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Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads

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Abstract. From various oxic or anoxic habitats several strains of bacteria were isolated which in the absence of molecular oxygen oxidized phenol to CO_2 with nitrate as the terminal electron acceptor. All strains grew in defined mineral salts medium; two of them were further characterized. The bacteria were facultatively anaerobic Gram-negative rods; metabolism was strictly oxidative with molecular oxygen, nitrate, or nitrite as electron acceptor. The isolates were tentatively identified as pseudomonads. Besides phenol many other benzene derivatives like cresols or aromatic acids were anaerobically oxidized in the presence of nitrate. While benzoate or 4-hydroxybenzoate was degraded both anaerobically and aerobically, phenol was oxidized under anaerobic conditions only. Reduced alicyclic compounds were not degraded. Preliminary evidence is presented that the first reaction in anaerobic phenol oxidation is phenol carboxylation to 4-hydroxybenzoate.

Key words: Anaerobic degradation – Aromatic compounds – Phenol – Cresol – 4-Hydroxybenzoate – Denitrification – *Pseudomonas* sp.

Anaerobic degradation of aromatic acids was first reported by Tarvin and Buswell (1934). Pioneering studies by Evans and coworkers mainly focussed on anaerobic degradation of benzoate as a model substrate (for reviews see Evans 1977; Sleat and Robinson 1984). It is well known that the aerobic breakdown of aromatic ring structures requires oxygenases for activation and ring cleavage (Dagley 1975). Clearly, the anaerobic degradation has to take a different route and therefore uses other mechanisms. In pure cultures of photosynthetic or denitrifying bacteria reduced cyclic intermediates were isolated and identified during anaerobic benzoate metabolism and thus a reductive degradation of benzoate was proposed (Dutton and Evans 1969; Williams and Evans 1975). This led to the general concept of reductive attack on the aromatic ring under anaerobic conditions.

Anaerobic degradation of non-carboxylated aromatic compounds is less studied and was reported first by Chmielowsky et al. (1965). Defined methanogenic cocultures degrading phenol could not be isolated until recently (for review see Young 1984). Nitrate dependent anaerobic phenol metabolism was reported by Bakker (1977a). Recently also sulfate-reducing bacteria were isolated which completely oxidize phenol (Bak and Widdel 1986). Here we describe newly isolated strains of facultatively anaerobic denitrifying bacteria which under anaerobic conditions completely oxidized phenol as well as about 15 other aromatic compounds. Experiments are presented indicating that carboxylation of phenol to 4-hydroxybenzoate is the initial reaction in anaerobic phenol degradation in these strains.

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Materials and methods

Sources of organisms

The enrichment cultures were inoculated with either anaerobic sludge from the municipal sewage plant in Konstanz or anaerobic sediment samples from a polluted creek near Konstanz, FRG. Activated sludge from the municipal sewage plant in Ulm or soil samples of a field near Ulm, FRG, were used as inoculum from aerobic habitats.

Medium and growth conditions

The basal medium had the following composition (values in $g\,l^{-1}$): $Na_2HPO_4 \cdot 2\,H_2O$ (2.0), KH_2PO_4 (1.0), NH_4Cl (0.5), K_2SO_4 (0.06). The autoclaved medium was cooled under an atmosphere of N_2 and the following components were added from steril stock solutions: $MgCl_2 \cdot 6\,H_2O$ (200 g/l) + $CaCl_2 \cdot 2\,H_2O$ (50 g/l) solution (0.5 ml/l), trace element solution SL 10 (1 ml/l) (Widdel et al. 1983), selenite-tungstate solution (1 ml/l) (Tschech and Pfennig 1984), seven vitamins solution (10 × concentrated, 0.5 ml/l) (Pfennig 1978), 1 M $NaHCO_3$ solution (5 ml/l). The pH was adjusted to 7.2–7.4 with 1 M HCl or NaOH. The medium was dispensed à 50 ml or à 100 ml into 125 ml-infusion bottles (Müller + Krempel AG, Bülach, Switzerland), gassed with N_2, and sealed with Latex rubber septa (Maag Technic AG, Dübendorf, Switzerland). Sterile substrate solutions were added by syringes. Neutral stock solutions (0.25 M) of di- or trihydroxybenzenoid substrates were prepared and stored oxygen-free in Latex rubber sealed infusion bottles. All growth tests were carried out at 28° C.

Isolation and characterization

Pure cultures were obtained by repeatedly applying the agar shake culture method as described by Pfennig (1978). Tubes were gassed with N_2 and sealed oxygentight with rubber stoppers. Purity of liquid cultures was checked microscopically after growth in phenol-containing medium as well as

in medium to which glucose or fructose had been added; the phenol oxidizing bacteria were not able to utilize carbohydrates. For confirmation of the Gram stain the KOH test was performed (Gregerson 1978). Poly-β-hydroxybutyrate was identified as β-hydroxybutyric acid methyl ester by gas chromatography (Braunegg et al. 1978).

Determination of growth parameters

Samples of the cultures were taken by syringe. Optical density was determined in 1 cm cuvettes at 578 nm, consumption or conversion of aromatic substrates was followed by taking UV-absorption spectra of the centrifuged and 1:10 diluted culture liquid. Quantitation of nitrate and nitrite was done by colorimetric tests.

Fixation of [^{14}C]bicarbonate

To determine the fixation of [^{14}C]bicarbonate into the carboxyl group of 4-hydroxybenzoate, cells of strain K 172 or S 100 were grown anaerobically with either phenol or 4-hydroxybenzoate. Cells were centrifuged and resuspended in the same volume of fresh anaerobic medium lacking any electron acceptor. To these cell suspensions 5 mM 4-hydroxybenzoate plus labelled bicarbonate was added and the fixation of [^{14}C]carbon into acid stable compound, i.e. 4-hydroxybenzoate, was followed with time. Samples of the suspensions were withdrawn by syringe, acidified to pH 2 and gassed with N_2/CO_2 (80%/20%) gas mixture for 15 min. Labelling of 4-hydroxybenzoate was confirmed by isolating 4-hydroxybenzoate by HPLC: all acid stable label was contained in the 4-hydroxybenzoate peak.

Chemical analyses

Volatile fatty acids, methylated dicarboxylic or aromatic acids, phenol, and cresols were assayed by gas chromatography. The samples were analyzed in a gas chromatograph with flame ionisation detector equipped with a 1.8 m × 3 mm glass column packed with SP 1000 + 1% H_3PO_4 on Chromosorb WAW, 80–100 mesh; carrier gas was nitrogen with a flow rate of 30 ml/min; oven temperature was isothermal between 130° C and 200° C. Reverse phase HPLC with UV detection (254 nm) was used to isolate and identify p-cresol, 4-hydroxybenzylalcohol, 4-hydroxybenzaldehyde and 4-hydroxybenzoate. Samples were analyzed under isocratic conditions on a μ-Bondapak C18-column (Waters) with isopropanol-ammoniumacetate buffer (100 mM, pH 4.5 in water) (15 vol %/85 vol %) as solvent at a flow rate of 1 ml min^{-1}. The retention times were 5.3 min (4-hydroxybenzylalcohol), 6.6 min (4-hydroxybenzoate), 10.6 min (4-hydroxybenzaldehyde), and 21.0 min (p-cresol).

Results

Enrichments and isolation

Enrichments of phenol-degrading, denitrifying bacteria were done anaerobically under a nitrogen gas phase in a mineral salts medium supplemented with vitamins. This medium contained 1 mM phenol and 5 mM nitrate as substrates and was inoculated with 10%–20% (v/v) of aerobic or anaerobic soil samples or sludges. After 1 day of incubation

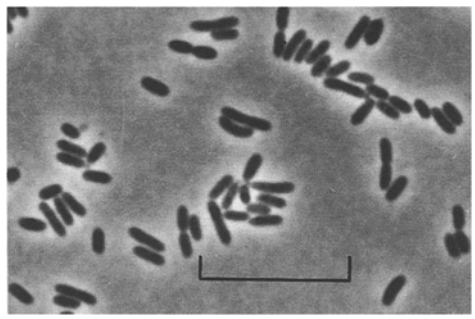

Fig. 1. Phase contrast photomicrograph of strain S 100 growing with phenol. Bar equals 10 μm

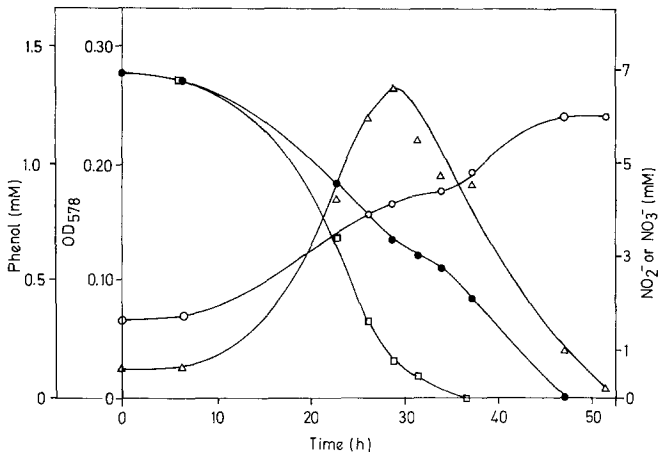

Fig. 2. Substrate dependent growth curve of strain S 100 growing anaerobically with phenol. Experiments were performed at 28° C in 125 ml infusion bottles sealed with Latex rubber septa. Samples were taken by syringe at the times indicated. The headspace was N_2. ○ Cell density, ● phenol, □ nitrate, △ nitrite

at 28° C gas production started in all enrichments and 2 days later ceased because nitrate was completely reduced to nitrogen gas. The phenol concentration remained unchanged. Only after a third addition of 5 mM nitrate was phenol oxidized. In subcultures on the same medium 1 mM phenol was degraded within 2–4 days, and after five transfers into fresh medium isolation of the phenol-degrading bacteria was attempted in agar shake series. From tubes containing only up to about 30 colonies visibly different types of colonies were separated by following shake series. After 3–4 repetitions of this procedure several strains of phenol-oxidizing denitrifying bacteria were isolated in pure culture and strains K 172 and S 100 were further characterized.

Characterization of strain S 100

Cells of strain S 100 were motile, straight rods with round ends which were 0.5 μm to 0.6 μm in diameter and 1.1 μm–2.2 μm long (Fig. 1). The cells were Gram-negative and KOH-positive. After aerobic growth oxidase and catalase reaction were positive. Cells grew well in mineral salts medium; vitamins and ammonium that were present in the isolation medium were not required; strain S 100 assimilated nitrate. The pH optimum for anaerobic growth with phenol was between 7.0 and 7.4 with limits at 6.5 and 9.0; the

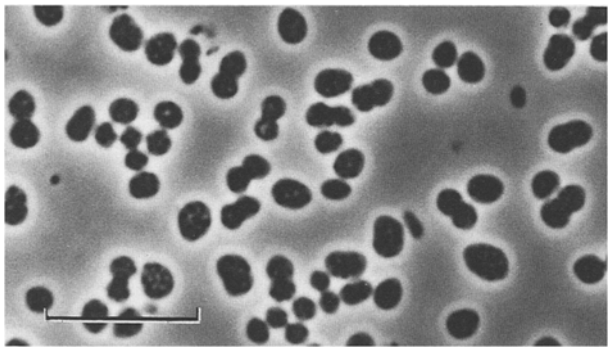

Fig. 3. Phase contrast photomicrograph of strain K 172 growing with phenol. Note the different cell shapes due to accumulation of storage material. Bar equals 10 μm

optimal temperature was around 28° C and no growth occurred below 4° C or above 40° C. Under optimal conditions the doubling time in static culture was about 20 h. The bacteria first reduced nitrate almost quantitatively to nitrite before reduction to N_2 occurred (Fig. 2). At a nitrite concentration of 5 mM growth of the bacteria was inhibited. The growth curve never was exponential possibly due to the toxicity of phenol (see Discussion). At 2 mM phenol concentration the lag phase of freshly transferred cultures lasted 1 – 3 days; with 5 mM phenol cultures did not grow.

Besides phenol, strain S 100 grew anaerobically with various aromatic and aliphatic compounds when nitrate was present as electron acceptor. The results are summarized in Table 1. Metabolism was strictly oxidative. While benzoate, 3- or 4-hydroxybenzoate or protocatechuate was oxidized both under aerobic and anaerobic conditions, phenol only was oxidized in the absence of molecular oxygen with nitrate or nitrite as electron acceptor. With phenol the molar growth yield of strain S 100 was 40 g per mol. Per mol phenol degraded about 4 mol nitrate was reduced to N_2. The theoretical stoichiometry of phenol oxidation to CO_2

$$C_6H_6O + 11\ H_2O \rightarrow 6\ CO_2 + 28\ [H]$$

coupled to reduction of nitrate to N_2

$$5.6\ HNO_3 + 28\ [H] \rightarrow 2.8\ N_2 + 16.8\ H_2O$$

would be 1 : 5.6. Since about one third of phenol metabolized was used as carbon source the experimentally determined value 1 : 4 corresponds well with the theoretical stoichiometry when phenol assimilation is considered in calculation.

Characterization of strain K 172

Cells of strain K 172 appeared in varying forms and sizes. Both straight motile rods with round ends, 0.5 μm wide and 1.0 μm – 2.0 μm long, and coccoid, mostly immotile rods of varying width 0.75 μm – 1.5 μm and length 1.0 μm – 2.5 μm were observed (Fig. 3). In young cultures the little motile rods dominated whereas in ageing cultures cells began to swell as a consequence of poly-β-hydroxybutyrate accumulation. Polysaccharides were not detected. The Gram reaction was negative and the KOH test positive. After aerobic growth catalase and oxidase reaction were positive. Cells grew well in mineral salts medium without additions of vitamins and ammonium. Strain K 172 grew best at 28° C and pH 7.0 – 7.4 with a doubling time of about 18 h. As

Table 1. Carbon and energy sources tested for growth of the newly isolated strains K 172 and S 100

Aromatic and aliphatic compounds tested as electron donors and carbon sources for anaerobic growth in the presence of nitrate (5 mM). All substrate were provided at a concentration of 1 mM.

Utilized[a]
Phenol (K, S), m-cresol (S), p-cresol (K, S), benzoate (K, S), benzaldehyde (K, S), benzylalcohol (K), 2-hydroxybenzoate (S), 3-hydroxybenzoate (S), 4-hydroxybenzoate (K, S), 4-hydroxybenzaldehyde (K, S), 4-hydroxybenzylalcohol (K, S), 2-aminobenzoate (K), protocatechuate (S), vanillate (S), phenylacetate (K, S), 4-hydroxyphenylacetate (K, S), mandelate (S), phenylpropionate (K, S), cinnamate (S), indole (K), indolylacetate (K, S), adipate (K), pimelate (K), succinate (K, S), fumarate (K, S), malate (K, S), heptanoate (K), caproate (K), butyrate (K, S), propionate (K, S), acetate (K, S), ethanol (K, S), pyruvate (K, S), lactate (K, S), casamino acids (K, S)[b].

Not utilized
Pyridine, aniline, toluene, o-cresol, catechol, 3- and 4-aminobenzoate, 3-hydroxybenzylalcohol, 3-hydroxybenzaldehyde, resorcinol, hydroquinone, 2,3-, 2,4-, 2,5-, 2,6-, 3,5-dihydroxybenzoate, pyrogallol, gallate, phenylalanine, tyrosine, tryptophane, 2-, 3-, 4-chlorobenzoate, o-phthalate, cyclohexanol, cyclohexanone, cyclohexane-1,2-diol, cyclohexanecarboxylate, cyclohexaneacetate, glucose, fructose, xylose, maltose, citrate, arginine, betain, glycerol, methanol, formate, H_2/CO_2 (80%/20%; v/v).

Electron acceptors tested with benzoate as carbon and energy source.
Utilized: nitrate, nitrite, oxygen.
Not utilized: sulfate, thiosulfate, sulfite, fumarate.

[a] (K), (S), or (K, S) indicates utilization of the substrate by strain K 172, strain S 100, or both strains
[b] Only poor growth

observed with strain S 100, growth of strain K 172 also gave no exponential growth curve and intermediately nitrite accumulated. Metabolism was strictly oxidative. Anaerobic metabolism of aromatic compounds was somewhat restricted in strain K 172 as compared to S 100 (Table 1). Phenol only could be degraded anaerobically with nitrate or nitrite as electron acceptor, whereas benzoate was degraded both anaerobically and aerobically. With strain K 172 the molar growth yield amounted to 42 g cell dry matter per mol phenol. About 4 mol nitrate was reduced to N_2 during degradation of 1 mol phenol.

Anaerobic degradation of p-cresol

Both strains K 172 and S 100 grew faster with p-cresol than with phenol (data not shown). 2-Fluoracetate was added to p-cresol degrading cultures to block acetyl-CoA oxidation. Under these conditions p-cresol was oxidized to 4-hydroxybenzoate which accumulated in the medium; 4-hydroxybenzylalcohol and 4-hydroxybenzaldehyde were detected as intermediates by HPLC. Metabolism of m-cresol by strain S 100 seemed not to proceed via oxidation of the methylgroup.

Anaerobic degradation of phenol

Both strains K 172 and S 100 oxidized phenol only under anaerobic conditions; presence of oxygen inhibited phenol metabolism. Anaerobic phenol degradation was dependent

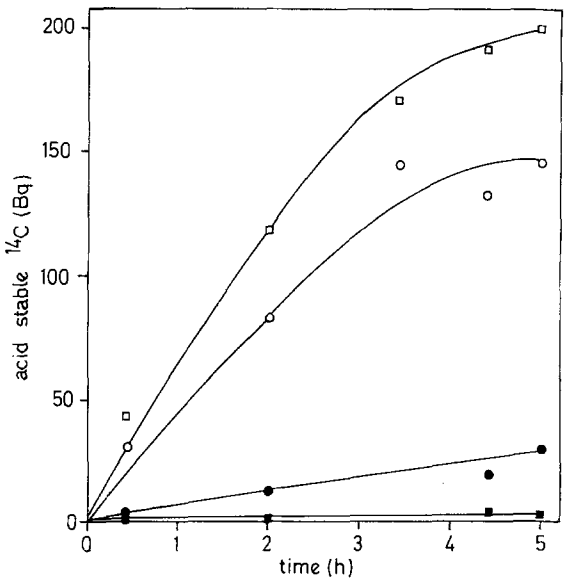

Fig. 4. Isotope exchange between the carboxyl group of 4-hydroxybenzoate and $^{14}CO_2$ catalized by cell suspensions of strain K 172 or S 100. Cells were grown with either phenol or 4-hydroxybenzoate. The cell suspensions contained 5 mM 4-hydroxybenzoate plus 4 mM [^{14}C]bicarbonate with a specific radioactivity of 750 Bq/μmol (CO_2 plus bicarbonate). No electron acceptor was added. At times indicated, samples were taken by syringe, acidified, gassed with N_2/CO_2 (80%/20%; v/v) and the acid stable ^{14}C was counted. It was controlled by HPLC that the acid stable ^{14}C was incorporated into 4-hydroxybenzoate. ○ Strain K 172 grown with phenol, ● strain K 172 grown with 4-hydroxybenzoate, □ strain S 100 grown with phenol, ■ strain S 100 grown with 4-hydroxybenzoate

on bicarbonate; if bicarbonate was omitted from the culture medium growth in freshly inoculated cultures started only very slowly but could be accelerated by later addition of bicarbonate. This CO_2 effect did not apply to growth on 4-hydroxybenzoate. As decarboxylation of 4-hydroxybenzoate under anaerobic conditions has repeatedly been reported we assumed that carboxylation of phenol to 4-hydroxybenzoate might be the initial reaction of anaerobic phenol degradation by our isolates. Strains K 172 and S 100 were anaerobically grown in a fed-batch culture with phenol or 4-hydroxybenzoate in the presence of [^{14}C]bicarbonate. Approximately the same growth yields were observed with phenol and 4-hydroxybenzoate; consequently [^{14}C]bicarbonate was diluted to the same extent by $^{12}CO_2$ derived from substrate oxidation. After three generations, phenol-grown cells had incorporated twice as much ^{14}C from $^{14}CO_2$ per mg dry weight, compared with 4-hydroxybenzoate-grown cells.

In a second experiment it was tested whether phenol-grown cells were able to incorporate ^{14}C from [^{14}C]bicarbonate into 4-hydroxybenzoate. Cells of both strains grown with phenol or with 4-hydroxybenzoate were suspended in fresh anaerobic medium lacking nitrate. Phenol-grown cells incorporated rapidly ^{14}C into an acid-stable compound when 4-hydroxybenzoate (5 mM) was present (Fig. 4). This was not observed when 4-hydroxybenzoate or when phenol-grown cells were omitted. Interestingly, ^{14}C incorporation was not catalyzed by 4-hydroxybenzoate-grown cells. The specific rate of ^{14}C incorporation presumably into the carboxyl of 4-hydroxybenzoate was about 20 nmol · min^{-1} · mg^{-1} protein. The acidified, ^{14}C-containing culture supernatant was analyzed by HPLC: all acid stable label was obtained in the 4-hydroxybenzoate fraction. This indicated that phenol-grown cells – and only those – catalyzed an ^{14}C isotope exchange from $^{14}CO_2$ and the carboxyl of 4-hydroxybenzoate.

Discussion

We have reported on the isolation of denitrifying bacteria which anaerobically grew well with phenol or other aromatic compounds (generation time ≈ 20 h). The phenol degrading strains isolated by Bakker (1977b) grew only very slowly (generation time ≈ 150 h). Therefore, anaerobic phenol metabolism has mainly been examined in acclimated anaerobic sludges or in enrichment cultures (Balba and Evans 1980; Knoll and Winter 1987). The first obligately anaerobic bacterium, which completely oxidizes phenol with sulfate as terminal electron acceptor, *Desulfobacterium phenolicum*, was recently isolated by Bak and Widdel (1986). Phenol and phenolic compounds are biogenic intermediates in the carbon cycle, but also enter nature from many anthropogenic sources. This may explain why phenol degrading denitrifiers could easily be isolated from very different oxic or anoxic habitats.

The newly isolated phenol-degrading bacteria strains K 172 and S 100 quantitatively reduced nitrate to nitrite before nitrite reduction started. More often nitrate is directly reduced to N_2 or NH_3 without intermediary accumulation of nitrite. This may indicate that different enzyme systems in nitrate reduction were differently induced. Strains K 172 and S 100 were tentatively identified as pseudomonads because they possessed a variety of typical characteristics, viz. Gram-negative cell wall, motility, strictly oxidative metabolism with oxygen, nitrate or nitrite as the respective terminal electron acceptor, and positive oxidase and catalase reaction. Besides the enrichment substrate phenol the new *Pseudomonas* sp. K 172 and S 100 were able to anaerobically degrade many different aromatic compounds in the presence of nitrate. Benzoate was oxidized both under aerobic and anaerobic conditions – but certainly via different pathways (Taylor et al. 1970). Notably, degradation of phenol by both strains only occurred in the absence of molecular oxygen. Studies on growth parameters of aerobic phenol oxidizing bacteria in continuous culture showed that the growth rate was decreased to one half already at 1 mM phenol concentration (Jones et al. 1973). In future studies the growth-inhibitory effects of NO_2^- and phenol may be overcome by chemostate cultures. The free energies of aerobic or anaerobic, nitrate dependent (N_2 product) phenol oxidation only differ by about 200 kJ:

$$C_6H_6O + 3\,H_2O + 7\,O_2 \rightarrow 6\,HCO_3^- + 6\,H^+$$

$$\Delta G_o' = -2996\ \text{kJ mol}^{-1}$$

$$5\,C_6H_6O + H_2O + 28\,NO_3^- \rightarrow 30\,HCO_3^- + 2H^+ + 14\,N_2$$

$$\Delta G_o' = -2806\ \text{kJ mol}^{-1}.$$

The molar growth yield of about 40 g determined for anaerobic phenol degradation by strain K 172 or S 100 corresponded well with yield values of aerobic phenol oxidation by *Pseudomonas putida* (48 ± 7 g mol^{-1}; Hill and Robinson 1975). P-Cresol was metabolized by the *Pseudomonas* sp. K 172 and S 100 probably via anaerobic oxidation of the methyl group via the alcohol and aldehyde to the carboxyl

level, yielding 4-hydroxybenzoate. This is concluded from the intermediary formation of these compounds when further metabolism of 4-hydroxybenzoate to CO_2 was blocked. This oxidation sequence of the methyl group of p-cresol recently has been shown in another denitrifying bacterium (Bossert and Young 1986). The question arises how phenol is metabolized anaerobically. Bakker (1977a) and Balba and Evans (1980) proposed phenol reduction to cyclohexanone. Although the present study was not aimed at clarifying this point, some observations were made which direct to a different mechanism. First, the hypothetical intermediate of phenol degradation, cyclohexanone, could not be detected in phenol degrading cultures nor were cyclohexane-derivatives degraded. Second, denitrifying bacteria enriched and isolated under anaerobic conditions with cyclohexanol or cyclohexanone were not able to degrade phenol anaerobically (unpublished results). *Desulfobacterium phenolicum* is also unable to grow with cyclohexanone (Bak and Widdel 1986).

An interesting alternative mechanism of phenol activation is phenol carboxylation in para position yielding 4-hydroxybenzoate. Phenol carboxylation is industrially used (Kolbe-Schmitt reaction). During aerobic degradation *Rhodococcus erythropolis* carboxylates aniline to 2-aminobenzoate, which is then degraded via catechol (Aoki et al. 1984, 1985). Decarboxylation of 4-hydroxybenzoate to phenol and CO_2 occurs under anaerobic conditions (Scheline 1966; Curtius et al. 1976; Tschech and Schink 1986). A first indication for phenol carboxylation by the two strains K 172 and S 100 was the finding that phenol degrading cultures fixed twice as much $^{14}CO_2$ into cell carbon compared with 4-hydroxybenzoate degrading cells. A second, stronger argument is the observed isotope exchange between $^{14}CO_2$ and the (carboxyl of) 4-hydroxybenzoate. This exchange reaction implies (a) that 4-hydroxybenzoate is enzymatically cleaved to CO_2, which exchanges with $^{14}CO_2$, and to an enzyme-bound phenol, and (b) that this reaction is reversible under cellular conditions. The finding that 4-hydroxybenzoate-grown cells dit not catalyze this isotope exchange shows that this enzyme system is well controlled. This biological specificity is a further argument for the role of the carboxylating/decarboxylating enzyme in anaerobic phenol degradation by the two strains.

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