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Biodegradation of 4-nitroanisole by two *Rhodococcus* spp.

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Abstract

Two *Rhodococcus* strains, *R. opacus* strain AS2 and *R. erythropolis* strain AS3, that were able to use 4-nitroanisole as the sole source of carbon and energy, were isolated from environmental samples. The first step of the degradation involved the *O*-demethylation of 4-nitroanisole to 4-nitrophenol which accumulated transiently in the medium during growth. Oxygen uptake experiments indicated the transformation of 4-nitrophenol to 4-nitrocatechol and 1,2,4-trihydroxybenzene prior to ring cleavage and then subsequent mineralization. The nitro group was removed as nitrite, which accumulated in the medium in stoichiometric amounts. In *R. opacus* strain AS2 small amounts of hydroquinone were produced by a side reaction, but were not further degraded.

Introduction

Nitroaromatic compounds have entered nature in large amounts as a result of their extensive use in the production of dyes, plastics, and explosives and their application as insecticides and herbicides. Since these chemicals are toxic and mutagenic, it is important to understand their fate once they enter the environment.

Although the nitro group renders the aromatic ring more resistant to biodegradation (Alexander 1994), several nitroaromatic compounds have been shown to be degraded by microorganisms (Higson 1992; Marvin-Sikkema & de Bont 1994; Spain 1995). There are five ways of attacking the nitro group of nitroaromatic compounds. First, the nitro group can be removed as nitrite by a monooxygenase as has been described for a number of nitrophenols (Spain et al. 1979; Zeyer & Kocher 1988). Second, the nitro group can be eliminated as nitrite, following a dioxygenase-catalyzed insertion of two hydroxyl groups (Spanggord et al. 1991; Haigler et al. 1994). Third, the nitro group can be reduced to an amino group, giving rise to aromatic amines which can or cannot be further degraded (Zeyer & Kearney 1984; Preuss et al. 1993). Fourth, the nitro group can be eliminated reductively as nitrite (Lenke et al. 1992; Duque et al. 1993). Fifth, it can be partially reduced to hydroxylamines followed by a replacement reaction (Groenewegen et al. 1992; Haigler & Spain 1993; Rhys-Williams et al. 1993) or Bamberger-like rearrangement reaction (Nishino & Spain 1993) yielding catechols or phenols, respectively.

Nitroanisoles are of great importance in the chemical industry and have caused one of the biggest and most important accidents in the history of the German chemical industry (Heudorf & Peters 1993). Nitroanisoles have been shown to disappear in bacterial mixed cultures (Wellens 1990) and to be transformed unspecifically to the corresponding highly toxic nitrophenols (Edelson & McMullen 1977), but no complete mineralization has been reported yet. In this paper we describe the isolation of two *Rhodococcus* spp. strains that are able to use 4-nitroanisole as the sole source of carbon and energy. Additionally, we propose a pathway for the degradation of 4-nitroanisole by these two microorganisms.

Materials and methods

Chemicals

4-Nitroanisole and 1,2,4-trihydroxybenzene were obtained from Aldrich Chemical Co. (Buchs, Switzerland). 4-Nitrophenol, 4-nitrocatechol, catechol, hydroquinone, and 2-nitroanisole were from Fluka (Buchs, Switzerland). 3-Nitroanisole and 3-nitrophenol were purchased from Merck (Dietikon, Switzerland), and 2-nitrophenol from Riedel-deHaen (Berlin, Germany).

Isolation and identification of bacteria

A mixture of soil samples of different origins was used to inoculate a mineral salts medium (Harms & Zehnder 1994) supplemented with 4-nitroanisole (250 mg l^{-1}) as the sole source of carbon and energy. Batch enrichment cultures were incubated at 25°C on a rotary shaker. After several transfers of the enrichment culture (10%; vol/vol), samples of the culture were plated on solid medium, which was prepared by adding 1.5% agar to the mineral salts medium. A few crystals of 4-nitroanisole were distributed evenly on the agar plates. Two pure cultures that were able to grow on 4-nitroanisole as single carbon source could be isolated and were named AS2 and AS3. The 16S rRNA of both isolates was partially sequenced by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, Germany).

Growth of bacteria

R. opacus strain AS2 and *R. erythropolis* strain AS3 were cultivated in the mineral salts medium with 100 mg l^{-1} or 50 mg l^{-1} 4-nitroanisole, respectively. The concentration of 4-nitroanisole was specifically lower for *R. erythropolis* strain AS3 than for *R. opacus* strain AS2 because concentrations of 100 mg l^{-1} of nitroanisole were found to be toxic for the AS3 strain. Cultures were inoculated with 2% (vol/vol) nutrient broth-grown precultures and incubated at 30°C on a rotary shaker at 180 rpm. During the growth experiments, the optical density at 546 nm was followed photometrically. Samples were taken once every hour, centrifuged (3 min at 14,000 x *g* and 20°C) and the supernatant stored at –20°C until HPLC analysis and nitrite determination were performed.

For the respirometric assays, cells were grown overnight in the mineral medium containing one of the following C-sources: 4-nitroanisole (*R. opacus* strain AS2: 100 mg l^{-1}; *R. erythropolis* strain AS3: 50 mg l^{-1}), 4-nitrophenol (addition of 7 mg l^{-1} every six to fourteen hours), ethanol (2.88 ml l^{-1}), or glucose (2 g l^{-1}). For 4-nitroanisole and 4- nitrophenol as C-sources, the medium was supplemented with 100 mg l^{-1} yeast extract. Bacteria were harvested by centrifugation at 4°C and 8,500 x *g*. The cells were washed with 100 mM pH 7.2 phosphate buffered saline (PBS) that contained 84.4 mM of NaCl, 2.1 mM of KH_2PO_4 and 6.8 mM of K_2HPO_4. The bacteria were resuspended in a small volume of PBS and stored on ice until the measurements were performed.

Substrate utilization

Utilization of aromatic substrates was tested on solid media. Cells were inoculated on mineral salts medium agar in petri dishes. Some substrate crystals were distributed on the agar plates and the plates were incubated at 25°C for up to two weeks. We followed bacterial growth qualitatively and, in case of the colored substrates 4-nitrophenol and 4-nitrocatechol, observed the decolorization of the solid media.

Metabolite production and isolation

R. opacus strain AS2 and *R. erythropolis* strain AS3 were grown in 300 ml of medium in 500ml-bottles at room temperature. Aeration was achieved by stirring vigorously with magnetic stir bars. 4-Nitroanisole was added as the sole carbon source (*R. opacus* strain AS2: 100 mg l^{-1}; *R. erythropolis* strain AS3: 50 mg l^{-1}). Disappearance of 4-nitroanisole and formation of metabolites were followed by HPLC analysis. When all substrate was consumed and a considerable amount of metabolite had been formed, cells were removed by centrifugation, and the supernatants were extracted twice with half the volume of ethyl acetate. After drying the extract with anhydrous sodium sulfate and filtering it through a glass filter, the solvent was evaporated and the residue was dissolved in a small volume of methanol.

Analytical methods

HPLC analysis of the culture fluids was performed on a LiChrospher® 100 RP–8 column (5 μm), 215 mm by 4 mm internal diameter (Merck, Dietikon, Switzerland). The mobile phase was methanol-water (60:40; vol/vol) with 0.05% phosphoric acid. The flow rate was set to

1 ml min^{-1}. 4-Nitroanisole and 4-nitrophenol were detected and quantified by their absorbance at 317 nm with a diode array detector 440 (Kontron Instruments, Schlieren, Switzerland). 4-Nitroanisole and 4-nitrophenol exhibit UV-adsorption maxima at 316 nm (Haderlein & Schwarzenbach 1993; Schwarzenbach et al. 1988). Both compounds were identified by comparison of retention times and UV spectra with those of authentic standards.

Mass spectra were obtained with a Finnigan MAT ITD 800 (ion trap detection) mass spectrometer (Finnigan MAT, San Jose, Calif.) coupled to a Carlo Erba HRGC 5160 Mega Series gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a 15m PS086 (88% Dimethyl, 12% Diphenyl) glass capillary column. Electron ionization was used. The injection (0.7 μl) was administered on column at 70°C. The temperature program was run from 70 to 230°C with a rate of 10°C min^{-1}. Samples were dissolved in ethyl acetate and injected without derivatization.

Nitrite was determined colorimetrically according to the method of Moir et al. (1993). Oxygen uptake rates were determined with a Clark-type oxygen electrode at 30°C. Reaction mixtures contained cells (OD_{546} = 0.7–1.0) in 3 ml of 100 mM PBS. After recording the endogenous oxygen uptake, 50 μM of the aromatic substrate were added and the oxygen uptake was measured for another ten to twenty minutes. Oxygen uptake rates were calculated by subtracting the endogenous oxygen consumption. Protein contents of the reaction mixture were determined with the Bio-Rad Protein Microassay (Bio-Rad, Glattbrugg, Switzerland) after the cells had been broken by a cell homogenizer (Braun, Melsungen, Germany).

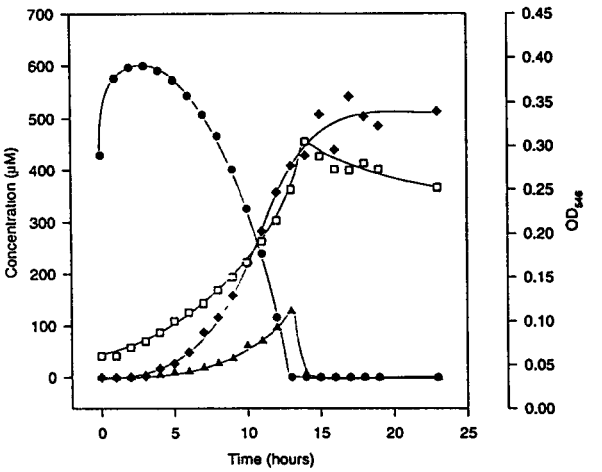

Figure 1. Growth of *R. opacus* strain AS2 on 4-nitroanisole. Symbols represent 4-nitroanisole (●), 4-nitrophenol (▲), nitrite (♦), and the OD_{546} (□).

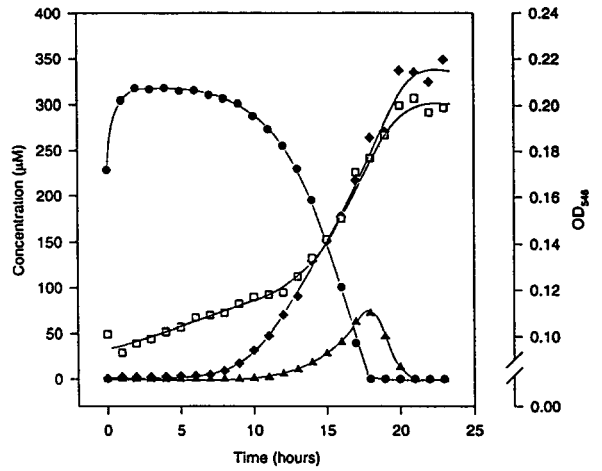

Figure 2. Growth of *R. erythropolis* strain AS3 on 4-nitroanisole. Symbols represent 4-nitroanisole (●), 4-nitrophenol (▲), nitrite (♦), and the OD_{546} (□).

Results

Characterization of the bacterial strains

Identification of the strains was based on the following characteristics:

- both strains are gram-positive,
- they possess a pronounced cell cycle, and
- both strains contain *m*-diaminopimelic acid as diamino acid in the cell wall (data not shown).

We tentatively identified the strains as *Rhodococcus* spp. Partial 16S rRNA sequencing revealed 100% sequence homology with *Rhodococcus opacus* for strain AS2 and 99.5% sequence homology with *Rhodococcus erythropolis* for strain AS3. Because the species level is reached at 99% 16S rRNA sequence homology, both strains can be designated to the respective species.

Growth of R. opacus strain AS2 and R. erythropolis strain AS3 on 4-nitroanisole

The growth of *R. erythropolis* strain AS2 and *R. opacus* strain AS3 with 4-nitroanisole as the only source of carbon and energy is shown in Figures 1 and 2. The con-

centration of 4-nitroanisole increased during the first 3 hours because it had not yet dissolved completely at the beginning of the experiment. The amount of substrate added corresponded to 653 μM for *R. opacus* strain AS2 and 326 μM for *R. erythropolis* strain AS3. After five to ten hours the substrate concentration began to decrease rapidly. Complete degradation was achieved after 13 and 18 hours for AS2 and AS3, respectively. 4-Nitrophenol (for identification see below) transiently accumulated in the medium during growth of both strains, but disappeared soon after the complete degradation of 4-nitroanisole. 4-Nitrophenol never appeared in high concentrations indicating its subsequent metabolization during the degradation of 4-nitroanisole. The fact that 4-nitrophenol was only released transiently in minor amounts is further confirmed by the growth of cells which paralleled the decrease of nitroanisole. A small peak whose retention time corresponded to that of 4-nitrocatechol appeared transiently in both growth experiments (data not shown). The OD_{546} increased until all 4-nitroanisole had disappeared. The subsequent decrease in OD_{546} might be explained by the lysis of a fraction of the cells or by changes of the cell shape as a result of the cell cycle. The nitro group of 4-nitroanisole was released into the medium as nitrite in stoichiometric amounts by *R. erythropolis* strain AS3, while only 80% of the expected nitrite was detected for *R. opacus* strain AS2. This deficiency can be explained by the fact that both strains are able to use nitrite as nitrogen source (data not shown). The doubling times of *R. opacus* strain AS2 and *R. erythropolis* strain AS3 were 5.9 h and 10.4 h, respectively. Maximal specific degradation rates of 4-nitroanisole were 18.4 μmol mg protein^{-1} h^{-1} for *R. opacus* strain AS2 and 12.1 μmol mg protein^{-1} h^{-1} for *R. erythropolis* strain AS3.

Utilization of nitroaromatic substrates

R. opacus strain AS2 grew on solid media with 4-nitroanisole, 4-nitrophenol, 4-nitrocatechol, and catechol as the sole sources of carbon and energy, but not with hydroquinone, 2-nitroanisole, 3-nitroanisole, 2-nitrophenol, or 3-nitrophenol. *R. erythropolis* strain AS3 showed the same substrate spectrum. Growth on 1,2,4-trihydroxybenzene could not be investigated with the method applied here since the compound spontaneously transforms to the red hydroxy–1,4-benzoquinone in aqueous media (Jain et al. 1994). Degradation of in aqueous solution colored substrates 4-nitrophenol and 4-nitrocatechol by both strains could be seen from the decolorization of the plates. Growth free zones around the crystals of 4-nitroanisole and catechol indicated inhibition of growth by high concentrations of these compounds.

Identification of metabolites

The ethylacetate extracts of the supernatants of *R. opacus* strain AS2 and *R. erythropolis* strain AS3 both contained mainly one compound with an HPLC retention time and a UV-Vis spectrum identical to those of 4-nitrophenol (data not shown). In aqueous solution the metabolite exhibited a bright yellow color and an absorption maximum at 400 nm corresponding to the properties of 4-nitrophenolate. The mass-spectra (not shown) were identical to those of authentic 4-nitrophenol. A small amount of hydroquinone could be detected by GCMS analysis in the ethylacetate extract of *R. opacus* strain AS2. In addition, ethylacetate extracts of both strains contained small quantities of a compound with the same retention time and UV-Vis spectrum as 4-nitrocatechol.

Oxidation of aromatic compounds by resting cells

The substrate specific oxygen consumption rates for a variety of substrates were measured in 4-nitroanisole-, 4-nitrophenol-, ethanol-, and glucose-grown cells (Table 1). Absolute oxygen consumption rates differed from experiment to experiment, though the normalized consumption rates were rather similar. This may be due to the fact that depending on the age of the preculture, the bacterial cultures grew with slightly different rates, and therefore, were harvested in slightly different physiological states. In Table 1 typical results for oxygen uptake rates are shown.

Oxygen consumption of 4-nitroanisole-grown cells of both strains was stimulated by 4-nitroanisole, 4-nitrophenol, 4-nitrocatechol, and 1,2,4-trihydroxybenzene. Catechol and 3-nitroanisole also stimulated the oxygen consumption significantly, although the rates were lower. 2-Nitrophenol was only oxidized by *R. opacus* strain AS2, and hydroquinone, 2-nitroanisole, and 3-nitrophenol were not oxidized at all. 4-Nitrophenol-grown cells generally showed lower oxygen consumption rates than 4-nitroanisole-grown cells which may reflect the high toxicity of nitrophenol. Normalized oxygen consumption rates of 1,2,4-trihydroxybenzene were higher for 4-nitrophenol-grown cells than for 4 -nitroanisole-grown cells. None of the nitroaromatic compounds were oxidized by cells grown on glucose or ethanol.

Table 1. Specific oxygen uptake rates by resting cells of *R. opacus* strain AS2 and *R. erythropolis* strain AS3.

Strain and substrate	Specific oxygen rates (nmol O_2 min^{-1} mg protein $^{-1}$) after growth with					
	4-Nitroanisole		4-Nitrophenol		Glucose	Ethanol
AS2						
4-Nitroanisole	568	(100%)	192	(100%)	< 5	ND[a]
4-Nitrophenol	249	(44%)	91	(48%)	< 5	ND
Hydroquinone	< 5		< 5		< 5	ND
1,2,4-Trihydroxybenzene	86	(15%)	70	(36%)	18	ND
4-Nitrocatechol	159	(28%)	74	(38%)	< 5	ND
Catechol	12	(2%)	8	(4%)	< 5	ND
2-Nitroanisole	< 5		< 5		< 5	ND
3-Nitroanisole	10	(2%)	< 5		< 5	ND
2-Nitrophenol	24	(6%)	< 5		< 5	ND
3-Nitrophenol	< 5		< 5		< 5	ND
AS3						
4-Nitroanisole	390	(100%)	101	(100%)	ND	< 5
4-Nitrophenol	154	(39%)	18	(18%)	ND	< 5
Hydroquinone	< 5		< 5		ND	< 5
1,2,4-Trihydroxybenzene	111	(28%)	102	(101%)	ND	5
4-Nitrocatechol	66	(17%)	18	(18%)	ND	< 5
Catechol	27	(7%)	< 5		ND	< 5
2-Nitroanisole	< 5		< 5		ND	< 5
3-Nitroanisole	20	(4%)	< 5		ND	< 5
2-Nitrophenol	< 5		< 5		ND	< 5
3-Nitrophenol	< 5		< 5		ND	< 5

[a]ND = Not Determined.

Discussion

We have been able to isolate two *Rhodococcus* strains that are capable of using 4-nitroanisole as the sole source of carbon and energy. A metabolite that accumulated transiently in the medium during growth of both strains was identified as 4-nitrophenol by GCMS. This suggests an *O*-demethylation of 4-nitroanisole to 4-nitrophenol. *Escherichia coli* has been reported to *O*-demethylate 4-nitroanisole to 4-nitrophenol (Edelson & McMullen 1977), but was not able to metabolize 4-nitrophenol any further. *O*-Demethylation of methoxylated aromatic compounds to the corresponding phenols has been described for a number of compounds under aerobic (Bernhardt et al. 1977; Cartwright & Smith 1967) and anaerobic conditions (Bache & Pfennig 1981; Frazer & Young 1986). According to existing reports, the aerobic removal of the methyl group is conducted by a monooxygenase. Iron-sulfur proteins (Bernhardt et al. 1975) and cytochromes P–450 (Sutherland 1986; Karlson et al. 1993) have been shown to be responsible for this reaction. Cartwright & Smith (1967) reported that the methyl group is removed as formaldehyde and converted via formate to carbon dioxide under aerobic conditions.

For the degradation of 4-nitrophenol, two possible pathways have been described. One pathway involves the transformation of 4-nitrophenol to hydroquinone via 4-benzoquinone and then subsequent conversion to γ-hydroxymuconic semialdehyde and maleylacetate (Spain et al. 1979; Spain & Gibson 1991). Alternatively, 4-nitrophenol can be converted to maleylacetate via 4-nitrocatechol and 1,2,4-trihydroxybenzene (Jain et al. 1994). Oxygen uptake of whole cells of *R. opacus* strain AS2 and *R. erythropolis* strain AS3 could be increased by 4-nitrocatechol or 1,2,4-trihydroxybenzene, but not by hydroquinone (Tab.1). The nitrophenol-degrading strains both exhibited increased oxygen uptake rates for 4-nitrocatechol and 1,2,4-trihydroxybenzene as the substrates (Spain et al. 1979; Spain & Gibson 1991). However, in case of degradation of 4-nitrophenol via hydroquinone, large oxygen uptake rates with hydroquinone as the substrate were reported (Spain & Gibson 1991), whereas for degradation of 4-nitrophenol via 4-nitrocatechol, no oxygen uptake with hydroquinone was detected (Jain

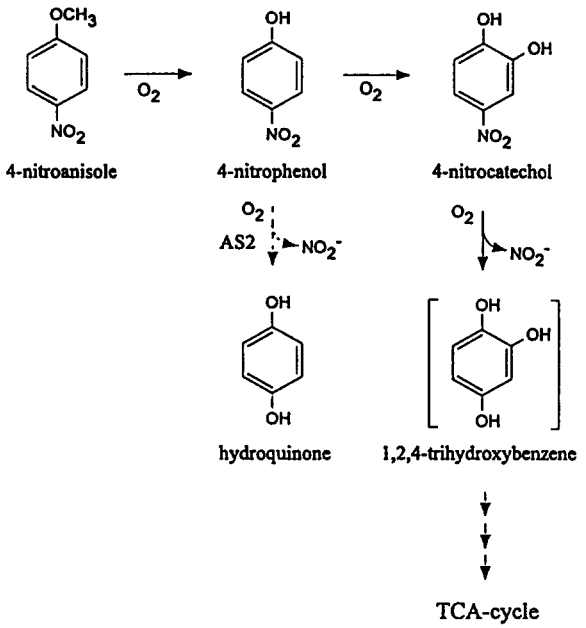

Figure 3. Proposed pathway for the degradation of 4-nitroanisole by *R. opacus* strain AS2 and *R. erythropolis* strain AS3.

et al. 1994). For our isolates, *R. opacus* strain AS2 and *R. erythropolis* strain AS3, the absence of any activity towards hydroquinone strongly indicates the degradation of 4-nitrophenol via 4-nitrocatechol and 1,2,4-trihydroxybenzene as depicted in Figure 3. This assumption is supported by the fact that traces of 4-nitrocatechol could be detected in ethylacetate extracts of both strains.

4-Nitroanisole is demethylated by an oxygenase to 4-nitrophenol. The methyl group is probably removed as formaldehyde, that may be further oxidized to carbon dioxide (Cartwright & Smith 1967). 4-Nitrophenol is hydroxylated to 4-nitrocatechol. Subsequently, the nitro group is removed by an oxygenase and released as nitrite. The stoichiometric release of nitrite confirms this nitro group elimination. The resulting 1,2,4-trihydroxybenzene can be cleaved and fed into the tricarboxylic acid cycle. A minor metabolic side reaction led to the formation of small amounts of hydroquinone by *R. opacus* strain AS2. Because *R. opacus* strain AS2 is unable to grow on hydroquinone on solid medium, or to degrade hydroquinone in liquid medium (data not shown), and its oxygen uptake rate is not stimulated by hydroquinone, it was assumed that the hydroquinone detected is a dead end product.

We have shown that the degradation of 4-nitroanisole by two *Rhodococcus* spp. involves the transient accumulation of 4-nitrophenol in the medium. 4-Nitrophenol is much more toxic than 4-nitroanisole because it can uncouple the proton transport in the membrane and thereby inhibit ATP-production. As a consequence, when the prediction of the fate of nitroanisoles in the environment is intended, one has to take into account that bacteria can not only metabolize chemicals in a beneficial way, but can also generate even more hazardous compounds. These compounds may accumulate under certain conditions and prevent further degradation.

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