Microbial transformation of nitroaromatic compounds under anaerobic conditions

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The transformation of several mono- and dinitroaromatic compounds (tested at 50–200 µM) by methanogenic bacteria, sulphate-reducing bacteria and clostridia was studied. Some of the nitroaromatics tested were transformed chemically by 1.5 mM quantities of culture media reducing agents, like cysteine or sulphide. This abiotic reduction occurred at the *o*-nitro-groups preferentially. Nitrophenols, *p*-nitroaniline and *p*-nitrobenzoic acid were completely transformed biologically into the corresponding amino derivatives. The nitroaromatics were transformed by all of the bacterial strains tested. While growing cells of sulphate-reducing bacteria and *Clostridium* spp. carried out nitroreduction, methanogen cells lysed in the presence of nitroaromatics. Nevertheless these culture suspensions converted nitroaromatics to the corresponding amino derivatives. This was also confirmed by crude cell extracts of methanogenic bacteria. The rate of nitroreduction by sulphate-reducing bacteria depended on the electron donors supplied and the cell density, with molecular hydrogen being the most effective donor of reducing equivalents. The toxicity of *p*-nitrophenol to some of the organisms tested depended on the concentration of the nitroaromatic compound and the type of organism.

Introduction

Nitroaromatic compounds are released into the environment in large quantities, since they are widely used not only as pesticides, but also as explosives and precursors for dyes, pharmaceuticals and plastics. They are also formed during incineration of organic compounds and synthesized photochemically in the atmosphere (Gibson, 1982). Some nitroaromatic compounds, such as 1-nitropyrene, are highly mutagenic and/or carcinogenic (Thiem *et al.*, 1979), and several nitroaromatics are potent uncouplers of oxidative and photosynthetic phosphorylation (Terada, 1981; Shea *et al.*, 1983). Once released into the environment, nitroaromatics underlie complex physical, chemical, and biological processes. Depending on the prevailing conditions they may be transformed photochemically (Nakagawa & Crosby, 1974), or reduced electrochemically (Macalady *et al.*, 1986). Nitroaromatics can also be transformed by micro-organisms under aerobic

conditions (Germanier & Wuhrmann, 1963; Zeyer & Kearney, 1984; Schackmann & Müller, 1991; Spain & Gibson, 1991). Little is known about the transformation of nitroaromatics by growing cultures of anaerobic bacteria. Transformation reactions have been examined mostly in mixed cultures and enzyme preparations (Boyd *et al.*, 1983; Hallas & Alexander, 1983; Zeyer & Kearney, 1984; Blotevogel & Butte, 1989). Specific nitroreductases catalysing the reduction of nitro-groups of aromatics to amino-groups have been isolated and characterized (Kinoushi & Ohnishi, 1983; Bryant & DeLuca, 1991; Rafii *et al.*, 1991). One of these enzymes acts as an electron carrier protein for the artificial electron-acceptor menadione, indicating quinone reductase activity (Bryant & DeLuca, 1991).

The present study was performed to investigate the behaviour of nitroaromatics in the presence of pure cultures of sulphate-reducing bacteria, methanogenic bacteria, and *Clostridium* spp., as well as the effect of nitroaromatics on these bacteria.

Methods

Strains of bacteria. The following strains of bacteria were used: *Methanobacterium formicicum* (DSM 1535), *Methanobacterium thermo-*

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Abbreviations: p-NP, *p*-nitrophenol; p-AP, *p*-aminophenol; m-NP, *m*-nitrophenol; p-NB, *p*-nitrobenzoic acid.

autotrophicum (DSM 1053), *Methanosarcina barkeri* (DSM 800), *Methanosarcina frisia* (DSM 3318), *Methanosarcina* sp. KS2002 (isolated from sewage sludge), *Methanospirillum hungatei* (DSM 864), *Methanogenium tationis* (DSM 2702) and *Methanoculleus oldenburgensis* (DSM 6216); *Desulfovibrio desulfuricans* (DSM 642), *Desulfovibrio gigas* (DSM 496), *Desulfovibrio* sp. AS (isolated from sediment from the river Amazon, Brazil), *Desulfovibrio* sp. HB (isolated from sewage sludge), *Desulfococcus multivorans* (DSM 2059), *Desulfotomaculum orientis* (DSM 765), *Desulfotomaculum* sp. GROL (isolated from a ditch sediment at Bremen, FRG); *Clostridium pasteurianum* (DSM 525) and *Clostridium* sp. W1 (isolated from soil).

Chemical transformation. An appropriate amount of each nitroaromatic compound was placed into serum flasks containing 50 ml potassium phosphate buffer (50 mM; pH 7.0) to give a final concentration of 0.2 mM. Then the reducing agents Ti(III) nitrilotriacetate, sodium dithionite, sodium sulphide, sodium sulphite, and cysteine. HCl were added to give a final concentration of between 1.5 and 10.0 mM. Before analysis the reaction mixtures were incubated at 37 °C for one week.

Media and culture conditions. The methanogenic bacteria were cultivated and maintained as previously described (Balch *et al.*, 1979; Blotevogel *et al.*, 1986), with molecular hydrogen and carbon dioxide as energy and carbon sources. The sulphate-reducing bacteria were cultivated and maintained as described by Widdel & Bak (1992). Unless otherwise indicated, the sulphate-reducing bacteria were grown with sulphate (20 mM) and lactate (20 mM) except for *Desulfotomaculum* sp. GROL, which was grown with butanol (20 mM) as energy and carbon source. The clostridia were grown in the medium described by Widdel & Bak (1992), with 10 mM-fructose as energy and carbon source. Experiments with added nitroaromatics were also performed under these conditions, while transformation experiments with p-NP were carried out in fed-batch culture. Samples were withdrawn every 24 h and the losses of p-NP, the production of p-AP, and the growth parameters were measured. All experiments were carried out at 37 °C in triplicate.

Analytical procedures. The optical density was measured spectrophotometrically at 580 nm. The protein content was determined as described by Bradford (1976) with bovine serum albumin as standard. Methane was analysed by gas chromatography with a Varian model 6000 gas chromatograph equipped with a thermal conductivity detector. The injector and the detector were heated to 70 °C and 180 °C, respectively; the oven temperature was 50 °C. The gases were separated from each other in a 2 m glass column packed with Porapak Q (Supelco) with 30 ml N₂ min⁻¹ as a carrier gas.

The nitroaromatic and aminoaromatic compounds were measured by means of UV/VIS-spectrophotometry based on their specific absorbance at a certain wavelength (Alexander & Lustigman, 1966; Hallas & Alexander, 1983). Absorption spectra were taken from 200 to 500 nm after precipitating the hydrogen sulphide in the 1 ml sample with 0.1 ml 2% (w/v) zinc acetate solution. Reference spectra of the nitro- and aminoaromatics (20–1000 µM) were determined in phosphate-buffered standard solutions (10 mM; pH 7.2). Disappearance of nitrophenols and production of aminophenols was determined by reverse-phase HPLC [LiChrosorb C18 (10 µm); eluent H₂O/methanol/glacial acetic acid (65:30:5 by vol.), pH 3.0; flow rate 2.0 ml min⁻¹; detection UV 254 nm].

Chemicals. All chemicals were of reagent grade and were obtained from Serva, Fluka or Janssen. For the preparation of 100 mM stock solutions the nitroaromatics and aminoaromatics were dissolved in deionized distilled water with the exception of 2,4-dinitrophenol, which was dissolved in methanol (97%, v/v).

Results

Chemical reduction of nitroaromatics by medium constituents and possible metabolic products

In order to differentiate chemical from biological transformation reactions it was first necessary to study the behaviour of the nitroaromatics in the presence of typical reducing agents which are used for the preparation of media for anaerobic bacteria (Table 1). *o*-Nitrophenol (*o*-NP) was completely reduced chemically to its corresponding amino derivative by the reducing agents sulphide and/or cysteine. Therefore it was not used to study biological transformation. The transformation of 2,4-dinitrophenol (2,4-DNP) by sulphide and cysteine resulted in the formation of 2-amino-4-nitrophenol; *p*-nitroaniline (*p*-NA) was transformed to an unidentified product under the same conditions. Ti(III) nitrilotriacetate (Ti-NTA) and sodium dithionite caused a complete transformation of every nitroaromatic compound tested into the corresponding amino- or diamino-derivative; therefore these compounds were not used in biological transformation experiments.

Reduction of nitroaromatics by growing cultures of bacteria

Uninoculated and autoclaved controls showed no transformation of the nitroaromatics tested. Only 2,4-dinitrophenol and *p*-nitroaniline were partially transformed by media reducing agents. All of the organisms studied were able to perform the transformation of nitroaromatic compounds and to complete the partial transformation of 2,4-dinitrophenol and *p*-nitroaniline (Table 2). *Desulfococcus multivorans* and *Desulfotomaculum orientis* only transformed *p*-nitrophenol. Transformation always resulted in the formation of amino-

Table 1. Transformation of several nitroaromatic compounds by reducing agents

Experiments were carried out at pH 7.2 and 37 °C for 7 d. Each reducing agent was tested at 1.5–10 mM.

Nitroaromatic compound (0.5 mM)	Reducing agent*				
	Ti-NTA	Dithionite	Sulphite	Sulphide	Cysteine
p-NP	+	+	–	–	–
m-NP	+	+	–	–	–
o-NP	+	+	–	+	+
2,4-DNP	+	+	–	±	±
p-NB	+	+	–	–	–
p-NA	+	+	–	±	±

* Ti-NTA, Ti(III) nitrilotriacetate; +, complete transformation into the corresponding amino derivative; ±, partial transformation; –, no transformation

Table 2. Transformation of several nitroaromatic compounds by growing cultures of methanogenic bacteria, sulphate-reducing bacteria and *Clostridium*

The compounds were used at 50 μM , 100 μM and 100 μM for the three groups of bacteria respectively. Experiments were carried out in typical media for 7 d (except *Ms. barkeri* and *Methanosarcina* sp. KS2002, which were incubated for 14 d at pH 7.0–7.2 and 30 °C/37 °C. Complete transformation of the nitroaromatics was ascertained by UV/VIS and HPLC. The abiotically transformed nitroaromatics 2,4-dinitrophenol and *p*-nitroaniline were investigated for complete transformation by the listed organisms.

Organism	Nitroaromatic compound*				
	p-NP	m-NP	2,4-DNP	p-NB	p-NA
<i>Methanobacterium formicum</i>	+	+	+	+	+
<i>thermoautotrophicum</i>	+	+	+	+	+
<i>Methanospirillum hungatei</i>	+	+	+	+	+
<i>Methanosarcina barkeri</i>	+	+	+	+	+
<i>frisia</i>	+	+	+	+	+
sp. KS2002	+	+	+	+	+
<i>Methanogenium tationis</i>	+	+	+	+	+
<i>Methanoculleus oldenburgensis</i>	+	+	+	+	+
<i>Desulfovibrio desulfuricans</i>	+	+	+	+	+
<i>gigas</i>	+	+	+	+	+
sp. AS	+	+	+	+	+
sp. HB	+	+	+	+	+
<i>Desulfococcus multivorans</i>	+	—	—	—	—
<i>Desulfotomaculum orientis</i>	+	—	—	—	—
sp. GROL	+	+	+	+	+
<i>Clostridium pasteurianum</i>	+	+	+	+	+
sp. W1	+	+	+	+	+

* +, 100 % transformation into the corresponding amino derivative; —, no transformation.

aromatics as ascertained by UV/VIS-spectrophotometry and HPLC.

As a typical representative of the sulphate-reducing bacteria, *Desulfovibrio gigas* was studied more extensively. The nitro-group reduction (0.1 mM each maximally) by growing cultures of *Desulfovibrio gigas* was strongly correlated with the growth phase (Fig. 1). Controls without added nitroaromatics showed the same course of growth. The maximum rate of p-NP transformation during the exponential phase of growth was around 0.28 $\mu\text{mol (mg protein)}^{-1} \text{ h}^{-1}$.

The two *Clostridium* species studied were also able to completely transform all the nitroaromatics tested to their corresponding amino derivatives (Table 2). The *Clostridium* spp. showed the same transformation behaviour as *Desulfovibrio gigas*. Again the course of growth was not affected by the nitroaromatic compound. The maximum rate of p-NP transformation during the exponential phase of growth was about 0.42 $\mu\text{mol (mg protein)}^{-1} \text{ h}^{-1}$.

The transformation of nitroaromatic compounds by methanogenic bacteria only occurred while cells lysed. Therefore it was necessary to pregrow the cells to an optical density of about 0.15 before adding the nitroaromatics. As shown in Table 2, all of the methanogens tested were able to perform a complete transformation of the nitroaromatics. *Methanosarcina frisia* was investigated in more detail. The results in Fig. 2 indicate that p-NP was transformed by growing cultures of *Methanosarcina frisia* (OD_{580} 0.15) accompanied by a decrease in the optical density. As long as p-NP was present, the methane production ceased entirely. When transformation had been completed, growth and methane production of *Methanosarcina frisia* recovered.

Effect of different electron donors on the rate of nitroaromatic reduction by *Desulfovibrio gigas*

Further experiments with *Desulfovibrio gigas* showed that different electron donors affected the p-NP trans-

Fig. 1. Repeated transformation of p-NP (100 μ M) by cultures of *Desulfovibrio gigas* with lactate and sulphate (each 20 mM) as carbon and energy sources. $\circ$, OD₅₈₀; $\blacksquare$, p-NP recovery (%); $\downarrow$ addition of p-NP (100 μ M).

Fig. 2. Repeated transformation of p-NP (50 μ M) by cultures of *Methanosarcina frisia* with molecular hydrogen and carbon dioxide as carbon and energy sources. $\circ$, OD₅₈₀; $\blacksquare$, p-NP recovery (%); $\downarrow$ addition of p-NP (50 μ M).

formation differently (Fig. 3a). The following sequence of transformation velocity could be observed: molecular hydrogen > pyruvate > lactate > ethanol. Except in the case of molecular hydrogen the transformation velocity was correlated with the specific growth rates and the biomass yields (Fig. 3b). With molecular hydrogen the transformation was completed within the early stages of the exponential-phase of growth.

Effect of nitro- and aminoaromatics on growth

Concentrations of p-NP higher than 0.2 mM impaired the growth of *Desulfovibrio gigas*, which became apparent by an extended lag phase. A concentration of 1.0 mM led to complete inhibition of growth. The transformation

Fig. 3. (a) Influence of different carbon and energy sources on the reduction of p-NP (200 μ M) by growing cultures of *Desulfovibrio gigas*. (b) Growth of *Dv. gigas* with different energy and carbon sources in the presence of 200 μ M p-NP. $\circ$, Control; $\bullet$, ethanol (30 mM); $\square$, lactate (20 mM); $\blacksquare$, pyruvate (20 mM); and ∇ , H₂/CO₂ plus acetate (20 mM).

product *p*-aminophenol (p-AP; 0.1 to 10 mM) did not influence growth. Similar results were obtained with *Clostridium pasteurianum*, but this organism tolerated higher concentrations of p-NP than did *Desulfovibrio gigas*. Complete inhibition of growth of *Clostridium pasteurianum* was observed in the presence of 5.0 mM-p-NP. The transformation product p-AP did not influence the growth of this organism nor the growth of *Methanosarcina frisia*.

Discussion

Our results conclusively show that under anaerobic conditions nitroaromatics were converted into the corresponding amino compounds by living bacteria as well as abiotically. Chemical transformation occurred in the presence of media-reducing compounds like sulphide or Ti(III) nitrilotriacetate (Table 1). In the case of *o*-nitrophenol and 2,4-dinitrophenol, nitro-group transformation occurred only in the ortho-position; the transformation product of *p*-nitroaniline is not known.

In contrast to recent findings (Macalady *et al.*, 1986), our results clearly demonstrate that nitroaromatics (0.2 mM) can be reduced directly by certain reductants (1.5 mM). A chemical transformation of 2,4,6-trinitrotoluene to 2,4-diamino-6-nitrotoluene by sulphide ions was also observed (Preuss *et al.*, 1990).

Biological transformation of certain nitroaromatics by cell-free extracts of anaerobic bacteria has been observed in *Clostridium* spp. (O'Brien & Morris, 1971; Angermaier & Simon, 1983; Raffi *et al.*, 1991), *Bacteroides fragilis* (Kinoushi & Ohnishi, 1983), *Veillonella alcalescens* (McCormick *et al.*, 1976) and *Enterobacter cloacae* (Bryant & DeLuca, 1991). Our results extend this range of species for which transformation has been demonstrated to some strains of sulphate-reducing and methanogenic bacteria (Table 2). According to other authors (Boyd *et al.*, 1983; Battersby & Wilson, 1989) the reductive transformation of nitroaromatic compounds leads to a detoxification of the substance.

Methanogenic bacteria lysed in the presence of nitroaromatics. This was correlated with the reduction of the nitro-substituent to the corresponding amino-group. In Fig. 2 the behaviour of *Methanosarcina frisia* in the presence of 50 µM p-NP is shown. Cell lysis does not depend on the type of nitroaromatic compound. This may be the reason why methanogenesis is inhibited by nitroaromatics during anaerobic sewage treatment (Horowitz *et al.*, 1982; Boyd *et al.*, 1983; Johnson & Young, 1983). However, Fedorak *et al.* (1990) found no evidence that anilines negatively influenced methanogenesis. This was also confirmed in our experiments: after the reduction of the nitro-substituent, the resulting aminoaromatic molecule no longer hindered cell growth. Additionally, other aminoaromatics did not cause cell lysis. Therefore we assumed that nitroaromatics or intermediates of the reduction process like nitroso- or hydroxylamines are the real toxicants, since they may react with the unique cell membrane components of the methanogens.

A second toxic function of a nitroaromatic compound might be determined by the fact that it acts as an 'electron trap'. This has already been assumed for the oxygen sensitivity of methanogens (Jarrell, 1985), consequently leading to the breakdown of ATP synthesis. A third possibility might be the uncoupling activity of nitroaromatics on electron transport chains thus inhibiting ATP synthesis, followed by cell lysis. Although information about the biochemistry of nitro-group reduction under methanogenic conditions is rather poor, it cannot be excluded that the coenzyme F₄₂₀ is involved in such a reaction due to its low redox potential ($E'_0 = -340$ mV). Apparently the methanogens were more sensitive to the presence of nitroaromatics than were the sulphate-reducing bacteria and clostridia. This

is probably due to the destruction of the twofold function of the cell membranes of most methanogens as a physiological barrier and as the cell skeleton. In sulphate-reducing bacteria and clostridia such a destructive effect of nitroaromatics might be prevented by the presence of a murein-containing cell wall and a different composition of the cell membrane.

In contrast to the methanogens, *Clostridium* spp. and sulphate-reducing bacteria reduced the nitro-substituents without cell lysis (Table 2). It is not known why *Desulfococcus multivorans* and *Desulfotomaculum orientis* only transformed p-nitrophenol. As shown in Fig. 1, there is a strong correlation between the growth phase and the reduction process. In addition, p-NP reduction by *Desulfovibrio gigas* is also dependent on the kind of electron donor. Due to the electron donor applied and the biomass yielded the following sequence of the rate of transformation of p-NP could be measured: ethanol < lactate < pyruvate < hydrogen (Fig. 3a, b). To our knowledge there are no specific nitroreductases in sulphate-reducing bacteria. Hydrogenase, together with a ferredoxin-like protein, was demonstrated to function as a nitroreductase in *Veillonella alcalescens* and *Clostridium pasteurianum* (McCormick *et al.*, 1976). A mechanism for the reduction of aromatic nitro-compounds catalysed by hydrogenase and ferredoxin was also proposed for *Clostridium* (Angermaier & Simon, 1983). But other enzymes like pyruvate:ferredoxin oxidoreductases, sulphite reductases, nitrite reductases, or quinone reductases are able to transform nitroaromatics as well (Kinoushi & Ohnishi, 1983; Bryant & DeLuca, 1991).

No degradation of the amino-substituted aromatics was observed with the above-mentioned micro-organisms. Therefore we conclude that the reduction of nitro-substituents in these organisms is an unspecific detoxification reaction mediated by certain enzymes and/or cofactors.

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