation. The assay tested positive for all of the known monooxygenase-expressing strains that degraded NDMA and negative for both the dioxygenase-expressing strains as well as the monooxygenase-expressing strains that did not degrade NDMA. However, the assay also tested positive for strain G4 expressing the T2MO that did not degrade NDMA. Further, strain RR1 degraded NDMA but did not test positive for the naphthol assay. Although a valuable tool for assessing specific types of monooxygenase activity, these results suggest that this technique cannot be used as a specific screen for NDMA degradative capabilities.

While the results reported here support a prior conclusion (Yoshinari and Shafer, 1990) that sMMO was most likely involved in NDMA degradation by the methanotroph OB3b, this study goes further to confirm the role of monooxygenases using a suite of methods, as well as describing a variety of other monooxygenase-expressing bacteria that are capable of NDMA degradation. Further, these results differ significantly from previous reports by axenic cultures

in both the concentrations of NDMA evaluated and the extent of NDMA biodegradation observed. Specifically, the experiments in this study were conducted at the upper bounds of environmental relevance, with initial concentrations of 200 µg/L NDMA, while the previous studies were conducted in the 2.2–740 mg/L range (Rowland and Grasso, 1975; Yoshinari and Shafer, 1990). In addition, the extent of NDMA degradation reported here ranges from 40% to >99% for the tested strains, compared to less than 4% transformation as reported previously (Rowland and Grasso, 1975; Yoshinari and Shafer, 1990).

Given the low concentrations of NDMA likely encountered in the environment and the proposed regulatory standard of 10 ng/L (Cal DHS, 2002), attention to relevant environmental concentrations is of particular importance for NDMA attenuation. Further, at these low environmental NDMA concentrations, which are still considered high with respect to potential human health effects, it is unlikely that organisms would be able to sustain growth on NDMA as primary substrate. This suggests that cometabolic rather than metabolic degradation may be the dominant attenuation mechanism.

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