Biodegradation of 4-nitrobenzoate, 4-aminobenzoate and their mixtures: new strains, unusual metabolites and insights into pathway regulation

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Abstract

Strains PB4 (Burkholderia cepacia) and SB4 (Ralstonia paucula) were isolated on 4-aminobenzoate and found to also grow on 4-nitrobenzoate. Nevertheless, and although reduction of a nitro group into an amino function is a thermodynamically favorable reaction, the main 4-nitrobenzoate degradation pathway used by these bacteria did not involve 4-aminobenzoate as an intermediate. Rather strains PB4 and SB4 used the previously described partial reduction into 4-hydroxylaminobenzoate, subsequently converted into protocatechuate. Remarkably, both microorganisms also harbored a mutase, through which two dead-end metabolites, 3-hydroxy-4-aminobenzoate and 3-hydroxy-4-acetamidobenzoate, were produced. Regulation of the pathways appeared to differ in both strains. In strain PB4, both 4-nitro- and 4-aminobenzoate were able to induce their own degradation as well as the degradation of the corresponding aminated or nitrated derivative. On the other hand, when strain SB4 was incubated with mixtures of 4-nitro- and 4-aminobenzoate, 4-aminobenzoate strongly interfered with the degradation of 4-nitrobenzoate, even when the nitro compound was the inducer. Since aminoaromatic compounds are common co-contaminants of nitroaromatic-polluted sites, bacteria such as strains PB4 and SB4, which can mineralize both a nitroaromatic compound and its corresponding amino derivative, are relevant subjects of investigation in the interest of a complete site remediation. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Nitroaromatic biodegradation; Aminoaromatic biodegradation; 4-Nitrobenzoate; Reductase; Mutase

1. Introduction

Most nitroaromatic compounds present in our environment are the result of industrial and agricultural activities. As an example, 4-nitrobenzoate is an intermediate in the synthesis of dyes and pharmaceuticals [1]. Despite the toxicity [2] and the xenobiotic character of nitroarenes, some bacteria have been found to harbor the enzymes for the complete metabolism of certain nitroarenes [3]. However, due to the thermodynamically favorable reduction of the nitro group into an amino function [4], this reduction, either biotic or abiotic, is the reaction most commonly undergone by nitroaromatic compounds. In most cases, the resulting aminoaromatic compound is not further degraded [5,6]. As a consequence, aminoaromatic compounds are often present at sites contaminated with nitroaromatic compounds [7,8]. In a sample from a former ammunition plant in Germany, several aminobenzoic acids were detected near nitroaromatic compounds [9]. The fact that certain aminoaromatic compounds are toxic should not be neglected. 4-Aminobenzoate, in the 0.1 mM range, is a precursor of folic acid [10] but can inhibit bacteria at concentrations a hundred times higher [11]. A realistic decontamination scheme should involve the removal of both amino- and nitroaromatic compounds.

Several research groups have isolated microbial strains that mineralize 4-nitrobenzoate by a reductive pathway [12–17]. However, these strains achieved only a partial reduction of 4-nitrobenzoate to 4-hydroxylaminobenzoate,
which was in turn converted into protocatechuate by the intervention of a lyase. 4-Aminobenzoate was not an intermediate and in most cases it was not even a growth substrate. In the present study, strains PB4 and SB4, isolated on 4-aminobenzoate, were found to be able to also grow on 4-nitrobenzoate. 4-Aminobenzoate might hence be an intermediate in 4-nitrobenzoate degradation. Bacteria degrading a nitroaromatic compound via the amino intermediate, if they exist, remain elusive.

Strains PB4 and SB4 belong to the species *Burkholderia cepacia* and *Ralstonia paucula*, respectively. Although isolated from different environments, they are closely related. Members of *Burkholderia* and *Ralstonia* species are soil bacteria, occasionally plant or human pathogens [18,19], classified in the β-subgroup of the Proteobacteria.

The purpose of this study was to clarify the degradation pathway of 4-nitrobenzoate by strains PB4 and SB4 and to investigate the degradation of 4-nitro- and 4-aminobenzoate in mixtures. This second part is justified by the widespread co-contamination of nitroaromatic-polluted sites by aminoaromatic compounds.

2. Materials and methods

2.1. Microorganisms and culture conditions

4-Aminobenzoate served as the sole source of carbon, nitrogen and energy for the isolation of *B. cepacia* strain PB4 (from the soil of a former ammunition plant, Germany) ([20]; DSM 12775) and *R. paucula* strain SB4 (from the sand of a playground, Stuttgart, Germany) [21]. A substrate screening revealed that strains PB4 and SB4 were also able to grow on 4-nitrobenzoate. Using the Biolog GN assay (Hayward, CA, USA), strain SB4 was identified as belonging to the CDC group 1Ve-2 (which was recently named *R. paucula* sp. nov. [22]) with a similarity index of 0.831. Both bacteria grew on 4-nitrobenzoate, 4-aminobenzoate and 2-aminobenzoate. Strain PB4 but not SB4 was also able to grow on 4-hydroxybenzoate. Neither of them grew on 4-nitrotoluene, 2-nitrobenzoate, 3-nitrobenzoate or 3-aminobenzoate.

The microorganisms were grown in a nitrogen-free medium [23] supplemented with 1–4 mM 4-nitro- or 4-aminobenzoate in order to obtain induced cells. Non-induced cells were grown in a basal mineral salts (BMS) medium [24] supplemented with a mixture of 13.33 mM glucose and 6.67 mM sodium succinate (molar ratio 2:1) plus ammonium chloride (16 mM). Induced and non-induced cells were harvested at mid-exponential phase and washed twice in phosphate buffer before being inoculated into the nitrogen-free medium for aromatic degradation studies. Batch reactors (250-ml conical flasks, containing 100 ml of medium) were incubated at 30°C and 100 rpm on an orbital incubator (Gallenkamp, Leicester, UK). All cultures were carried out in duplicate.

2.2. Oxygen uptake experiments

Oxygen uptake by concentrated suspensions of induced and non-induced cells was measured at 19°C with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, USA). The final volume of 1.8 ml contained 55 µM substrate, cells (0.3–1.8 mg of protein) and phosphate buffer (0.02 M, pH 7.0).

2.3. Preparation of cell extracts and enzyme assays

Washed cells were broken by two passages through a French press at 6.9×10⁷ N m⁻². The slurry was centrifuged at 50 000 rpm for 0.5 h at 4°C. The pellet was discarded and the supernatant stored on ice until use.

The decrease in optical density at 340 nm, due to the oxidation of NAD(P)H by 4-nitrobenzoate reductase [12] or 4-hydroxybenzoate 3-hydroxylase (EC 1.14.13.2) [25] in the presence of the appropriate substrate, was used to measure the activity of these enzymes. Reaction mixtures for the reductase contained 0.2 µmol substrate, 0.2 µmol NAD(P)H, 47 µmol phosphate buffer (pH 7.4) and 40–400 µg protein in a total volume of 1 ml. Reaction mixtures for the hydroxylase contained 0.3 µmol 4-hydroxybenzoate, 0.01 µmol FAD, 0.2 µmol NAD(P)H, 46.4 µmol Tris–HCl buffer (pH 8.0) and 40–400 µg protein in a total volume of 1 ml. A molar extinction coefficient εNADP⁺H of 6220 M⁻¹ cm⁻¹ was used to calculate specific activities. One unit (U) is defined as the oxidation of 1 µmol NAD(P)H min⁻¹.

Activities of protocatechuate 3,4-dioxygenase (EC 1.13.11.3) [26] were measured by following the consumption of protocatechuate at 290 nm in a reaction mixture containing 0.2 µmol protocatechuate, 48 µmol Tris–HCl buffer (pH 8.0) and 40–400 µg protein in a total volume of 1 ml. A molar extinction coefficient of 2300 M⁻¹ cm⁻¹ was used, which is the difference between εS₉₀ of protocatechuate (3890 M⁻¹ cm⁻¹) and εS₉₀ of the product 3-carboxy-muconate (1590 M⁻¹ cm⁻¹). In order to determine protocatechuate 4,5-dioxygenase (EC 1.13.11.8) activity [27], the formation of 2-hydroxy-4-carboxymuconic semialdehyde was measured at 410 nm (εS₄₁₀ = 9700 M⁻¹ cm⁻¹). The reaction mixture contained 0.3 µmol protocatechuate, 48 µmol Tris–HCl buffer (pH 8.0) and 40–400 µg protein in a total volume of 1 ml. For both protocatechuate dioxygenases, one unit (U) of enzyme activity is defined as the oxidation of 1 µmole of protocatechuate per minute.

2.4. Anaerobic conditions

Anaerobic incubations were done in 100-ml serum flasks containing 25 ml of medium and closed tightly by a rubber stopper. The atmosphere of the headspace was evacuated and a mixture of 80% N₂ and 20% CO₂, first passed over a heated copper wire, was flushed through the solution. This
procedures was repeated three times before inoculation with washed and flushed cells of strains PB4 or SB4 grown on 4-nitrobenzoate.

2.5. Identification of 3-hydroxy-4-acetamidobenzoate

Culture fluid containing the metabolite produced by strain PB4 was acidified to pH 4 with HCl, saturated with NaCl and extracted once with a double volume of ethyl acetate. After evaporation, it was resuspended in H$_2$O and analyzed by liquid chromatography/mass spectrometry (LC/MS). Samples were analyzed on a TSP (Thermo Separation Products, San Jose, CA, USA) HPLC coupled to a Finnigan MAT TSQ 7000 (San Jose, CA, USA) triple quadrupole mass spectrometer with atmospheric pressure chemical ionization (APCI). LC conditions were identical to those described below with the exception that H$_2$O replaced phosphate buffer in the mobile phase. For the MS, temperatures of the capillary and of the vaporizer chamber were 220°C and 400°C, respectively. Pressures were 70 psi for the sheath gas and 10 psi for the auxiliary gas. The corona discharge was 5 µA.

2.6. Analytical methods

Quantitative determination of 4-nitrobenzoate, 4-amino-benzoate and other aromatic compounds was done by HPLC on an Alltima C$_{18}$ column (5 µm; 4.6 × 250 mm; Alltech, Deerfield, IL, USA). The mobile phase was an isocratic solvent system consisting of phosphate buffer (25 mM, pH 3.2): acetonitrile 75:25 (v/v) at 1 ml min$^{-1}$. Compounds were monitored at UV OD$_{230}$ with a Waters 996 diode array detector (Waters, Milford, MA, USA). Intermediates and products were identified by comparison of retention times and UV spectra with those of authentic standards. Ammonia was quantified enzymatically (Kit Sigma No. 171-UV, St. Louis, MO, USA). Microorganism concentration was determined through optical density at 540 nm (OD$_{540}$) or by protein measurement (Kit Sigma BCA-1, St. Louis, MO, USA). UV/visible spectra were recorded on a spectrophotometer PU8720 (Philips, Eindhoven, The Netherlands).

2.7. Chemicals

3-Hydroxy-4-acetamidobenzoate was synthesized by reaction of 3-hydroxy-4-aminobenzoate with acetic anhydride [28]. All other chemicals were of the highest purity commercially available and were obtained from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, USA), Fluka (Buchs, Switzerland) or Aldrich (Milwaukee, WI, USA).

3. Results

3.1. Growth with 4-nitrobenzoate

Growth of strain SB4 on 4 mM 4-nitrobenzoate followed the regular bacterial growth cycle. However, a decrease of protein concentration and the appearance of a yellow-orange color in the medium (previously observed by Rhys-Williams et al. [14] and Michan et al. [16]) accompanied further additions of 4-nitrobenzoate. UV/visible spectroscopy of the supernatant revealed a broad absorbing band with two maxima at 425 nm and 466 nm, responsible for the yellow-orange color. Two new peaks (peak A and peak B, A being more polar) were detected by HPLC when strain SB4 was incubated with 4-nitrobenzoate. The compound representing peak A did not seem to be responsible for the color of the culture since its UV/visible spectrum, as determined by the photodiode array detector, showed no absorption in the visible range. Comparison of HPLC retention time and UV spectrum with those of a standard allowed the identification of peak A as 3-hydroxy-4-aminobenzoate. When a solution of 3-hydroxy-4-aminobenzoate in phosphate buffer was left standing in the presence of O$_2$ for some days, it became progressively bright orange. HPLC analysis of this orange solution showed the presence of peak B, and its UV/visible spectrum was the same as described above (a broad band in the visible spectrum). These observations suggest that the compound responsible for peak A (3-hydroxy-4-aminobenzoate) was converted into the compound responsible for peak B by an abiotic reaction.

A new HPLC peak also appeared when strain PB4 metabolized 4-nitrobenzoate. The shape of the UV spectrum was indicative of a disubstituted benzoic acid (data not shown) [29]. The mass spectrum revealed a compound with a molecular mass of 195 Da and the loss of fragments of 42 and 44 Da, consistent with the presence of an acetyl and a carboxyl group, respectively. Moreover, the compound obtained by acetylation of 3-hydroxy-4-aminobenzoate had the same retention time, UV spectrum and mass spectrum as the product accumulated by strain PB4. The new HPLC peak was presumably caused by 3-hydroxy-4-acetamidobenzoate. This metabolite did not seem to have any adverse effect on strain PB4. Ammonia was released by both strains degrading both 4-nitro- and 4-aminobenzoate, alone or in mixture.

3.2. Anaerobic conversion of 4-nitrobenzoate by resting cells of strain PB4 or SB4

When cells of 4-nitrobenzoate-grown strain PB4 were incubated with 4-nitrobenzoate under anaerobic conditions, conversion of the latter was comparable to that under aerobic conditions and protocatechuate was the main product formed. Some traces of 3-hydroxy-4-acetamidobenzoate and of 4-aminobenzoate (less than 0.01
mM) were also detected. 4-Nitrobenzoate remained largely unconverted by 4-nitrobenzoate-grown cells of strain SB4 under anaerobic conditions. The converted part (5%) gave rise to protocatechuate and to some 3-hydroxy-4-amino-benzoate.

3.3. Oxygen uptake rate

The rate of oxygen consumption by induced and non-induced concentrated cell suspensions of strain PB4 or SB4 was measured in the presence of potential intermediates of the 4-nitrobenzoate or 4-aminobenzoate degradation pathway (Table 1). Non-induced cells showed no aromatic substrate-dependent oxygen uptake (data not shown).

3.4. Resting cell suspensions

The conversion of the compounds used in the oxygen uptake experiment by resting cell suspensions of induced or non-induced strain PB4 or SB4 was investigated by HPLC (data not shown). Conversion results were consistent with oxygen uptake measurements, i.e. rapid conversion was observed with combinations of inducer and substrate that caused increased oxygen uptake. Neither of the strains was able to convert 3-hydroxy-4-aminobenzoate. Conversion of 4-nitro- or 4-aminobenzoate by non-induced cells started after a lag phase, which was peculiarly short for the degradation of 4-aminobenzoate by strain SB4.

3.5. Enzymatic activities of cell-free extracts

Enzymatic activities were measured in cell-free extracts of strains PB4 and SB4 (Table 2). Oxidation of NADPH in the presence of 4-nitrobenzoate was catalyzed by cell extracts of 4-nitrobenzoate-grown strains PB4 and SB4, indicating a 4-nitrobenzoate reductase activity. Moreover,

![Diagram](https://example.com/diagram.png)

Fig. 1. Degradation of 1 mM 4-nitrobenzoate alone (□), 1 mM 4-aminobenzoate alone (○) and equimolar mixtures (1 mM of each) thereof (■, ■) by strain PB4 grown on 4-nitrobenzoate (A), strain PB4 grown on 4-aminobenzoate (B), strain SB4 grown on 4-nitrobenzoate (C), strain SB4 grown on 4-aminobenzoate (D). Initial protein content, PB4 grown on 4-nitrobenzoate: 140 mg l⁻¹; PB4 grown on 4-aminobenzoate: 155 mg l⁻¹; SB4 grown on 4-nitrobenzoate: 80 mg l⁻¹; SB4 grown on 4-aminobenzoate: 130 mg l⁻¹.

### Table 1

| Substrate                  | Oxygen uptake rate (nmol (mg protein)⁻¹ min⁻¹) after growth of PB4 on: | Oxygen uptake rate (nmol (mg protein)⁻¹ min⁻¹) after growth of SB4 on: |
|----------------------------|-----------------------------------------------------------------------|-----------------------------------------------------------------------|
|                            | 4-Nitrobenzoate | 4-Aminobenzoate | 4-Nitrobenzoate | 4-Aminobenzoate |
| 4-Nitrobenzoate            | 28.8           | 11.7            | 15.4            | <5              |
| 4-Aminobenzoate           | 10.3           | 35.6            | 17.7            | 88.7            |
| 4-Hydroxybenzoate         | <5             | 22.5            | <5              | <5              |
| Protocatechuate           | 52             | 33.0            | 35.5            | 73.5            |
| 3-Hydroxy-4-aminobenzoate | <5             | <5              | <5              | <5              |
| 4-Nitrophenol             | <5             | <5              | ND*             | <5              |

*ND, not determined.
in agreement with the oxygen uptake measurements, such an activity was also measured with cell extracts of 4-aminobenzoate-grown strain PB4. 4-Nitrobenzoate reductase activity was not detected in non-induced cells. 4-Hydroxybenzoate 3-hydroxylase was detected only in cell extracts of strain PB4 grown on 4-aminobenzoate. The conditions required to measure the initial oxidation of 4-aminobenzoate in strain PB4 and in strain SB4 could not be determined. Protocatechuate 3,4-dioxygenase activity, but no protocatechuate 4,5-dioxygenase activity, was detected in strains PB4 and SB4 induced on either 4-nitrobenzoate or 4-aminobenzoate.

3.6. Degradation of 4-nitrobenzoate or 4-aminobenzoate by induced and non-induced cells of strain PB4 and strain SB4

The kinetics of degradation of 1 mM 4-nitrobenzoate or 4-aminobenzoate (as sole compound in the medium; open symbols) by strain PB4 (Fig. 1A,B) or strain SB4 (Fig. 1C,D) grown on 4-nitrobenzoate (Fig. 1A,C) or 4-aminobenzoate (Fig. 1B,D) are shown in Fig. 1. 4-Nitrobenzoate-grown cells of strain PB4 or strain SB4 started to degrade 4-nitrobenzoate immediately, as did 4-aminobenzoate-grown cells with 4-aminobenzoate. 4-Aminobenzoate-grown cells of SB4 did not significantly transform 4-nitrobenzoate for the first 4 h of the incubation (Fig. 1D), neither did non-induced cultures (data not shown). However, noticeable is the fact that 4-aminobenzoate-grown cells of PB4 started to consume 4-nitrobenzoate immediately (Fig. 1B), whereas non-induced cells needed more than 4 h to start the degradation. Although the degradation of 4-aminobenzoate by 4-nitrobenzoate-grown cells of PB4 was slow at the beginning, there was no definite lag before the degradation started. The degradation of 4-aminobenzoate by 4-nitrobenzoate-grown cells of SB4 was rapid (Fig. 1C). However, it was not significantly different from that observed with non-induced cells, which was consistent with observations made in resting cell experiments (see above). Protocatechuate was observed transiently during the degradation of 4-nitro- and 4-aminobenzoate by strain SB4 irrespective of the inducer.

3.7. Degradation of an equimolar mixture of 4-nitrobenzoate and 4-aminobenzoate by induced and non-induced cells of strain PB4 and strain SB4

The cultures used in the experiment described in Section 3.6 were also inoculated in a medium containing an equimolar mixture (1 mM of each) of 4-nitro- and 4-aminobenzoate (Fig. 1, solid symbols). The fact that the inoculum was exactly the same for the mixtures as for each compound alone (see Section 3.6) allowed us to make a comparison of the kinetics observed. For both inducers, the degradation of 4-nitrobenzoate by strain PB4 was slowed down by the presence of 4-aminobenzoate. In contrast, the rate of 4-aminobenzoate degradation was almost unaltered by the presence of 4-nitrobenzoate (Fig. 1A,B). Irrespective of the inducer molecule, strain SB4 started by degrading 4-aminobenzoate while 4-nitrobenzoate concentration remained fairly stable (Fig. 1C,D). When all 4-aminobenzoate was consumed, the concentration of 4-nitrobenzoate started to decrease. In comparison with the kinetics observed with a compound alone in the medium, the degradation curve of 4-aminobenzoate was left unchanged by the presence of 4-nitrobenzoate. The presence of 4-aminobenzoate clearly hindered the degradation of 4-nitrobenzoate by 4-nitrobenzoate-grown cells of strain SB4 (Fig. 1C).

Degradation patterns of an equimolar mixture of 4-nitro- and 4-aminobenzoate by non-induced cells of strains PB4 and SB4 are presented in Fig. 2. After a lag phase of approx. 3 h, 4-nitro- and 4-aminobenzoate started to be degraded by strain PB4 (Fig. 2A) and the degradation kinetics of both compounds matched each other closely. 1.25 moles ammonia were released by the metabolism of a total of 2 moles of aromatic compounds. With strain SB4 (Fig. 2B), degradation of 4-aminobenzoate started after a lag of around 5 h, followed by degradation of 4-nitrobenzoate (lag of ±7 h). A mixed substrate growth rather than...
a diauxic growth was observed in cultures of each of the two strains grown on both compounds together.

4. Discussion

4.1. Strain description

*B. cepacia* PB4 as well as *R. paucula* SB4 have been isolated with 4-aminobenzoate as sole carbon and nitrogen source and found to be able to grow on 4-nitrobenzoate as well. The nitro group is mostly a xenobiotic substituent and it was surprising that bacteria isolated for their ability to degrade 4-aminobenzoate, a molecule common in nature [10], were also able to degrade the corresponding nitroaromatic compound. Therefore, one can wonder if the pathway is intended for 4-nitrobenzoate or is the result of a relaxed enzyme specificity. Cloning and sequencing of the gene coding for the lyase activity as well as its neighbors would hopefully give clues on the evolutionary origin of the lyase step.

It is certainly not the first time that *Burkholderia* and *Ralstonia* strains are shown to use an aromatic compound as sole carbon source. To our knowledge, all 4-nitrobenzoate degraders studied to date are, like strains PB4 and SB4, pseudomonads. This group is known for its broad metabolic diversity [18], comprising efficient aromatic degraders [12–16,30]. However, this is the first report of xenobiotic degradation by *R. paucula*.

4.2. Degradation pathways of 4-nitrobenzoate and 4-aminobenzoate

To our knowledge, aerobic strains proven to degrade a nitro compound via its amino counterpart are unknown at the present time. Even though such mechanisms were postulated in the past, the aminoaromatic compound was not rigorously shown to be an intermediate [31–34].

Identification of the main product of anaerobic conversion of 4-nitrobenzoate by whole cells is one way to clarify the degradation pathway of 4-nitrobenzoate. The formation of protocatechuate but only insignificant amounts of 4-aminobenzoate showed that 4-aminobenzoate was not the major intermediate of the 4-nitrobenzoate degradation pathway of each strain. These results, reinforced by the physiological and enzymatic analyzes, indicate that strains PB4 and SB4 used the well-known step from 4-hydroxylaminobenzoate to protocatechuate (Fig. 3), requiring a lyase. Since all recently described 4-nitrobenzoate-degrading strains harbor this pathway [12–16], it does not seem surprising that PB4 and SB4 join this general tendency, although, contrary to the strains described earlier, they were isolated on 4-aminobenzoate. It appears to be a generality that nitroaromatics are not degraded via their amine derivatives. One reason may be the additional reducing equivalents needed for the reduction of hydroxylamine to amine. The partial (chemoselective) reduction allows an economy of these reducing equivalents [3,35]. Another reason could be an economy of oxygen which is of limited availability in soils.

However, our results could not firmly exclude that mineralization of 4-nitrobenzoate in strain PB4 proceeds by two parallel pathways, a major one and a minor one involving 4-aminobenzoate. The formation of traces of 4-aminobenzoate was most likely catalyzed by a non-specific nitroreductase, similarly to what has been reported by Cartwright and Cain [31].

From [21] and from the present study, the metabolic sequence of 4-aminobenzoate degradation was determined to be as follows (Fig. 3): in strain PB4, 4-aminobenzoate is attacked by a first monooxygenase to produce 4-hydroxylaminobenzoate, a molecule common in nature [10], and was also able to degrade the corresponding nitroaromatic compound. Therefore, one can wonder if the pathway is intended for 4-nitrobenzoate or is the result of a relaxed enzyme specificity. Cloning and sequencing of the gene coding for the lyase activity as well as its neighbors would hopefully give clues on the evolutionary origin of the lyase step.

It is certainly not the first time that *Burkholderia* and *Ralstonia* strains are shown to use an aromatic compound as sole carbon source. To our knowledge, all 4-nitrobenzoate degraders studied to date are, like strains PB4 and SB4, pseudomonads. This group is known for its broad metabolic diversity [18], comprising efficient aromatic degraders [12–16,30]. However, this is the first report of xenobiotic degradation by *R. paucula*.

4.3. Mutase-catalyzed formation of dead-end metabolites

3-Hydroxy-4-aminobenzoate, found in cultures of strain SB4 growing on 4-nitrobenzoate, might have been formed by a mutase-catalyzed Bamberger-like rearrangement starting from 4-hydroxylaminobenzoate (Fig. 3). The abiotic transformation of 3-hydroxy-4-aminobenzoate produced a yellow-orange compound present in cultures of
strain SB4. It seemed to be highly toxic to the SB4 bacteria. This compound might be an oxidative dimerization product, as observed with 6-amino-m-cresol [36]. 3-Hydroxy-4-acetamidobenzoate, found in cultures of strain PB4, is likely produced by the acetylation of 3-hydroxy-4-aminobenzoate (Fig. 3). Acetylation is a common cellular way to detoxify an aminoaromatic compound [37].

3-Hydroxy-4-aminobenzoate has been proposed as a possible intermediate in the degradation pathway of 4-nitrobenzoate [17]. Our experimental results suggest that it is more likely a dead-end product. The observation of hydroxylaminobenzoate mutase activity is a result of interest. A mutase has recently been shown to be involved in 4-nitrotoluene degradation [36]. Mutase activities on hydroxylaminobenzoates have been characterized by Davis et al. [38]. These authors found two genes encoding hydroxylaminobenzene mutase in nitrobenzene-degrading Pseudomonas pseudoalcaligenes JS45. Schenzle et al. [39] described amino acid sequences of the NH$_2$-terminus and of two internal sequences of a 3-hydroxylaminophenol mutase from Ralstonia eutropha JMP134. These sequences have no homologies to the deduced amino acid sequence of the two hydroxylaminobenzene mutases in P. pseudoalcaligenes JS45 [38]. Neither the enzymes of P. pseudoalcaligenes JS45, nor the enzyme of R. eutropha JMP134 transformed 4-hydroxylaminobenzoate, indicating some specificity for the proposed 4-hydroxylaminobenzoate mutases of strains PB4 and SB4. Overall this suggests that enzymes which can act as mutases on hydroxylaminobenzenes are prevalent. They may have different primary metabolic functions and descend from different ancestors.

4.4. Patterns of induction

From the observation of the degradation curves for strain PB4, it appears that 4-aminobenzoate inhibits slightly 4-nitrobenzoate degradation, whereas 4-aminobenzoate degradation is not influenced by the presence of 4-nitrobenzoate. With strain SB4, irrespective of the pre-culturing conditions, the presence of 4-aminobenzoate inhibits totally one or several enzymes of the degradation pathway of 4-nitrobenzoate.

Surprising was the ability of 4-aminobenzoate-grown cells of strain PB4 to degrade 4-nitrobenzoate without a
lag. Strain PB4 showed the unusual property of cross-inducing 4-nitro- and 4-aminobenzoate degradation. This attribute, resulting in the simultaneous utilization of both compounds, may confer an advantage to strain PB4 over other strains in the site contaminated by nitroaromatic compounds from which it was isolated. Given the low concentrations of carbon compounds found in oligotrophic environments, a microorganism which is able to simultaneously metabolize different carbon and/ or energy sources may have a competitive advantage compared to other, less versatile organisms [40].

5. Summary

This study extends the description of strains harboring a partial reduction pathway for the degradation of a nitroaromatic compound to bacteria isolated on an amino instead of a nitro compound. In addition to the lyase catalyzing the conversion of 4-hydroxylaninobenzoate to protocatechuic acids, strains PB4 and SB4 also synthesize an enzyme able to act as a mutase. Although using a similar degradation pathway, their behavior towards the degradation of aromatic compounds in mixtures reflects different mechanisms of regulation, the study of which would provide clues for the engineering of biodegradative pathways. The novel features of cross-induction by, and simultaneous degradation of, 4-nitro- and 4-aminobenzoate by strain PB4 make it an interesting microorganism model for bioremediation.

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References

[1] Merck and Co., Inc. (1996) The Merck Index. An Encyclopedia of Chemicals, Drugs, and Biologicals, 12th edn. Merck and Co., Inc., Rahway, NJ.
[2] Zeiger, E., Anderson, B., Haworth, S., Lawlor, S., Mortelmans, K. and Speck, W. (1987) Salmonella mutagenicity tests. III. Results from testing of 225 chemicals. Environ. Mutagen. 9, 1–109.
[3] Spain, J.C. (1995) Bacterial degradation of nitroaromatic compounds under aerobic conditions. In: Biodegradation of Nitroaromatic Compounds (Spain, J.C., Ed.), pp. 19–35. Plenum Press, New York.
[4] Corbett, M.D. and Corbett, B.R. (1995) Bioorganic chemistry of the arylhydroxylamine and nitrosoarene functional groups. In: Biodegradation of Nitroaromatic Compounds (Spain, J.C., Ed.), pp. 151–182. Plenum Press, New York.
[5] McCormick, N.G., Feeherry, F.E. and Levinson, H.S. (1976) Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Microbiol. 31, 949–958.
[6] Schackmann, A. and Müller, R. (1991) Reduction of nitroaromatic compounds by different Pseudomonas species under aerobic conditions. Appl. Microbiol. Biotechnol. 34, 809–813.
[7] Spanggord, R.J., Gibson, B.W., Keck, R.G., Thomas, D.W. and Barkley, J.J. (1982) Effluent analysis of wastewater generated in the manufacture of 2,4,6-trinitrotoluene. 1. Characterization study. Environ. Sci. Technol. 16, 229–232.
[8] Swindoll, C.M., Perkins, R.E., Gannon, J.T., Holmes, M. and Fisher, G.A. (1995) Assessment of bioremediation of a contaminated wetland. In: Intrinsic Bioremediation, Vol. 1 (Hinchee, R., Wilson, J.C. and Downey, D.C., Eds.), pp. 163–169. Battelle Press, Columbus, OH.
[9] Schmidt, T.C., Petersmann, M., Kaminski, L., von Löw, E. and Stork, G. (1997) Analysis of aminobenzoic acids in waste water from a former ammunition plant with HPLC and combined diode array and fluorescence detection. Fresenius J. Anal. Chem. 357, 121–126.
[10] Stanier, R.Y., Ingraham, J.L., Whelin, M.L. and Painter, P.R. (1987) General Microbiology. Macmillan, London.
[11] Richards, R.M.E., Xing, D.K.L. and King, T.P. (1995) Activity of p-aminobenzoic acid compared with other organic acids against selected bacteria. J. Appl. Bacteriol. 78, 209–215.
[12] Groenewegen, P.E.J. and de Bont, J.A.M. (1992) Degradation of 4-nitrobenzoate via 4-hydroxylaminobenzoate and 3,4-dihydroxybenzoate in Comamonas acidovorans NBA-10. Arch. Microbiol. 158, 381–386.
[13] Haigler, B.E. and Spain, J.C. (1995) Biodegradation of 4-nitrotoluene by Pseudomonas sp. strain 4NT. Appl. Environ. Microbiol. 59, 2239–2243.
[14] Rhys-Williams, W., Taylor, S.C. and Williams, P.A. (1993) A novel pathway for the catalolism of 4-nitrotoluene by Pseudomonas. J. Gen. Microbiol. 139, 1967–1972.
[15] Yabannavar, A.V. and Zylstra, G.J. (1995) Cloning and characterization of the genes for p-nitrobenzoate degradation from Pseudomonas putida YH105. Appl. Environ. Microbiol. 61, 4284–4290.
[16] Michan, C., Delgado, A., Had our, A., Lucchesi, G. and Ramos, J.L. (1997) In vivo construction of a hybrid pathway for metabolism of 4-nitrotoluene in Pseudomonas fluorescens. J. Bacteriol. 179, 3036–3038.
[17] Meulenber, R. and de Bont, J.A.M. (1995) Microbial production of catechols from nitroaromatic compounds. In: Biodegradation of Nitroaromatic Compounds (Spain, J.C., Ed.), pp. 37–52. Plenum Press, New York.
[18] Schlöter, M., Lebuhn, M., Heulin, T. and Hartmann, A. (2000) Ecology and evolution of bacterial microdiversity. FEMS Microbiol. Rev. 24, 647–660.
[19] Moissenet, D., Goujon, C.P., Garbarg-Chenon, A. and Vu-Thien, H. (1997) In vivo construction of a hybrid pathway for metabolism of 4-nitrotoluene in Pseudomonas fluorescens. J. Bacteriol. 179, 3036–3038.
[20] Peres, C.M., Van Aken, B., Naveau, H. and Agathos, S.N. (1999) Continuous degradation of mixtures of 4-nitrobenzoate and 4-aminobenzoate by immobilized cells of Burkholderia cepacia strain PB4. Appl. Microbiol. Biotechnol. 52, 440–445.
[21] Vandamme, P., Goris, J., Coenye, T., Hoste, B., Janssens, D., Kersters, K., De Vos, P. and Falsen, E. (1999) Assignment of Centers for Disease Control group IVc-2 to the genus Ralstonia as Ralstonia pickettii YH105. J. Gen. Microbiol. 145, 959–960.
[22] Lenke, H., Pieper, D.H., Bruhn, C. and Knackmuss, H.-J. (1992)
Degradation of 2,4-dinitrophenol by two Rhodococcus erythropolis strains, HL 24-1 and HL 24-2. Appl. Environ. Microbiol. 58, 2928–2932.

[23] Stanier, R.Y., Palleroni, N.J. and Doudoroff, M. (1966) The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43, 159–271.

[24] Hosokawa, K. and Stanier, R.Y. (1966) Crystallisation and properties of p-hydroxybenzoate hydroxylase from Pseudomonas putida. J. Biol. Chem. 241, 2453–2460.

[25] Stanier, R.Y. and Ingraham, J.L. (1954) Protocatechuic acid oxidase. J. Biol. Chem. 210, 799–808.

[26] Wheelis, M.L., Palleroni, N.J. and Stanier, R.Y. (1966) The metabolism of aromatic acids by Pseudomonas testosteroni and P. acidovorans. Arch. Microbiol. 59, 302–314.

[27] Corbett, M.D. and Corbett, B.R. (1981) Metabolism of 4-chloroanisole by the yeast Rhodosporidium sp. Appl. Environ. Microbiol. 41, 942–949.

[28] Scott, A.I. (1964) Interpretation of the Ultraviolet Spectra of Natural Products. Pergamon Press, Oxford.

[29] Laurie, A.D. and Lloyd-Jones, G. (2000) Quantification of phnAc and nahAc in contaminated New Zealand soils by competitive PCR. Appl. Environ. Microbiol. 66, 1814–1817.

[30] Durham, N.N. (1958) Studies on the metabolism of p-nitrobenzoic acid. Can. J. Microbiol. 4, 141–148.

[31] Cartwright, N.J. and Cain, R.B. (1959) Bacterial degradation of the nitrobenzoic acids. 2. Reduction of the nitro group. Biochem. J. 73, 305–314.

[32] Ke, Y-H., Gee, L.L. and Durham, N.N. (1959) Mechanism involved in the metabolism of nitrophenyl-carboxylic acid compounds by microorganisms. J. Bacterioli. 77, 593–598.

[33] Germanier, R. and Wurmann, K. (1963) Über den aeroben mikrobiellen Abbau aromatischer Nitroverbindungen. Pathol. Microbiol. 26, 569–578.

[34] Schenzle, A., Lenke, H., Spain, J.C. and Knackmuss, H.-J. (1999) Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by Ralstonia eutropha JMP134. Appl. Environ. Microbiol. 65, 2317–2323.

[35] Ross, R. (1991) Diplomarbeit. Institut für Mikrobiologie der Universität Stuttgart, Stuttgart.

[36] Spiess, T., Desiere, F., Fischer, P., Spain, J.C., Knackmuss, H.-J. and Lenke, H. (1998) A new 4-nitrotoluene degradation pathway in a Mycobacterium strain. Appl. Environ. Microbiol. 64, 446–452.

[37] Tweedy, B.G., Loepky, C. and Ross, J.A. (1970) Metobromuron: acetylation of the aniline moiety as a detoxification mechanism. Science 168, 482–483.

[38] Davis, J.K., Paoli, G.C., He, Z., Nadeau, L.J., Somerville, C.C. and Spain, J.C. (2000) Sequence analysis and initial characterization of two isozymes of hydroxylaminobenzene mutase from Pseudomonas pseudoalcaligenes JS45. Appl. Environ. Microbiol. 66, 2965–2971.

[39] Schenzle, A., Lenke, H., Spain, J.C. and Knackmuss, H.-J. (1999) 3-Hydroxylaminophenol mutase from Ralstonia eutropha JMP134 catalyzes a Bamberger rearrangement. J. Bacteriol. 181, 1444–1450.

[40] Egli, T. (1995) The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. In: Advances in Microbial Ecology, Vol. 14 (Jones, J.G., Ed.), pp. 305–386. Plenum Press, New York.