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Mineralization of 2,4,6-trinitrophenol (picric acid): characterization and phylogenetic identification of microbial strains

J Rajan[1,2], K Valli[1], RE Perkins[3], FS Sariaslani[1], SM Barns[4], A-L Reysenbach[4], S Rehm[4], M Ehringer[4] and NR Pace[4]

[1]*BioProcess Development Center;* [3]*Environmental Biotechnology, Central Research and Development, Dupont Co, Experimental Station, Wilmington, DE 19880-0173;* [4]*Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, IN 47405, USA*

Four bacterial strains that use picric acid as their sole carbon and energy source were isolated. Mineralization of ^{14}C-UL-picric acid showed that up to 65% of the radioactivity was released as $^{14}CO_2$. HPLC and UV/Vis spectral analyses indicated complete degradation of picric acid by these organisms. HPLC and LC/MS analyses showed transient formation of 2,4-dinitrophenol during picric acid degradation. Degradation of picric acid was concomitant with stoichiometric release of three moles of nitrite per mole of picric acid. The four picric acid degraders were identified as close relatives of *Nocardioides simplex* (ATCC 6946) based on their small subunit (16S) rRNA gene sequences.

Keywords: microbial degradation; nitroaromatic degradation; picric acid degradation; 2,4,6-trinitrophenol mineralization

Introduction

Nitroaromatic compounds are used as dyes, plastics, herbicides, pesticides, pharmaceuticals and explosives. Due to the presence of electron-withdrawing nitro groups on aromatic rings, such compounds are resistant to electrophilic attack by oxygenase-type enzymes that commonly initiate degradation. The degree of recalcitrance of these compounds is proportional to the number of nitro groups present on the aromatic ring. Industrial production of polynitroaromatics over several decades has resulted in contamination of soil, ground-, surface-, and wastewaters around manufacturing sites. In addition to their well-documented toxicity [12,15], some nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenol (picric acid) are explosive. Since decontamination of sites that have been polluted with polynitroaromatics is of high priority, microbial degradation of nitroaromatics has received considerable attention [15].

Recent advances in our understanding of the microbial degradation of nitroaromatics indicate that biodegradation could provide an efficient, cost effective and environmentally benign means of remediation of these often-hazardous chemicals. For example, the recalcitrant explosive TNT can be mineralized by a hybrid *Pseudomonas* strain [3]. In contrast, far less attention has been paid to the microbial degradation of picric acid, which is formed as the major by-product during manufacture of several nitroaromatic compounds. Initial reports on the possibility of microbial degradation of picric acid appeared in 1941 by Erikson [4] and in 1956 by Gundersen and Jensen [5]. Reductive conversion of picric acid by *Pseudomonas aeruginosa* to the direct mutagen picramic acid was reported in 1979 by Wyman *et al* [17]. However, detailed information about mineralization of picric acid is scarce. The only recent information on microbial degradation of picric acid has been presented by Lenke and Knackmuss [8] in which a *Rhodococcus erythropolis* strain, while growing on succinate, used picric acid as its nitrogen source.

In this communication we present the first example of isolation and characterization of microbial strains that use picric acid as their sole source of carbon and energy. These isolates were identified phylogenetically based on comparative analysis of their ribosomal RNA gene sequences.

Materials and methods

Microorganisms

A sample from an industrial waste treatment facility was used as the source of microorganisms. The minimal medium, pH 7.2, contained (g L^{-1}): $NaH_2PO_4 \cdot H_2O$, 0.6; K_2HPO_4, 1.8; and $(NH_4)_2SO_4$, 1.5. Yeast extract (0.5 mg ml^{-1}) was added during the first month of enrichment. An aliquot (10 ml L^{-1}) of a stock trace element solution containing (mg per 100 ml): $MgCl_2$, 2000; $CaCl_2$, 400; $MnCl_2 \cdot 4H_2O$, 80; $FeCl_3 \cdot 6H_2O$, 50; $Na_2MoO_4 \cdot 2H_2O$, 150; was added to the minimal salts medium. Picric acid (0.44 mM, 100 mg L^{-1}) was added from a 44 mM (1%) standard solution which was adjusted to pH 7.5 with 1 N NaOH prior to filter sterilization. Serial transfers (5% inoculum) into fresh medium were made every 72 h. The

Correspondence: FS Sariaslani, PhD, BioProcess Development Center, CR&D, Dupont Co, Experimental Station, Wilmington, DE 19880-0173, USA
[2]Present address: Zeneca, Biocides, 1800 Concord Pike, 248 Wilmington, DE 19897, USA
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concentration of picric acid was gradually raised to 4.4 mM over a period of several months.

Following several transfers onto fresh agar plates, three strains (Nb, Nd, and Nf) that used picric acid as their sole source of carbon and energy were isolated. Using a similar technique, a separate picric acid-degrading organism (FJ) was obtained, from the same source of inoculum. In this case, an industrial wastewater containing picric acid (2000–3000 mg L^{-1}) was used as the carbon source.

Disappearance of picric acid was monitored by measuring absorbance at 354 nm (ϵ = 14.3 mM^{-1} cm^{-1}) and also by high performance chromatography (HPLC). All dilutions for optical density measurements were carried out in 0.1 N HCl. This assay allowed accurate measurement of picric acid concentrations as low as 4.4 μM. Growth was monitored by dry weight determination.

Microorganisms grown for 72 h on minimal medium containing picric acid (1.1 mM) were harvested by centrifugation (6000 × *g*, 10 min). The cell pellet (~0.6–0.8 g dry wt L^{-1}) was resuspended in 250 ml of minimal medium. Two 25-ml aliquots were withdrawn for dry weight determinations. The remaining 200 ml suspension was dispensed as 25-ml aliquots in 125-ml flasks. Picric acid or other test compounds to be used (0.88–4.4 mM) were added, flasks were incubated with shaking (250 rpm, 30°C) and samples (1–2 ml) were withdrawn at intervals for various analyses.

When substrates other than picric acid were used for growth, sodium salts (1.1–2.2 mM) of the respective substrates were filter-sterilized and then added to the minimal medium.

High Performance Liquid Chromatography (HPLC) and Liquid Chromatography/Mass Spectrometry (LC/MS)

HPLC was performed on a Hewlett Packard model 1090 instrument (Hewlett Packard, Wilmington, DE, USA) equipped with a diode array detector. A Supelcosil LC8 column (25 by 4.6 mm, 5 μm packing) (Supelco, Inc, Bellefonte, PA, USA) was used. The solvent system contained de-ionized water with 0.1% acetic acid (solvent A) and methanol with 0.1% acetic acid (solvent B). Various compounds were eluted, at a flow rate of 1 ml min^{-1}, using a gradient starting with 20% B (0–5 min), increasing to 50% B (5–20 min) followed by gradual increase to 90% B (20–25 min) and stationary at 90% B (25–27 min). The data were analyzed by obtaining area under sample peaks at wavelengths of 280, 354 and 450 nm (reference at 550 nm). The retention volumes for picric acid, 2,4-dinitrophenol, 2,6-dinitrophenol, 2-nitrophenol and 4-nitrophenol were 3.5, 14.8, 10.4, 17.8 and 15.4 ml respectively. LC/MS was performed on a Hewlett Packard HP 1090 liquid chromatograph equipped with a Vydac C8 column (2.1 mm × 15 cm) (Vydac, Hesperia, CA). Compounds were eluted at a flow rate of 0.4 ml min^{-1} using the above solvent system. The gradient employed was from 0 to 18 min using 10% solvent B and from 18 to 38 min with 100% solvent B. Oven temperature was 40°C. The liquid chromatograph was connected to a Fisons TRIO-2000 Mass Spectrometer (Manchester, UK) with electrospray ionization source in negative ion mode. A split ratio of 50 : 1 using LC packing accurate splitter was used.

Radiotracer studies

Cell suspensions were prepared as described above. Aliquots (50 ml) of these suspensions were added to 250-ml flasks fitted with rubber stoppers containing two glass tubes connected to sealed tygon tubings. Picric acid (^{14}C-UL, 8.7 μCi $\mu$$mol^{-1}$) was prepared by NEN (NEN Research Products, Boston, MA, USA). ^{14}C-picric acid (~70 nCi) and 1.1–2.2 mM of unlabeled picric acid were added to the cell suspensions. Flasks were incubated with shaking and were, at various time intervals, flushed with oxygen. The carbon dioxide produced was adsorbed into a solution of ethanolamine/methanol/Ecolyte (15 : 35 :50) (ICN Chemicals, Aurora, OH, USA). The radioactivity was measured in a Beckman Liquid Scintillation Counter LS 3801 (Beckman Instruments, Fullerton, CA, USA). When the experiment was terminated, the amount of radioactivity in the supernatant medium (25–37%) and cell paste (11.5–15.2%) was also measured. The extent of quenching by the supernatant medium and biomass was calculated to be 48% and 32% respectively. At the end of the experiment, after appropriate corrections for quenching, a mass balance of up to 92.9% of the original radioactivity was achieved.

Analysis of nitrite

Nitrite was assayed using a previously-described colorimetric method [2]. Picric acid-grown cells were washed and resuspended in minimal medium with picric acid (1.75 mM) as the sole carbon source and degradation of picric acid and release of nitrite were measured.

Fatty acid analysis of various isolates

The fatty acid constituents of various isolates were analyzed using the Microbial Identification System (MIDI) (Microbial ID Inc, Newark, DE, USA) as described by Birnbaum *et al* [1].

DNA extraction and nucleic acid sequencing

Genomic DNA of strains Nb, Nd, Nf and FJ, was extracted and purified from approximately 0.1 g cell paste by lysis in 3% SDS and microwave heating, followed by phenol extraction, as described previously [14]. The polymerase chain reaction (PCR) was used to amplify 16S-like rRNA genes (rDNAs) from each DNA using primers which correspond to nucleotide positions 8 to 27 of *Escherichia coli* 16S rRNA (forward primer, 8FPL: 5′-GCGGATCCGCGGCCGCTGCAGAGTTTGATCCTGGCTCAG-3′) and to *E. coli* positions 1510 to 1492 (reverse primer, 1492RPL: 5′-GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACTT-3′). (Both primers contain polylinker sequences with restriction endonuclease sites to facilitate cloning of PCR products.) PCR reactions, purification of amplification products, and cloning into pBluescript KS-vector were performed as described previously [14]. Clones containing inserts approximately 1.5 kb in size were identified by agarose-gel electrophoresis of plasmid DNA prepared by alkaline lysis [11]. Plasmid sequencing was performed with Sequenase 2.0 (United States Biochemical Corp, Cleveland, OH, USA), according to manufacturer's protocols, using rRNA-specific primers [7] and on a Li-Cor automated sequencer using sequitherm sequencing reagents (Epicentre Technologies, Madison, WI, USA). Phylogenetic trees were con-

structed using maximum likelihood analysis (using the 'restart' option of fastDNAml) [13] with random order of addition of taxa. Trees were displayed using TREETOOL [10].

The GeneBank accession numbers for the isolates FJ and Nb are, U27856 and U27857 respectively.

Results

Growth on picric acid and structurally-related nitroaromatic compounds

All four picric acid-degrading isolates (Nb, Nd, Nf and FJ) use picric acid (up to 4.4 mM) as the sole source of carbon and energy. HPLC and LC/MS analyses have not shown production of any dead-end metabolites by any of these strains during growth on picric acid. These strains have somewhat different capabilities for degrading picric acid and other structurally related nitroaromatic compounds. When picric acid-grown cells were challenged with structurally-related nitroaromatics, distinct differences were observed as indicated in Table 1.

Nitrite release

All four picric acid-degrading isolates exhibited the stoichiometry of three moles of nitrite released per mole of picric acid degraded. Release of nitrite by strain Nf is depicted in Figure 1. These organisms also used 2,4-dinitrophenol (2,4-DNP) as their sole carbon source. Degradation of 2,4-DNP resulted in stoichiometric release of two moles of nitrite per mole of substrate (data not shown).

Mineralization of ^{14}C-picric acid

Conclusive proof of mineralization of picric acid was obtained through studies with ^{14}C-UL-picric acid (Figure 2).). Picric acid-grown cells of each isolate were resuspended in minimal medium with picric acid (1.1–2.2 mM) as the sole carbon source. Release of $^{14}CO_2$ was measured at intervals. These measurements indicated release of 40–47% of the original radioactivity as $^{14}CO_2$. When yeast extract (0.5 mg ml^{-1}) was added to the minimal medium as a growth stimulator, up to 60–65% of the radioactivity was released as $^{14}CO_2$. HPLC analysis of supernatant medium at the end of the experiment indicated absence of any aromatic compounds.

Dual substrates

Since HPLC and LC/MS analyses of supernatant medium indicated the transient formation of 2,4-DNP during degradation of picric acid, we examined the ability of the isolates to degrade 2,4-DNP, in the presence of picric acid. Picric acid-grown cells were challenged with picric acid (1.1 mM) and 2,4-DNP (1.4 mM). While metabolism of 2,4-DNP commenced immediately (Figure 3), degradation of picric acid was apparent only after most of the 2,4-DNP was consumed. Both substrates were depleted within 24 h.

Table 1 Degradation of structurally related nitrophenols by picric acid-degrading strains

Substrate	Degradation rates (μM h^{-1} g^{-1} cells) for strain:			
	Nb	Nd	Nf	FJ
Picric acid	175–210	160–175	306–350	306–350
2,4-Dinitrophenol	163–190	163–190	217–271	250–330
2,6-Dinitrophenol	22–27	16–22	22–27	16–27
2-Nitrophenol	0	0	22–35	14–35
4-Nitrophenol	0	0	86–101	57–86

Fatty acid analysis of the isolates

Automated quantitative analysis of fatty acid profiles (MIDI) [1] of the picric acid-degrading isolates indicated that each of the four isolates has a distinct fatty acid profile. Ratios of various signature fatty acids characteristic of actinomycetes, such as 10-methyl-pentadecanoic, -hexadecanoic, and -heptadecanoic acids, were distinctly different in all four strains. For example, the 10-methylheptadecanoic acid (tuberculosteric acid), a characteristic fatty acid among actinomycetes, comprised 3.0, 0.7, 16.6 and 4.7% of the total for strains Nd, Nf, FJ and Nb, respectively. These analyses indicated that strains Nb and Nd are linked at the 'species-level' (E.D. ~11). By the same criteria, strains Nf and FJ are similar to each other, and distinct from strains Nb and Nd. Comparison of the fatty acid profiles of the isolates with the database libraries failed to identify these strains conclusively.

Phylogenetic characterization of the picric acid-degrading isolates

A phylogenetic identification of the isolates was carried out by determining small subunit (16S) rRNA gene sequences and comparing them to those of known organisms available through the Ribosomal Database Project (approximately 2250 sequences) [10]. DNAs purified from isolates were subjected to polymerase chain reaction (PCR) using rRNA-specific primers complementary to universally conserved sequences. The PCR products were then cloned into a plasmid vector and the sequences determined. Phylogenetic affiliations of rDNA clones obtained are shown in Figure 4.

Comparison of approximately 300 nucleotides of sequence obtained from highly variable regions of the 16S rRNA gene sequence of picrate-degrading isolates Nb, Nd and Nf showed each to be identical (data not shown). Since this amount of sequence identity is likely to extend throughout the remainder of the molecule, complete rDNA sequence (approximately 1500 nucleotides) was determined for isolate Nb only. The rDNA sequence from isolate FJ differed from that of the other isolates, and so was determined in its entirety as well. Comparison of the Nb and FJ sequences with others available showed greatest similarity to that of the Gram-positive organism *Nocardioides simplex* (ATCC 6946) [16]. A summary of similarity values for the Nb, FJ and other Gram-positive bacterial sequences is given in Table 2. The 16S rDNA sequences of these isolates conformed to the secondary structure model common to Gram-positive bacteria (not shown) [6]. A phylogenetic tree constructed by maximum likelihood analysis of the isolate sequences, shows the close, specific relationship between the sequences of the Nb, Nd, Nf and FJ isolates, and that of *N. simplex* (Figure 4).

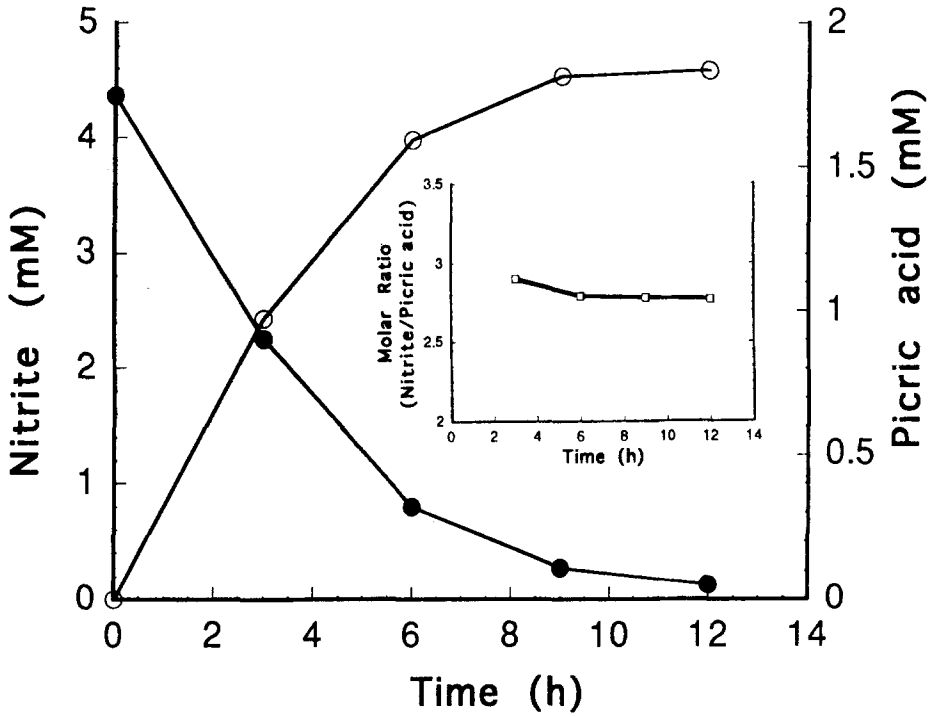

Figure 1 Release of nitrite (○) during degradation of picric acid (1.76 mM) (●) by strain Nf; (□) the ratio of moles of nitrite released per mole of picric acid degraded.

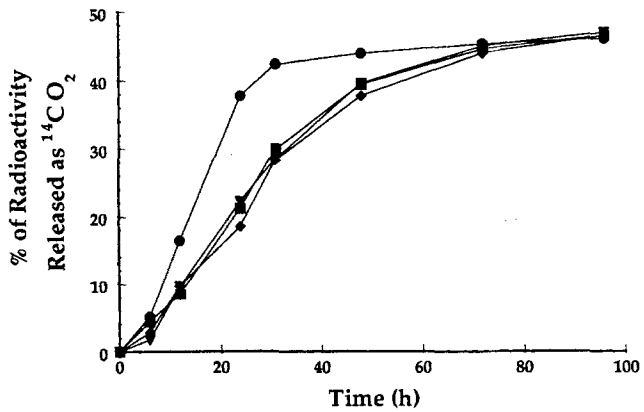

Figure 2 Mineralization of picric acid by isolates: strain FJ (●), strain Nf (▼), strain Nb (■), strain Nd (◆).

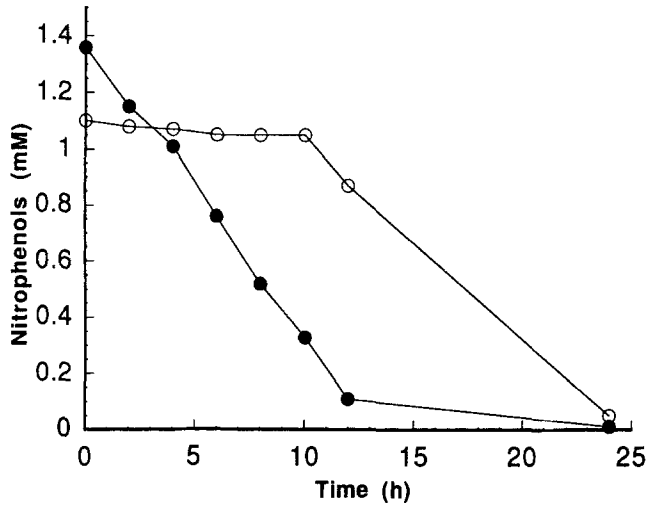

Figure 3 Degradation of picric acid (○) and 2,4-DNP (●) by strain Nf. Cells grown on picric acid were tested for their ability to degrade picric acid (1.1 mM) and 2,4-DNP (1.4 mM). Degradation of picric acid commenced only after a considerable amount of 2,4-DNP was consumed.

Discussion

In this communication, we describe isolation, identification and characterization of four bacterial strains that mineralize picric acid. The unique ability of these microorganisms to mineralize high concentrations of picric acid (4.4 mM) underlines their potential for biotreatment of waste streams or contaminated soils containing this 'high energy' compound. These picric acid-degraders exhibit some differences in their abilities to degrade picric acid and other structurally related nitrophenols. While fatty acid analysis did not provide conclusive identification for these organisms, it indicated that they belonged to different categories. Therefore, in order to provide conclusive evidence for their phylogenetic identity, we used analysis of rRNA gene sequences.

Although the three picrate-utilizing isolates (Nb, Nd, and Nf) that were originally isolated from the same sample using picric acid as the sole carbon source, differed to some extent in their biochemical capabilities and fatty acid compositions, all were identical in their rDNA sequence through the region of several hundred nucleotides inspected, and therefore probably represent different strains of the same species. The rDNA sequence of isolate FJ, which was obtained from a different inoculum, using an industrial effluent containing picric acid, is closely related to, but distinct from, the sequences of the three N strains.

Table 2 16S rDNA sequence similarity between picric acid-degrading isolates Nb and FJ and other bacteria

Organism	Percent similarity to organism:[a]											
	1	2	3	4	5	6	7	8	9	10	11	12
1) Nb[b]												
2) FJ	93.9											
3) *Nocardioides simplex* (6946)	94.6	97.2										
4) *Nocardioides plantarum*	93.8	94.3	93.6									
5) *Nocardioides luteus*	93.0	93.9	93.9	93.3								
6) *Nocardioides albus*	92.9	93.8	94.1	93.2	98.6							
7) *Nocardioides simplex* (8929)	92.7	93.0	93.2	92.7	94.2	94.6						
8) *Nocardioides fastidiosa*	92.1	92.1	92.8	92.4	92.4	92.5	94.1					
9) *Rhodococcus erythropolis*	89.0	89.2	90.0	89.3	89.4	89.2	90.8	91.3				
10) *Rhodococcus equi*	89.0	89.0	90.1	89.5	89.0	89.4	90.4	91.3	95.4			
11) *Rhodococcus fascians*	89.0	89.2	90.2	89.4	89.1	89.4	90.2	90.3	95.8	94.5		
12) *Nocardia otitidiscaviarum*	89.0	89.0	90.1	89.2	89.3	89.6	90.2	90.1	95.0	95.6	94.2	
13) *Propionibacterium acnes*	88.7	88.6	89.0	88.8	88.2	87.8	88.3	88.7	87.9	87.7	86.6	87.1

[a]Analysis restricted to approximately 1440 aligned sequence positions.
[b]Sequences of isolates Nd and Nf were 100% similar to that of Nb.

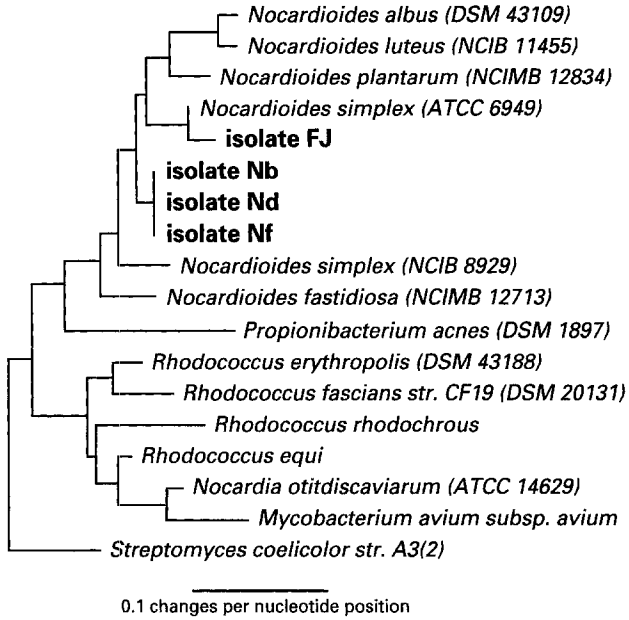

Figure 4 Phylogenetic analysis of rDNA sequences from the picric acid-degrading isolates representing Gram-positive bacteria. Trees were constructed by maximum likelihood analysis. The branching position of sequences Nb and FJ was inferred from analysis of 1245 nucleotides of sequence. The scale bar represents 0.1 fixed mutations per nucleotide position.

All these sequences are highly similar (>97.5%) to the rDNA of *N. simplex* (ATCC 6946).

Detailed studies on the mechanism of degradation of polynitroaromatics in general and picric acid in particular are scarce, partly due to the lack of microorganisms capable of mineralizing these compounds. Recently Lenke and Knackmuss [8] reported that a mutant strain of *Rhodococcus erythropolis* HL 24-2 uses picric acid (0.5 mM) as its nitrogen source when growing on succinate. They postulated involvement of two separate pathways and formation of an orange-colored hydride-Meisenheimer complex of picric acid as an intermediate during degradation of this compound. In the non-productive pathway, addition of two hydride ions to picric acid results in the formation of 2,4,6-trinitrocyclohexanone as a major dead-end metabolite. In the productive pathway, addition of one hydride ion yields a hydride-Meisenheimer complex, which regenerates aromaticity by elimination of nitrite and formation of 2,4-DNP.

The present study provides conclusive evidence for the ability of these strains related to *N. simplex* to degrade picric acid. The results indicate that all three nitro groups are eliminated during picric acid degradation. HPLC and LC/MS analyses showed that degradation of picric acid by any of these microorganisms does not lead to the formation of dead-end metabolites. Results of studies using ^{14}C-UL picric acid showed complete degradation of picric acid with release of up to 65% of the carbon as $^{14}CO_2$. These novel characteristics underline the potential of these isolates for biotreatment of industrial effluents containing picric acid.

While formation of an orange color was sometimes seen in some of these cultures, it remains to be proven if the color is indeed due to the formation of the Meisenheimer complex as previously described [8]. The transient formation of 2,4-DNP together with dual substrate studies and the ability of these organisms to rapidly degrade this compound imply involvement of 2,4-DNP as an intermediate in the degradation of picric acid. The detailed mechanism of picric acid degradation and whether all the strains use the same pathway for this degradation is currently being investigated in our laboratory.

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