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Degradation of 2,4-Dinitrophenol by Two *Rhodococcus erythropolis* Strains, HL 24-1 and HL 24-2

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Two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2, were isolated from soil and river water by their abilities to utilize 2,4-dinitrophenol (0.5 mM) as the sole source of nitrogen. Although succinate was supplied as a carbon and energy source during selection, both isolates could utilize 2,4-dinitrophenol also as the sole source of carbon. Both strains metabolized 2,4-dinitrophenol under concomitant liberation of stoichiometric amounts of nitrite and 4,6-dinitrohexanoate as a minor dead-end metabolite.

Nitroaromatic compounds are the building blocks of many pesticides and dyes. They are also used as precursors of arylamines. Therefore, nitroarenes are found as contaminants in waste waters, rivers, and groundwater, in pesticide-treated soils, and in the atmosphere (9, 21, 24, 27). A variety of nitroaromatic compounds are described as being toxic to microorganisms. 2,4-Dinitrophenol (2,4-DNP) is well known as an uncoupler of electron transport phosphorylation (15, 22, 30). Its structural analogs, 4,6-dinitro-2-methylphenol and 2-*sec*-butyl-4,6-dinitrophenol (Dinoseb), are important pesticidal chemicals (24).

Although a number of organisms that utilize DNPs as the sole source of carbon and nitrogen have been described, little is known about the metabolic pathways of these chemicals (7, 10, 12, 16, 17, 29, 31, 34). In most cases, decoloration of the culture fluid and accumulation of nitrite were observed. Tewfik and Evans (34) reported very briefly on a pseudomonad that metabolized 4,6-dinitro-2-methylphenol along a reductive pathway with 4-amino-2-methyl-6-nitrophenol, 5-amino-3-methylcatechol, and 2,3,5-trihydroxytoluene as metabolites.

As described by Bruhn et al. (2), under nitrogen-limiting conditions, nitroaromatic-degrading organisms can easily be enriched from soil in the presence of a readily degradable carbon source. Low concentrations of the nitrocompound were used as the selective substrate. Following this method, the present communication describes the isolation of bacteria with 2,4-DNP as the sole source of nitrogen and succinate as a carbon source.

MATERIALS AND METHODS

Isolation and characterization of organisms. Mixed samples of soil from the area of Wuppertal, Germany, and samples of water from the river Rhine were inoculated in 50 ml of nitrogen-free mineral medium supplemented with 0.5 mM 2,4-DNP as the nitrogen and 10 mM succinate as the carbon source. When the medium was incubated on a rotary shaker, its yellow coloration disappeared within 5 days. A 5-ml volume of the suspension was transferred into 50 ml of fresh medium. In order to isolate pure cultures, samples of the culture were plated on solid mineral media with 2,4-DNP and succinate. After an incubation time of 1 week, single colonies were tested for the ability to use 2,4-DNP as a nitrogen source. Two isolates, HL 24-1 and HL 24-2, were used for further studies. They grew on agar plates containing 2,4-DNP as the sole source of nitrogen but could not be subcultivated on control plates without 2,4-DNP. Both strains, HL 24-1 and HL 24-2, were identified by R. M. Kroppenstedt by biochemical reactions and on the basis of mycolic acid, menaquinone, and the fatty acid composition of the cell envelope (19a).

Culture conditions and measurements of growth. The *Rhodococcus erythropolis* strains HL 24-1 and HL 24-2 were grown in mineral medium containing 0.5 mM 2,4-DNP or 2 mM ammonia as a nitrogen source and 10 mM succinate as a carbon source. The mineral medium described by Bruhn et al. (2) was modified by increasing the concentration of buffer (50 mM [pH 7.4]) and reducing the concentration of $CaCl_2 \cdot H_2O$ (0.5 g/liter). Fluted Erlenmeyer flasks were incubated at 30°C on a rotary shaker at 150 rpm, and growth was monitored photometrically by measuring the turbidity at 546 nm (Spectrophotometer 810 P; Kontron Instruments, Zürich, Switzerland). The protein content was calculated indirectly by measurement of the dry weight on the assumption that 50% of the dry weight was protein (28). Solid media were prepared by addition of 1.5% (wt/vol) agar (No. 1; Oxoid Ltd., London, United Kingdom) to the mineral medium.

Analytical methods. For the determination of nitrite ion concentration in the culture fluid, the photometric method of Griess-Ilosvay as modified by Shinn was used (25). Ammonia ion concentration was estimated by the photometric Berthelot method (19) (Spectroquant; Merck, Darmstadt, Germany). Concentrations of 2,4-DNP, nitrite, succinate, and 4,6-dinitrohexanoate and its methyl ester were determined by reverse-phase high-performance liquid chromatography (HPLC) with Hyperchrome SC columns (length, 125 mm; diameter, 4.6 mm) filled with 5-μm particles of Lichrospher 100 RP8 (Bischoff, Leonberg, Germany) and by photometric detection at 210 nm. To quantify 2,4-DNP, nitrite, and 4,6-dinitrohexanoate and its methyl ester, the mobile phase was acetonitrile-water-H_3PO_4 (300:700:2.6 [vol/vol/vol]). To determine succinate, the mobile phase was composed of methanol-water-H_3PO_4 (100:900:2.6 [vol/vol/vol]).

Conversion of 2,4-DNP by resting cells of strain HL 24-1. As

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described above, cells of *R. erythropolis* HL 24-1 were grown in mineral medium with 2,4-DNP (0.5 mM) or ammonia (2 mM) as a nitrogen source and succinate as a carbon source. Fully induced 2,4-DNP-grown cells were obtained by addition of 0.5 mM 2,4-DNP during exponential growth 3 h before being harvested by centrifugation. The cells were resuspended in phosphate buffer (50 mM [pH 7.4]) and incubated with 2,4-DNP (0.5 mM) on a water bath shaker. The concentrations of 2,4-DNP, nitrite, and 4,6-dinitrohexanoate were determined by HPLC analysis. Additionally, nitrite was estimated photometrically.

For resting-cell experiments under anaerobic conditions, air was replaced by nitrogen and the cells resuspended in phosphate buffer (pH 7.4; 50 mM) were incubated with 0.5 mM 2,4-DNP in Hungate tubes according to standard procedures (14).

Isolation and identification of 4,6-dinitrohexanoate. Resting cells of *R. erythropolis* HL 24-1 were used to accumulate 4,6-dinitrohexanoate. These cells were obtained by growth, three times, in mineral medium (1,000 ml in 3-liter fluted Erlenmeyer flasks) with 2,4-DNP (0.5 mM) as a nitrogen source and succinate (10 mM) as a carbon source (see above). Cells were harvested, resuspended twice in 500 ml of phosphate buffer (optical density at 546 nm of 3 to 4), and incubated at 30°C with 0.5 mM 2,4-DNP. Disappearance of 2,4-DNP and formation of 4,6-dinitrohexanoate were determined by HPLC. To obtain larger amounts of 4,6-dinitrohexanoate after total consumption of the substrate, additional quantities of 2,4-DNP (0.5 mM) were supplied twice. The cells were removed by centrifugation, and the cell-free culture fluid was acidified to pH 3 with H_3PO_4 and extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, dried over $MgSO_4$, and evaporated to dryness. 4,6-Dinitrohexanoate was purified by semipreparative reverse-phase HPLC with a Hyperchrome SC column (length, 250 mm; diameter, 20 mm) filled with Lichrosorb 100 RP8 (Bischoff) by using an aqueous solvent system containing 50% acetonitrile (vol/vol) and 0.1% trifluoroacetic acid (vol/vol). 4,6-Dinitrohexanoate was recrystallized from water, and the melting point (corrected) was estimated with a heatable microscope (Reichert Thermovar).

A part of the extracted metabolite was derivatized with diazomethane (4). The HPLC analysis showed that only one product (methyl-4,6-dinitrohexanoate) was formed. The methyl ester was purified by semipreparative HPLC as described above by using an aqueous solvent system containing 45% acetonitrile (vol/vol) and 0.1% trifluoroacetic acid (vol/vol). The methyl ester was detected photometrically at 210 nm. The combined eluates were evaporated to dryness, and a nearly colorless oil of methyl-4,6-dinitrohexanoate was obtained. ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded on a Bruker CXP 300 spectrometer (7,046 T; Bruker, Rheinstetten, Germany). High-resolution ^1H NMR spectra were obtained at 300.13 MHz and decoupled ^{13}C NMR spectra were obtained at 75.47 MHz in $CDCl_3$ with tetramethylsilane as an internal standard. Positive-ion chemical ionization spectra were recorded on a mass spectrometer (70-250 S; VG Analytical Limited, Manchester, United Kingdom).

RESULTS

Isolation and characterization of bacterial strains. Two bacterial strains, HL 24-1 (originated from mixed soil samples) and HL 24-2 (obtained from Rhine water), were selected with 2,4-DNP (0.5 mM) as the sole nitrogen source and succinate as a carbon source. Both strains were identified as *R. erythropolis*.

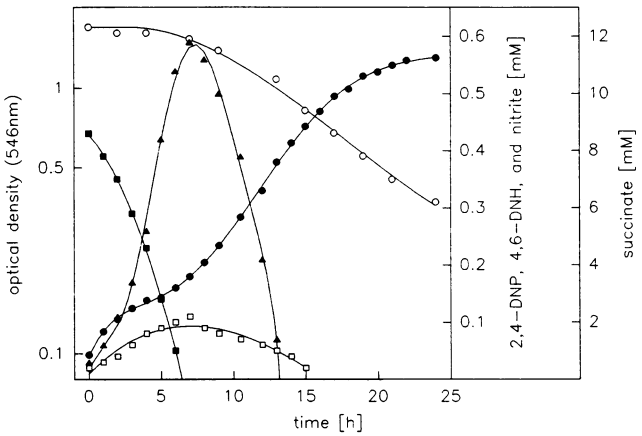

FIG. 1. Growth of *R. erythropolis* HL 24-1 with 2,4-DNP (0.5 mM) as a nitrogen source and succinate (10 mM) as a carbon source. The culture was incubated in a 500-ml fluted Erlenmeyer flask on a rotary shaker at 30°C. Increase in cell density (●) was determined photometrically at 546 nm. Concentrations of 2,4-DNP (■), 4,6-dinitrohexanoate (4,6-DNH [□]), and succinate (○) were measured by HPLC, and nitrite (▲) release was detected photometrically.

2,4-DNP as growth substrate. If *R. erythropolis* HL 24-1 or HL 24-2 was grown with 2,4-DNP as the sole source of nitrogen and succinate as a carbon source, 2,4-DNP was utilized without a lag phase. As shown for strain HL 24-1 (Fig. 1) during the initial growth period of 2,4-DNP consumption (t_d, approximately 7 h), only minor amounts of succinate were used. Major amounts of nitrite were accumulated in the culture fluid, which indicated that nitrite rather than ammonia was eliminated from 2,4-DNP or a metabolite. The bulk of succinate was utilized during the second growth phase (t_d, 4 h), and nitrite liberated during the first growth period served as a nitrogen source. Besides nitrite, HPLC analysis revealed a second metabolite from 2,4-DNP in the culture fluid (see below).

Concentrations of 2,4-DNP higher than 0.5 mM were toxic to the organisms and resulted in reduced growth rates. In order to confirm the utilization of 2,4-DNP as the sole source of carbon and energy, *R. erythropolis* HL 24-1 was inoculated into a mineral medium containing 0.5 mM 2,4-DNP without succinate. After depletion of the substrate, two additional portions of 2,4-DNP (ca. 0.5 mM each) were added. The consumption of 1.6 mM 2,4-DNP correlated with an increase of 0.23 in the optical density and the accumulation of 0.32 mM 4,6-dinitrohexanoate. During growth of *R. erythropolis* HL 24-1 with corresponding amounts of ammonia and succinate, the increase in the optical density was similar. Strain HL 24-2 could also utilize 2,4-DNP as the sole source of carbon and energy and exhibited similar growth characteristics.

Isolation and identification of metabolites. During growth of *R. erythropolis* HL 24-1 with 2,4-DNP as a nitrogen source, a second metabolite in addition to nitrite was accumulated in the medium. To isolate this product, 2,4-DNP was metabolized with resting cells pregrown with 2,4-DNP and succinate. After total conversion of 2,4-DNP, the metabolite was extracted from the acidified culture fluid with ethyl acetate. After evaporation of ethyl acetate, a slightly brown oil was obtained. Part of it was derivatized with diazomethane, and both preparations were purified by semipreparative HPLC.

TABLE 1. Characteristic signals from the chemical ionization mass spectrum of methyl-4,6-dinitrohexanoate

Mass (*m/z*)	Intensity (% of basic peak)	Fragment ion
238	100	$MH + NH_3\rceil^+$
221	8.2	$MH\rceil^+$
205	32.0	$(MH + NH_3)^+ - CH_3OH_2^+$
188	24.2	$MH^+ - CH_3OH_2^+$
174	100	$MH - HNO_2\rceil^+$
156	100	$MH - H_2O, - HNO_2\rceil^+$
142	98.9	$MH - HNO_2, - CH_3OH\rceil^+$
127	57.7	$MH^+ - CH_3NO_2, - CH_3OH_2^+$
110	55.9	$MH^+ - CH_3OH_2^+, - HNO_2, - HNO$

Recrystallization of the parent metabolite gave small colorless needles with a melting point of 69°C (corrected). The UV-visible light spectrum (in H_2O) showed an absorption maximum at 210 nm (ε = 2,300) and a broad band of low intensity at 255 nm (ε = 700). The methyl derivative (slightly colored oil) was used for NMR and mass spectroscopic analysis.

Chemical ionization (ammonia) was used to characterize the derivatized metabolite of 2,4-DNP. The mass spectrum revealed ion masses of *m/z* 221 and 238, which corresponded to the molecular ion (220) and the ammonium cluster of the molecular ion, respectively (Table 1). The elimination of HNO_2 and the sequential elimination of HNO and H_2O resulted in two major peaks at *m/z* 174 and *m/z* 156 (3). The fragment peak *m/z* 142 can be explained by elimination of HNO_2 and CH_3OH from the protonated parent molecule (26). The fragmentation pattern of the methyl ester is shown in Table 1 (3, 11). The molecular ion and the fragment ions revealed the following formula: $C_7H_{12}N_2O_6$. The number of hydrogen atoms clearly shows a reduction of the aromatic ring yielding a dinitrohexanoate. This structure explains the difficulties of obtaining a characteristic mass spectrum by conventional electron ionization of either the parent metabolite or the methyl derivative (data not shown). Similar observations were reported with other nitroalkanes (3).

For final identification of the metabolite, ^1H and ^{13}C NMR spectra were recorded. The integrals of the resonance lines added up to the expected 12 protons. In the ^1H NMR spectrum (Table 2), one singlet at 3.71 ppm is due to a methyl ester function (13). The remaining nine protons appeared as multiplets. Two multiplets corresponding to one and two protons, respectively, resonated at 4.73 ppm and 4.40 to 4.56 ppm. Because nitro groups have a high anisotropic effect, resonances of hydrogen atoms bound to nitro-substituted carbon atoms are shifted to low field (13). An aliphatic carbon skeleton with a terminal nitro substituent is in agreement with these data. The ^1H NMR data of the derivatized metabolite correspond to those of methyl-4,6-dinitro-5,5-dimethyl-hexanoate reported by Feuer et al. (6) and give further evidence for the postulated structure (Table 2). The ^{13}C decoupled magnetic resonance data of the derivatized metabolite (Table 2) are also compatible with the postulated structure of methyl-4,6-dinitrohexanoate (13, 18). Two signals correspond to the methyl ester function (C-1, 171.92 ppm; C-7, 52.08 ppm). The other five signals are due to the aliphatic structure with five sp^3 carbon atoms. Two of these signals were shifted to low field (C-4, 83.95 ppm; C-6, 71.01 ppm) because of the high anisotropic effect of the nitro groups (18).

Conversion of 2,4-DNP by resting cells of strain HL 24-1.

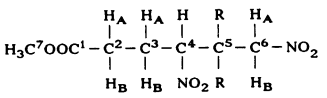

FIG. 2. Chemical structure for data in Table 2.

TABLE 2. ^1H NMR and ^{13}C NMR decoupled magnetic resonance data of methyl-4,6-dinitrohexanoate and methyl 4,6-dinitro-5,5-dimethylhexanoate

R = H		R = CH_3	
Nucleus	Chemical shift (ppm)	Nucleus	Chemical shift (ppm)[a]
2-H_A, H_B	2.13–2.69 (multiplet)	2-H_A, H_B	2.35 (multiplet)
3-H_A, H_B	2.13–2.69 (multiplet)	3-H_A, H_B	2.35 (multiplet)
5-H_A, H_B	2.13–2.69 (multiplet)	5-$(CH_3)_2$	1.30 (singlet)
4-H	4.73 (multiplet)	4-H	4.71 (triplet)
6-H_A, H_B	4.40–4.56 (multiplet)	6-$H_{(2)}$	4.50 (singlet)
7-$H_{(3)}$	3.71 (singlet)	7-$H_{(3)}$	3.73 (singlet)
C-1	171.92		
C-2	29.67[b]		
C-3	28.56[b]		
C-4	83.95		
C-5	30.39		
C-6	71.01		
C-7	52.08		

[a] Data are from Feuer et al. (6).
[b] Assignments to C-2 and C-3 are tentative.

Cells of *R. erythropolis* HL 24-1 and HL 24-2 grown with ammonia and succinate can convert 2,4-DNP only after prolonged incubation (≥1 h). This clearly showed that the initial enzymes of the pathway of 2,4-DNP were inducible.

To quantify the elimination of nitrite and the formation of 4,6-dinitrohexanoate, resting cells of *R. erythropolis* HL 24-1, pregrown with 2,4-DNP and succinate (optical density of 1.3), were incubated with 2,4-DNP (0.46 mM). After total conversion of 2,4-DNP (specific activity, 59 μmol/min/g of protein), 77% (±1%) of the organic nitrogen was recovered in the culture fluid as nitrite (0.71 mM) and 22% (±1%) was recovered as 4,6-dinitrohexanoate (0.1 mM) (Fig. 3). The nitrogen balance showed that other nitrogen-substituted metabolites were not accumulated at significant amounts. During further incubation, the concentration of 4,6-dinitrohexanoate slowly declined. This was due to spontaneous chemical decomposition of 4,6-dinitrohexanoate (0.49 mM), which was accompanied by liberation of nonstoichiometric amounts of nitrite (0.27 mM). The half-life of 4,6-dinitrohexanoate under physiological conditions was 2.7 h.

Under anaerobic conditions, resting cells of *R. erythropolis* HL 24-1 converted 2,4-DNP quantitatively into 4,6-dinitrohexanoate. The rate of this bioconversion was rather slow (6 μmol/min/g of protein). This activity could not be increased by addition of an alternative electron acceptor (nitrate [20 mM]) or a hydrogen donator (succinate, lactate, or glucose [10 mM]).

DISCUSSION

The two bacterial strains *R. erythropolis* HL 24-1 and HL 24-2 were selected under nitrogen-limiting conditions. Both strains metabolized 2,4-DNP with concomitant liberation of nitrite. With resting cells, almost 2 mol of nitrite per mol of 2,4-DNP was eliminated in view of the fact that approxi-

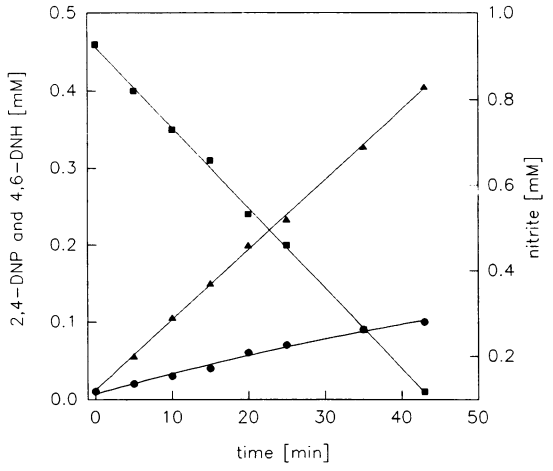

FIG. 3. Conversion of 2,4-DNP by resting cells of *R. erythropolis* HL 24-1. Resting cells were obtained by growth in mineral medium with 2,4-DNP (0.5 mM) and succinate (10 mM). The cells were harvested, resuspended in phosphate buffer (optical density at 546 nm of 1.3), and incubated at 30°C with 0.5 mM 2,4-DNP on a rotary shaker. Concentrations of 2,4-DNP (■) and 4,6-dinitrohexanoate (4,6-DNH [●]) were determined by HPLC. Nitrite (▲) was estimated photometrically.

mately 20% of the metabolized 2,4-DNP is accumulated as 4,6-dinitrohexanoate. Consequently, an initial reduction of the nitro groups, as postulated for the degradation of 4,6-dinitro-2-methylphenol by Tewfik and Evans (34), can be excluded.

Remarkably, this catabolic activity was also induced in the presence of a readily assimilable nitrogen source such as nitrite or ammonia. A similar observation has been made by Hess et al. (12) following 2,4-DNP mineralization by two different bacterial strains which were isolated under carbon-limiting conditions. Unexpectedly, both strains utilized 2,4-DNP not only as a source of nitrogen but also as the sole source of carbon and energy. Although 2,4-DNP is known as an efficient uncoupler of the oxidative phosphorylation, at concentrations of ≤0.5 mM growth yields with this substrate are high enough to exclude major energy loss. Furthermore, 2,4-DNP delayed the turnover of the additional carbon source, although liberated nitrite would allow preferential use. Consequently, these strains can be considered promising candidates for the elimination of 2,4-DNP from industrial waste streams containing additional nitrogen and carbon compounds.

Normally, the nitro groups of mononitrophenols were replaced by hydroxy groups prior to fission of the aromatic nucleus (1, 32, 33, 36–38). Enzymes converting 4-nitrophenol to hydroquinone and nitrite or 2-nitrophenol to catechol and nitrite have already been described in detail. The ability to eliminate nitrite ions from dinitrophenols by an oxidative mechanism was also demonstrated during mineralization of 2,6-DNP by *Alcaligenes eutrophus* JMP 134 (5). 4-Nitropyrogallol was identified as a major metabolite, indicating an initial attack by a dioxygenase.

Despite various attempts (data not shown) with both *R. erythropolis* HL 24-1 and HL 24-2, initial oxygenolytic enzymatic attack could not be demonstrated for the catabolism of 2,4-DNP. Instead of this, the identification of 4,6-dinitrohexanoate as the only organic metabolite of 2,4-DNP indicates that a reductive mechanism may be involved in the degradation pathway. The reduction of the aromatic ring gives evidence for a nucleophilic attack as the initial catabolic reaction. To our knowledge, this is the first report on an aerobic microbial reduction of the aromatic nucleus. Normally, the reduction of nitro groups giving rise to 2-amino-4-nitrophenol and 4-amino-2-nitrophenol as metabolites of 2,4-DNP has been reported (8, 23, 35).

Under anaerobic conditions, 2,4-DNP-grown cells of both strains quantitatively converted 2,4-DNP into 4,6-dinitrohexanoate. Compared with the transformation rate under aerobic conditions, the rate of 2,4-DNP turnover is rather slow and nitrite is not liberated. Whether 4,6-dinitrohexanoate is actually an intermediate of oxidative 2,4-DNP catabolism will be the subject of further investigations. Obviously, the highly electrophilic character of the aromatic nucleus of 2,4-DNP favors an initial reductive reaction. That the aromatic ring is subject to a nucleophilic rather than electrophilic attack is supported by investigations with 2,4,6-trinitrophenol (20).

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