Nitroaromatic compounds are common pollutants of natural environments due to abusive industrial and agricultural practices (7). These xenobiotic compounds are produced by industries manufacturing explosives, herbicides, pesticides, and dyes, and degradative pathways of these compounds are widely distributed among bacteria (for a review, see reference 15). Among these microorganisms, some Rhodococcus strains exhibit a high potential for the degradation of polynitroaromatic compounds (4). The biological hydride transfer to the aromatic ring with the concomitant formation of a Meisenheimer complex was shown to occur during the metabolism of picric acid by Rhodococcus erythropolis HL 24-2 (9). Further evidence for a reductive metabolic route was given in the same paper, showing the accumulation of 2,4-dinitrophenol (2,4-DNP) from picric acid, and in an accompanying communication reporting 4,6-dinitrohexanoate as the metabolite that accumulated under anaerobic conditions (11). A similar mechanism might account for the degradation of trinitrotoluene (TNT) by a Pseudomonas sp. strain able to convert TNT into toluene; the mineralization of the chemical was achieved by transferring the TOL catabolic plasmid to the recipient strain (3).

In this work, we report the isolation and characterization of a gram-positive bacterium, characterized as a new Rhodococcus strain on the basis of its 16S rRNA gene sequence, with a high biodegradative potential. As far as we know, there is, beside the identification of 4,6-dinitrohexanoate (11), no evidence for the metabolic fate of the putative Meisenheimer complex formed from 2,4-DNP. On the basis of our results and those cited above, we propose a pathway for the mineralization of 2,4-DNP with 3-nitroadipate as a key metabolic intermediate.

MATERIALS AND METHODS

Bacterial isolation, growth conditions, and biodegradative potential. Rhodococcus sp. strain RB1 was isolated by aerobic enrichment cultivation of an activated sludge from a wastewater plant in Alicante, Spain. The medium used was M9 mineral medium (13) with 0.5 mM 2,4-DNP as the sole nitrogen and carbon source. After enrichment (four reisolation steps), samples of the culture were plated on the same medium, which was solidified with 1.8% (wt/vol) Bacto Agar (Difco). One colony was purified and proved capable of 2,4-DNP degradation, releasing the two nitro groups from the compound as nitrite. The release of nitro groups from 2,4-dinitrophenol occurs in two steps. First, the 2-nitro group is converted to nitrite, with the production of an aliphatic nitro compound identified by 1H nuclear magnetic resonance and mass spectrometry as 3-nitroadipate. Then, this metabolic derivative is further metabolized, releasing its nitro group as nitrite. Full nitrite assimilation upon reduction to ammonia requires that an additional carbon source be supplied to the medium.

The bacterial strain RB1 has been isolated by enrichment cultivation with 2,4-dinitrophenol as the sole nitrogen, carbon, and energy source and characterized, on the basis of 16S rRNA gene sequence comparison, as a Rhodococcus species closely related to Rhodococcus opacus. Rhodococcus sp. strain RB1 degrades 2,4-dinitrophenol, releasing the two nitro groups from the compound as nitrite. The release of nitro groups from 2,4-dinitrophenol occurs in two steps. After enrichment, the second step is accelerated and nitrite release is complete. The released nitrite is assimilated by the bacteria, releasing nitrate as a key metabolite.

Received 1 June 1998/Accepted 21 October 1998
target compound also isolated two organic pollutants (8, 18). An independent research group
were grown under aerobic conditions with 50 mM sodium acetate and 0.5 mM
strain RB1 cultured with 2,4-DNP as the sole nitrogen source. Cells
Rhodococcus opacus
strain RB1 clustered phylogenetically with species of
strain RB1 grown under aerobic conditions with 50 mM sodium acetate and 0.5 mM
were measured at the indicated times.
were grown under aerobic conditions with 50 mM sodium acetate and 0.5 mM
strain RB1 cultured with 2,4-DNP as the sole nitrogen source. Cells
2,4-DNP. A
strain RB1 grown under aerobic conditions with 50 mM sodium acetate and 0.5 mM
strain RB1 cultured with 2,4-DNP as the sole nitrogen source. Cells

Chemical shifts are given in parts per million relative to tetramethylsilane and
constants in Hertz.

All reagents were of the maximal purity commercially available.

RESULTS AND DISCUSSION

(i) Isolation and characterization of the strain. A bacterium,
Rhodococcus sp. strain RB1 was isolated from activated sludge after incubation with this compound as the
sole carbon, nitrogen, and energy source. Amplification by

The bacterium used as nitrogen sources 2,4-DNP, ammo-
monium inhibited nitrite uptake. As a matter in fact, in the

FIG. 1. Time course of cell growth, 2,4-DNP uptake, and nitrite release by

Rhodococcus sp. strain RB1 grew aerobically in liquid mineral medium with 2,4-DNP as the nitrogen source at concent-
trations of up to 5 mM (50 mM acetate as the carbon source). The
maximal concentration of 2,4-DNP tolerated by the bac-
terium under the same conditions with 2,4-DNP as the sole
carbon and nitrogen source was 2 mM. No growth was ob-
served with 2,4-DNP under anaerobic conditions.
The degradation of 2,4-DNP as the sole carbon and nitrogen
source in liquid cultures was complete and took place with an
almost stoichiometric nitrite release (1 mol of 2,4-DNP to 1.65
mol of nitrite). The nitrite released into the medium was not
taken up by the bacterium unless acetate was added to the
carbon-starved cells (data not shown). The increment in optical
density at 600 nm (ΔA600) observed in cultures with 1 mM
2,4-DNP as the sole carbon and nitrogen source was 0.18 ±
0.02. The ΔA600 in cultures growing with 3 mM acetate as the
carbon source and 2 mM nitrite as the nitrogen source was
0.2 ± 0.03, thus indicating that 2,4-DNP was fully used as a
carbon source without major energy losses.
The utilization of 2,4-DNP as a nitrogen source by
Rhodococcus sp. strain RB1 showed a diauxic growth curve (Fig. 1).
After an initial adaptation period (cells precultured on acetate and nitrite), the first phase is characterized by a minute growth
doubling time, approximately 21 h) and a rapid transforma-
tion of 2,4-DNP with simultaneous release of nitrite (almost 1
mol of nitrite per mol of 2,4-DNP consumed). During the
second phase, the strain grew more rapidly (doubling time,
ammonia as the sole nitrogen source (5 mM concentration) in
liquid culture experiments with 2 mM ammonium as the
nitro-
rium as an inhibitor of nitrite assimilation (2). As expected,
ammonium inhibited nitrite uptake. As a matter in fact, in the

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source was complete and took place with an
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second phase, the strain grew more rapidly (doubling time,

(ii) Degradation pathway of 2,4-DNP by Rhodococcus sp.
strain RB1. An initial reductive pathway has been demonstrated
in some gram-positive bacteria that degrade nitroaromatic compounds (4). The transfer of a hydride to the aromatic
ring of picric acid and 2-chloro-4,6-DNP provokes the release
of the 2-nitro or 2-chloro group with the concomitant forma-
tion of 2,4-DNP (9, 10). We have also observed the formation
of 2,4-DNP from picric acid in anaerobic resting-cell experi-
ments with Rhodococcus sp. strain RB1 (data not shown), thus
suggesting a similar reaction. In addition, Rhodococcus sp.
strain RB1 cells induced with 2,4-DNP produced a brilliant red
compound when transferred to medium with picric acid (0.5
mM), indicating the putative formation of a Meisenheimer
complex. The formation of this compound from picric acid was
unambiguously demonstrated in R. erythropolis HL 24-2 (9),
and therefore, we have used it as the reference strain. The
product formed by both R. erythropolis HL 24-2 and Rhodo-
coccus sp. strain RB1 from picric acid coeluted during HPLC
analysis and exhibited the same UV-visible light spectrum (data not shown). As mentioned above, the hydrogenation of the aromatic ring may result in the rearomatization and concomitant liberation of nitrite or chloride (9, 10). Nevertheless, this mechanism cannot be excluded in Rhodococcus strain RB1, since it should yield a mononitrophenol derivative (probably 4-nitrophenol) which cannot be mineralized by the bacterium. In spite of that, this mechanism cannot be excluded in other bacteria, and in fact, 4-nitrophenol has been detected recently in small amounts during the degradation of picric acid (16).

The ratio of nitrite released to 2,4-DNP consumed showed a stoichiometry of 1/1 instead of 2/1 during the first growth phase of Rhodococcus strain RB1 with 2,4-DNP as the nitrogen source (Fig. 1). As very little growth had occurred at the end of this growth phase, it can be assumed that the second nitrogen source (Fig. 1) is bound to an intermediate. However, analysis of the supernatants of the culture medium from this phase by HPLC showed only minute peaks absorbing at 210 nm. To amplify the signals, these supernatants were extracted and concentrated as described in Materials and Methods. A complex mixture of compounds absorbing at 210 nm was detected by HPLC. The main compound (approximately 70% in relative units, at 5 min net retention time) was purified by preparative HPLC and showed a single absorption maximum at 204 nm. The product contained in this fraction was identified as 3-nitroadipate dimethyl ester according to GC–high-resolution MS analysis (Table 1). Nevertheless, we assume that a partial dimethylation of the chemical occurred due to the heating in the presence of methanol during the concentration step (see Materials and Methods). This assumption is based on the need for acidic conditions (pH 2) for the full extraction of the chemical from the supernatant. In addition, the 1H-NMR spectrum of the extracted compound corresponded to that of the free acid (Table 2) with a minute amount of the diester (estimated to be around 2% according to the two singlet signals at 3.71 and 3.73 ppm corresponding to the methyl groups), even though, as the free acid is not volatile, only the diester was detected by GC-MS. The 3-nitroadipate is obviously more oxidized than the proposed initial intermediate (Meisenheimer complex). The nature of the oxidant is unknown, but we suggest that it should be O2. The reason is that, under anaerobic conditions, R. erythropolis accumulates mainly 4,6-dinitrohexanoate from 2,4-DNP (11) or 1,3,5-trinitropentane from picric acid (9). The formation of 4,6-dinitrohexanoate may be achieved by the hydration of the double bond of a putative dihydro-2,4-DNP.

### Table 1. Characteristic signals in the mass spectrum of 3-nitroadipate dimethyl ester

<table>
<thead>
<tr>
<th>Ion mass (m/z)</th>
<th>Intensity (%)</th>
<th>Molecular structure</th>
<th>Structural interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>188.0541</td>
<td>21</td>
<td>C2H10O4N</td>
<td>[M]+</td>
</tr>
<tr>
<td>170.0451</td>
<td>12</td>
<td>C2H8O4N</td>
<td>[M-OCH3]+</td>
</tr>
<tr>
<td>141.0549</td>
<td>53</td>
<td>C2H6O4N</td>
<td>[M-OCH3.H2O]+</td>
</tr>
<tr>
<td>128.0364</td>
<td>12</td>
<td>C2H5O4N</td>
<td>[M-OCH3.HNO3]+</td>
</tr>
<tr>
<td>113.0621</td>
<td>52</td>
<td>C2H4O4</td>
<td>[M-OCH3.HCO2CH3]+</td>
</tr>
<tr>
<td>109.0288</td>
<td>28</td>
<td>C2H3O3</td>
<td>[M-CO2CH3.HNO3]+</td>
</tr>
<tr>
<td>81.0339</td>
<td>25</td>
<td>C2H2O</td>
<td>[M-OCH3.HOCH3.HNO3]+</td>
</tr>
<tr>
<td>71.0515</td>
<td>100</td>
<td>C2H1O</td>
<td>[C2H4O2]+</td>
</tr>
<tr>
<td>59.0157</td>
<td>60</td>
<td>C2H2O2</td>
<td>[CO2CH3]+</td>
</tr>
</tbody>
</table>

Table 2. Structure and 1H-NMR data of 3-nitroadipate

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical shift (ppm)</th>
<th>Coupling constant (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.13</td>
<td>J1,2 = 17.7 Hz, J1,3 = 10.0 Hz</td>
</tr>
<tr>
<td>2</td>
<td>2.87</td>
<td>J2,3 = 3.5 Hz</td>
</tr>
<tr>
<td>3</td>
<td>4.99</td>
<td>J3,4 = 7.0 Hz, J3,5 = 7.0 Hz</td>
</tr>
<tr>
<td>4</td>
<td>2.20</td>
<td>J4,6 = 6.3 Hz, J4,7 = 6.3 Hz</td>
</tr>
<tr>
<td>5</td>
<td>2.21</td>
<td>J5,6 = 8.1 Hz, J5,7 = 8.1 Hz</td>
</tr>
<tr>
<td>6</td>
<td>2.46</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.44</td>
<td></td>
</tr>
</tbody>
</table>

* The spectrum form indicated that H-4-H-5 and H-6/H-7 were nonequivalent. Spectral simulation and iteration using LAOCOON III allowed the shift differences shown to be estimated. As all the lower-intensity lines in the observed spectrum could not be determined accurately, only estimates (±0.3 Hz) of the couplings J1,6, J3,5, J1,6, J4,7, J5,6, and J5,7 were possible. The remaining couplings are ±0.1 Hz.
An intermediate, whereas the dioxygenolytic cleavage of the double bond would produce 3-nitroadipate (Fig. 3).

These results may be summarized in the degradation pathway depicted in Fig. 3. We propose that 2,4-DNP mineralization by *Rhodococcus* takes place by a metabolic pathway including three different phases: (i) reduction of the aromatic ring, as previously demonstrated (10–12), by two successive hydride transfers; (ii) aerobic *ortho* ring fission, production of 3-nitrocompounds, and concomitant release of the *ortho* nitro group as nitrite (nevertheless, the reductive formation of 4,6-dinitrohexanoate [11] and subsequent oxidation to 3-nitroadipate cannot be excluded); and (iii) further metabolism of 3-nitroadipate with release of a second mole of nitrite.

Experiments are in progress to determine if enzymes of the 3-oxoadipate pathway, actually involved in benzoate metabolism by *Rhodococcus* strain R1 (data not shown), are also involved in the metabolism of 3-nitroadipate by this bacterium.

**ACKNOWLEDGMENTS**

We thank the Spanish Dirección General de Investigación Científica y Técnica (DGICYT grant PB95 0554 CO2 02) and Plan Andaluz de Investigación for financial support. Postdoctoral fellowships from the Ministerio de Educación y Ciencia and European Environmental Research Organization to R.B. and from Alexander von Humboldt to F.C. are also gratefully acknowledged.

We also thank Rolf Wittich for critically reading the manuscript, R. Blanco for his help in HPLC analysis, and Manfred Nimtz for performing GC-MS analysis. We also thank H.-J. Knackmuss for kindly supplying *R. erythropolis* HL 24-2.

**REFERENCES**


