Reductive Dechlorination of 2,4-Dichlorobenzoate to 4-Chlorobenzoate and Hydrolytic Dehalogenation of 4-Chloro-, 4-Bromo-, and 4-Iodobenzoate by *Alcaligenes denitrificans* NTB-1

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*Alcaligenes denitrificans* NTB-1, previously isolated on 4-chlorobenzoate, also utilized 4-bromo-, 4-iodo-, and 2,4-dichlorobenzoate but not 4-fluorobenzoate as a sole carbon and energy source. During growth, stoichiometric amounts of halide were released. Experiments with whole cells and cell extracts revealed that 4-bromo- and 4-iodobenzoate were metabolized like 4-chlorobenzoate, involving an initial hydrolytic dehalogenation yielding 4-hydroxybenzoate, which in turn was hydroxylated to 3,4-dihydroxybenzoate. The initial step in the metabolism of 2,4-dichlorobenzoate was catalyzed by a novel type of reaction for aerobic organisms, involving inducible reductive dechlorination to 4-chlorobenzoate. Under conditions of low and controlled oxygen concentrations, *A. denitrificans* NTB-1 converted all 4-halobenzoates and 2,4-dichlorobenzoate almost quantitatively to 4-hydroxybenzoate.

Halogenated compounds have been produced synthetically on a large scale during the last few decades, and they have entered the biosphere either by accidental spillage or by deliberate release. In many cases these chemicals may be transformed or fully eliminated from the environment by microbial degradation.

Aerobically, the majority of haloaromatic compounds are degraded by microorganisms that leave the carbon-halogen bond intact until chlorocatechols are formed and the ring is cleaved by a dioxygenase (4, 5, 7, 8, 25, 28, 35, 36). However, aerobic catabolism of haloaromatic compounds may also proceed along an initial oxidative or hydrolytic dehalogenation reaction. For instance, 4-fluorophenylalanine was oxidized by a monoxygenase to tyrosine (12). In a similar way 2-fluorobenzoate (6) and 4-chlorophenylacetate (20) were oxidized by a dioxygenase, yielding catechol and 3,4-dihydroxyphenylacetate, respectively. An analogous reaction is probably involved in the metabolism of 2-chlorobenzoate (2-CBA) in *Pseudomonas cepacia* INM1KZ-2 (38). Hydrolytic dehalogenation reactions have been reported for the metabolism of 3- and 4-chlorobenzoates (3-CBA and 4-CBA) (11, 13, 14, 18, 26, 37). Aerobic metabolism of halogenated aliphatic compounds also may involve either oxidative or hydrolytic dehalogenation reactions. Methyl chloride was oxidized by a methanotroph via the methane monoxygenase activity present, although methyl chloride did not serve as a carbon source for growth (29).

Dichloromethane dehalogenation in a *Hyphomicrobium* strain was catalyzed by a reduced glutathione-dependent dehalogenase, with formaldehyde as a product (16). Glutathione-independent hydrolytic dehalogenation of halogenated aliphatic compounds has also been observed (10, 21). To our knowledge, however, there are no reports on the aerobic metabolism of halogenated aliphatic or aromatic compounds involving reductive dehalogenation.

Under anaerobic conditions, haloaromatic compounds are degraded by reductive dechlorination before ring cleavage, as shown for chlorinated phenols (3) and chlorinated benzoates (9, 27). Reductive dehalogenation was also responsible for the degradation of halogenated aliphatic hydrocarbons under methanogenic conditions (1, 34) and under denitrifying conditions (2).

Recently we reported on the bioformation of 4-hydroxybenzoate (4-HBA) from 4-chlorobenzoate (4-CBA) by *Alcaligenes denitrificans* NTB-1 (32). The results described in this paper demonstrate that this species also grows on 2,4-dichlorobenzoate (2,4-DCBA), 4-bromobenzoate (4-BBA), and 4-iodobenzoate (4-IBA), 4-CBA, 4-BBA, and 4-IBA were all hydrolytically dehalogenated to 4-HBA, and evidence will now be presented for a reductive dehalogenation reaction under aerobic conditions. This novel type of reaction is involved in the catabolism of 2,4-DCBA via 4-CBA to 4-HBA.

MATERIALS AND METHODS

Media and culture conditions. *A. denitrificans* NTB-1 was routinely grown in a chemostat (volume, 1.0 liter; dilution rate, 0.03 h⁻¹) under carbon-limited conditions. The continuous culture (30°C, pH 7) was supplied with a mineral salts medium (32) to which 2,4-DCBA, 4-CBA, or 4-BBA was added at 1 g/liter and 4-IBA was added at 0.5 g/liter.

Experiments with whole cells. Simultaneous adaptation experiments and incubation experiments at controlled oxygen concentrations were performed as described previously (32).

Enzyme assays. Cell extracts were prepared by ultrasonic treatment as described previously (31). All assays were performed at 30°C. Attempts to assay for 2,4-DCBA dehalogenase activity were done by measuring 2,4-DCBA concentrations in various incubation mixtures in the presence of cell extract and NADPH. 4-CBA dehalogenase was assayed by measuring the conversion of 4-CBA in 4-HBA. 4-HBA hydroxylase was determined by means of 4-HBA-dependent NADPH oxidation (18). Protocatechuate dioxygenase was measured by means of a polarographic oxygen probe (18).

Analytical methods. Chloride, bromide, and iodide concentrations were determined with a Micro-Chlor-o-Counter.
TABLE 1. Rates of oxygen uptake by washed-cell suspensions of *A. denitrificans* NTB-1 grown on various carbon sources

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Oxygen uptakea (nmol of O₂ consumed/min per mg of protein) with indicated carbon source</th>
<th>4-CBA</th>
<th>4-BBA</th>
<th>4-IBA</th>
<th>2,4-DCBA</th>
<th>4-HBA</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-FBA</td>
<td>&lt;5</td>
<td>10</td>
<td>&lt;5</td>
<td>5</td>
<td>5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>4-CBA</td>
<td>100</td>
<td>170</td>
<td>45</td>
<td>45</td>
<td>20</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>4-BBA</td>
<td>75</td>
<td>140</td>
<td>60</td>
<td>50</td>
<td>25</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>4-IBA</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>35</td>
<td>15</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>2,4-DCBA</td>
<td>50</td>
<td>80</td>
<td>30</td>
<td>5</td>
<td>&lt;5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-HBA</td>
<td>200</td>
<td>220</td>
<td>55</td>
<td>40</td>
<td>10</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>Protocatechuate</td>
<td>20</td>
<td>60</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>&lt;5</td>
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<tr>
<td>Succinate</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
</tbody>
</table>

* No activity was observed with the following substrates: 2-chloro-4-hydroxybenzoate, 3,5-dichlorocatechol, gentisate, benzoate, 2- or 3-HBA, 2- or 3-CBA, 2-FBA, 3-BBA, 2,6-DCBA, 3,4-DCBA, 3,5-DCBA, catechol, or 2,3-dihydroxybenzoate.

(Marius, Utrecht, The Netherlands). Protein contents of whole cells and cell extracts were determined by the method of Lowry et al. (17) with crystalline bovine serum albumin as the standard. Concentrations of 4-fluorobenzoate (4-FBA), 4-CBA, 4-BBA, 4-IBA, 2,4-DCBA, and 4-HBA were quantitatively analyzed by reverse-phase high-pressure liquid chromatography (HPLC) with a C-18 column (200 by 3 mm; Chrompack, Middelburg, The Netherlands). The mobile phase was methanol-water-acetic acid (60:40:1); the flow rate was 0.4 ml/min, and detection was by UV absorbance at 254 nm. Under these conditions the following retention times (in minutes) were observed: 4-FBA, 4.1; 4-CBA, 8.5; 4-BBA, 11.4; 4-IBA, 10.6; 2,4-DCBA, 9.8; and 4-HBA, 2.9. Accumulating metabolites were initially identified by comparison of retention times with those of authentic samples and by in situ scanning of the UV spectra after the flow had been stopped. Ether extraction of the accumulating products was done as described by Marks et al. (19). The resulting ether extracts were methylated with diazomethane (33) and analyzed by gas-liquid chromatography–mass spectrometry (GC-MS). Samples were analyzed on a capillary column (SIL19CB; 26 m by 0.22 mm inner diameter; Chrompack). The temperature was programmed to rise from 120°C (2 min) to 260°C at a rate of 4°C/min. The methyl esters of 2,4-DCBA, 4-CBA, and 4-HBA under these conditions had retention times of 6.95, 4.43, and 12.52 min, respectively. Mass spectra of these compounds were obtained on a VG M7070F mass spectrometer at 70 eV electron impact. The oxygen concentration in the gas phase was measured as described previously (32).

**Chemicals.** All biochemicals were from Boehringer, Mannheim, Federal Republic of Germany. 2-Chloro-4-hydroxybenzoate and 2,3-dichlorobenzoate (2,3-DCBA) were from ICN Biomedicals Inc., Plainview, N.Y. 4-CBA, 4-FBA, and 2,4-DCBA were purchased from Janssen Chimica, Beerse, Belgium. 4-BBA was from Fluka, Buchs, Switzerland. All other chemicals were of commercially available analytical grade and were used without further purification.

**RESULTS**

**Growth of *A. denitrificans* NTB-1 on various substrates.** *A. denitrificans* NTB-1 was able to use the following compounds as sole carbon and energy sources: 4-CBA, 2,4-DCBA, 4-BBA, 4-IBA, 4-HBA, 3-hydroxybenzoate (3-HBA), benzoate, phenylacetate, glucose, succinate, pyruvate, acetate, and ethanol. No growth was observed with 2-CBA, 3-CBA, 2-HBA, 3,5-DCBA, 3,4-DCBA, 2,5-DCBA, 2,6-DCBA, 4-FBA, 4-chloro- or 4-fluorophenylacetate, or 2-chloro-4-hydroxybenzoate as substrates (substrate concentration, 2 mM). Previous results have shown that both the lag phase and the growth rate of *A. denitrificans* NTB-1 during growth on 4-CBA were dependent on the concentration of 4-CBA (32). Similar results were obtained during growth on 4-BBA and 2,4-DCBA. At a 2 mM concentration of these two compounds, doubling times of 12 and 13 h, respectively, were observed, while with 4 mM 4-BBA or 2,4-DCBA, strain NTB-1 doubled in 68 and 26 h, respectively. No growth of NTB-1 on 4-IBA (2 mM) was observed in batch culture. However, by growing NTB-1 in a chemostat under carbon-limited conditions (dilution rate, 0.03 h⁻¹), good growth was obtained. During all growth experiments the halide was quantitatively released, as determined by measuring halide accumulation.

**Simultaneous adaptation experiments.** To investigate the metabolism of 4-CBA, 4-IBA, and 2,4-DCBA, cells were grown on several substrates and incubated with possible intermediates, and rates of oxygen uptake were recorded. Cells grown on halobenzoates oxidized 4-CBA, 4-IBA, 4-BBA, 4-DCBA, 4-HBA, and 3,4-dihydroxybenzoate but not 4-FBA (Table 1). Succinate-grown cells did not oxidize 4-CBA, 4-IBA, 4-IBA, or 4,2-DCBA, but surprisingly, 4-HBA-grown cells oxidized these halogenated compounds. 2-Chloro-4-hydroxybenzoate, an intermediate of 2,4-DCBA metabolism in initial hydrolytic dehalogenation at the 4-position, was not oxidized by 2,4-DCBA-grown cells, nor was 3,5-dichlorocatechol, an intermediate in initial dioxygenation, oxidized by washed cells grown on 2,4-DCBA (Table 1).

**Catabolism of halobenzoates and excretion of intermediary products from these halobenzoates by washed cell suspensions.** To study the metabolism of the halobenzoates more directly, the disappearance of various halobenzoates from incubation mixtures was studied by HPLC. 4-CBA-grown cells readily catabolized 4-CBA, 4-IBA, 4-IBA, and 2,4-DCBA but not 4-FBA (Table 2). Similar results were obtained for cells grown on 4-BBA, 4-IBA, 2,4-DCBA, and 4-HBA (Table 2). Although the activities varied depending on the carbon source used for growth, no significant variation was observed in the relative rates of consumption of 4-CBA, 4-IBA, and 4-HBA (Table 2). Furthermore, the ratio of 4-haloacetobenate to 2,4-DCBA consumption for cells grown on various haloaromatics was in all cases about 0.65. An interesting observation was made with 4-HBA-grown cells. These cells, when incubated with 2,4-DCBA, transiently accumulated a product (Fig. 1) which, when analyzed by HPLC, had the same retention time and UV characteristics as authentic 4-CBA. After methylation of the reaction mixture sampled at 60 min (Fig. 1), two compounds were identified by comparison of the UV spectra with authentic samples.

**TABLE 2. Dehalogenation rates of various substrates by *A. denitrificans* NTB-1 cells grown on various carbon sources**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dehalogenation rate (nmol of substrate consumed/min per mg of protein) with indicated carbon source</th>
<th>4-CBA</th>
<th>4-BBA</th>
<th>4-IBA</th>
<th>2,4-DCBA</th>
<th>4-HBA</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-FBA</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>4-CBA</td>
<td>18</td>
<td>11</td>
<td>12</td>
<td>28</td>
<td>8</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>4-IBA</td>
<td>15</td>
<td>11</td>
<td>12</td>
<td>30</td>
<td>8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4-BBA</td>
<td>18</td>
<td>9</td>
<td>10</td>
<td>28</td>
<td>7</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2,4-DCBA</td>
<td>11</td>
<td>7</td>
<td>6</td>
<td>20</td>
<td>5</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

* ND, Not determined.
detected by GC-MS analysis. One compound showed the mass spectrum of methylated 2,4-DCBA, and the other compound showed a mass spectrum (Fig. 2A) identical to the mass spectrum of authentic 4-chlorobenzoic acid methylester. This accumulation of 4-CBA from 2,4-DCBA was not expected since the rate of 4-CBA consumption by whole cells was greater than the rate of 2,4-DCBA consumption (Table 2). However, when 4-CBA and 2,4-DCBA were added simultaneously, it was observed that the 4-CBA concentration remained constant until 2,4-DCBA was metabolized to leave a concentration of approximately 0.25 mM (Fig. 3).

Previous results for the metabolism of 4-CBA by *A. denitrificans* NTB-1 had shown that 4-CBA was almost stoichiometrically converted to 4-HBA under conditions of low oxygen concentration (32). Similar experiments have now been done with cells grown on 4-BBA, 4-IBA, and 2,4-DCBA with the same substrates (Fig. 4). In addition, the product obtained from the 2,4-DCBA incubation mixture was methylated, and GC-MS analysis showed that this methylated product was indeed 4-hydroxybenzoic acid methyl ester (Fig. 2B). The effect of the oxygen concentration in the gas phase on the rate of 2,4-DCBA consumption and the rate of 4-HBA formation was identical to the effect of oxygen on 4-CBA consumption and 4-HBA formation, as shown previously (32), in that conversion was maximal at about 1.2% oxygen in the gas phase, while under anaerobic conditions almost no 2,4-DCBA consumption and hence no 4-HBA formation was obtained.

No decrease in concentration as measured by HPLC and no halide release was observed when 2,4-DCBA-grown NTB-1 cells were incubated under normal atmospheric conditions with 2-chloro-4-hydroxybenzoate, 4-chlorophenylacetate, 2-CBA, 3-CBA, 4-FBA, 2,6-DCBA, 2,5-DCBA, 3,4-DCBA, or 3,5-DCBA.

**Experiments with cell extracts.** The feasibility of a degradation route of 2,4-DCBA involving 4-CBA and subsequent metabolism of 4-CBA was further investigated, as was the metabolism of 4-BBA and 4-IBA, by measuring specific enzyme activities in cell extracts. NTB-1 cells grown on either 4-HBA, 4-CBA, 4-BBA, 4-IBA, or 2,4-DCBA had an NADPH-dependent 4-hydroxybenzoate hydroxylase and

![Graph](https://via.placeholder.com/150)

**FIG. 1.** Formation of 4-CBA (○) and chloride (□) from 2,4-DCBA (●) by 4-HBA-grown *A. denitrificans* NTB-1 cells. Total volume of the reaction mixture was 10 ml, containing 17.5 mg of protein.

![Diagram](https://via.placeholder.com/150)

**FIG. 2.** MS of (A) methylated 4-CBA produced by 4-HBA-grown cells from 2,4-DCBA and (B) methylated 4-HBA formed from 2,4-DCBA at 1.2% oxygen in the gas phase.

![Graph](https://via.placeholder.com/150)

**FIG. 3.** Simultaneous consumption of 4-CBA (○) and 2,4-DCBA (●) by 4-HBA-grown NTB-1 cells; □, chloride release. Total volume of the reaction mixture was 10 ml, containing 17.5 mg of protein.
also a 3,4-dihydroxybenzoate dioxygenase, whereas succinate-grown cells did not contain these enzymes (Table 3).

Several attempts were made to show the presence of a reductive 2,4-DCBA dehalogenase and a hydrolytic 4-CBA dehalogenase in extracts of 2,4-DCBA-grown cells, but no activities were detected.

**DISCUSSION**

Aerobic microorganisms isolated on 4-CBA hydrolytically dehalogenate this substrate to 4-HBA (13, 14, 18, 26, 37), in contrast to the dioxygenase reactions used for the initial step in the catabolism of various other halobenzoates in several organisms (5, 7, 36). Microorganisms which degrade 4-CBA via 4-chlorocatechol so far have not been isolated by classical enrichment techniques, probably because the benzoate 1,2-dioxygenase of ordinary benzoate degraders is not active on 4-CBA (15, 23). Some years ago Hartmann et al. (8) isolated a *Pseudomonas* sp. by continuous enrichment which degraded 3-CBA, 4-CBA, and 3,5-DCBA via the corresponding chlorocatechols. They assumed that this strain was naturally evolved from two defined strains, *Pseudomonas* sp. strain B13 and *Pseudomonas* sp. strain mt-2, which were used as inocula. Later on, Reineke and Knackmuss (24) confirmed this hypothesis by constructing in vitro a 4-CBA degrader by combining the genes of the 3-CBA pathway of *Pseudomonas* sp. strain B13 and some genes of the TOL plasmid of *Pseudomonas* sp. strain mt-2.

*A. denitrificans* NTB-1 is no exception to the general rule for 4-CBA metabolism (32), and apart from 4-CBA it also metabolized 4-BBA and 4-IBA to 4-HBA. 4-FBA, however, did not serve as a growth substrate, nor was it deflourinated by cells grown on 4-CBA. In contrast, *Arthrobacter* sp. strain TM-1 grown on 4-CBA was able to deflourinate 4-FBA (18).

Strain NTB-1 was also able to grow on 2,4-DCBA. Bacteria degrading 2,4-DCBA have also been isolated by Vandenbergh et al. (30), but in these strains the catabolic route of 2,4-DCBA has not been studied. Zaitsev and Karasevich (39) isolated a *Corynebacterium* sp. which was able to convert 2,4-DCBA to 4-HBA. They considered three possible pathways for 2,4-DCBA metabolism: (i) reductive dechlorination to 4-CBA, which in turn would be dechlorinated to 4-HBA; (ii) hydrolytic dechlorination, yielding 2-chloro-4-hydroxybenzoate, followed by reductive dechlorination; and (iii) simultaneous dechlorination of both chlorine atoms, yielding 4-HBA. Zaitsev and Karasevich (39) suggested that the third pathway was the most probable, although on the basis of their results the two other pathways cannot be excluded.

Experiments with whole NTB-1 cells showed that 2-chloro-4-hydroxybenzoate was not an intermediate in the metabolism of 2,4-DCBA in our strain, nor was 2,4-DCBA degraded via 3,5-dichlorocatechol, a theoretical route resembling the aerobic degradation pathway of many haloaromatic compounds.

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**TABLE 3.** Specific activities of the 4-HBA hydroxylase (NADPH dependent) and the 3,4-dihydroxybenzoate dioxygenase of *A. denitrificans* NTB-1 after growth on various substrates

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>4-HBA hydroxylase (nmol/min per mg of protein)</th>
<th>3,4-Dihydroxybenzoate dioxygenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-CBA</td>
<td>20</td>
<td>175</td>
</tr>
<tr>
<td>4-BBA</td>
<td>30</td>
<td>180</td>
</tr>
<tr>
<td>4-IBA</td>
<td>15</td>
<td>175</td>
</tr>
<tr>
<td>2,4-DCBA</td>
<td>15</td>
<td>210</td>
</tr>
<tr>
<td>4-HBA</td>
<td>15</td>
<td>190</td>
</tr>
<tr>
<td>Succinate</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
compounds (4, 5, 7, 8, 25, 28, 35, 36). Instead, from incubation experiments with 4-HBA-grown cells, it is concluded that 4,4-DCBA is reduced to 4-CBA. To our knowledge this is the first report on aerobic metabolism of halogenated compounds involving reductive dehalogenation. Washed cells grown on 4-HBA had a higher consumption rate for 4-CBA than for 2,4-DCBA but nevertheless accumulated 4-CBA from 2,4-DCBA. Inhibition of the 4-CBA dehalogenase by 2,4-DCBA (Fig. 3) may explain this anomaly. Surprisingly, however, no 4-CBA accumulated from 2,4-DCBA with NTB-1 cells grown on 4-CBA, 4-BBA, 4-IBA, or 2,4-DCBA, although the ratio of 2,4-DCBA to 4-CBA consumption in these cells was comparable to this ratio in 4-HBA-grown cells (Table 2). We have not yet been able to measure a 2,4-DCBA reductive dehalogenase in cell extracts, although cell extracts have been prepared and tested in the presence and absence of oxygen with both NADH and NADPH as reductants.

4-HBA was produced from 2,4-DCBA when NTB-1 cells were incubated under conditions of low oxygen concentrations, which is in keeping with 4-CBA dehalogenation as a hydrolytic process (19, 22). Surprisingly, however, under anaerobic conditions almost no dehalogenation was observed, and presently it is being investigated whether this behavior is due to energy-dependent active transport of 2,4-DCBA.

On the basis of the results presented, a degradation route (Fig. 5) is proposed for halobenzoate metabolism in A. denitrificans NTB-1.

ACKNOWLEDGMENTS

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LITERATURE CITED


