Transformation of Phenol into Phenylalanine by a Methanogenic Consortium

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The conversion by a methanogenic consortium of phenol into phenylalanine, with benzoic and phenylpropionic acid as intermediates, was investigated. When ^{14}C -labelled phenol was fed to the consortium, the radioactivity was mostly transferred into methane and CO_2 , but 4% of the radioactivity was found in the water fraction after extraction of the culture medium with an organic solvent. Utilization of labelled compounds and analysis by gas chromatography coupled with mass spectrometry revealed that a fraction of the benzoic acid produced was transformed into 3-phenylpropionic acid. When fully ^{13}C -labelled acetic acid was fed to the consortium, the labels were incorporated at the 1 and 2 positions of 3-phenylpropionic acid. When deuterium-labelled 3-phenylpropionic acid was fed to the consortium, part of the phenylalanine of the biomass was labelled. These metabolic transformations are reversible, since deuterium-labelled phenylalanine generated labelled 3-phenylpropionic acid. Cinnamic acid was also transformed into 3-phenylpropionic acid.

Phenol is one of the most widely used chemicals and is found in many wastewaters of industrial origin. The degradation of phenol by methanogenic bacterial consortia has been reported by many investigators (1, 11, 14, 15, 19, 23, 25). We have extensively studied the methanogenic consortium isolated by Beaudet et al. (1). This consortium can degrade phenol at concentrations up to 1 g/liter (3) and transforms a variety of *ortho*-substituted phenolic compounds (5, 6). It was also very efficient in the treatment of wastewaters from a petroleum refinery containing high concentrations of phenolic compounds (8). The bacterium responsible for the transformation of phenol into benzoic acid via carboxylation and dehydroxylation is heat resistant and has been isolated recently (17, 18). As part of our continuing effort to better characterize the metabolism of this consortium, we report here a new, although minor, metabolic pathway of benzoic acid, which is an intermediate in the degradation of phenol.

The degradation pathways of benzoic acid under methanogenic conditions are well documented (9). The aromatic ring of benzoic acid is reduced and cleaved to produce medium-chain fatty acids, which are finally degraded into methane and CO_2 . Bécard et al. and Bisaillon et al. (2, 4) have suggested that, in the consortium of Beaudet et al., benzoic acid was transformed through 1-cyclohexene carboxylic acid into heptanoic acid and finally into acetic acid and methane. In the present study, we describe the transformations of benzoic acid into 3-phenylpropionic acid (PPA) and phenylalanine. This conversion of benzoic acid into an amino acid is of interest because this consortium was found to be very dependent on amino acids or proteose peptone as a nutrient for the cometabolism of phenol (4).

MATERIALS AND METHODS

Bacterial consortium. The anaerobic consortium of Beaudet et al. (1) was used in this study. A stock consortium was maintained at 37°C in the minimal medium of Boyd et al. (7) supplemented with 0.05% (wt/vol) proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.) and 1.6 mM phenol. Most experiments were performed with 100-ml anaerobic vials and a 12.5% inoculum as described previously (2).

Metabolite analysis. Metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS) with a Varian 3500 gas chromatograph coupled to a Finnigan Ion Trap 800. A 30-m DB-5 column (inside diameter, 32 mm; film thickness, 320 μm) was used. The injector temperature was 250°C, and the temperature gradient started at 70°C, increased to 210°C at 4°C/min, and then increased to a final temperature of 310°C at 20°C/min.

Extraction of nonpolar metabolites was performed with 1 ml of the culture medium to which was added 20 μl of a 7,500 ppm solution of *m*-cresol as an internal standard. This solution was then extracted with 1 ml of ethyl ether, and 150 μl of the organic phase was evaporated with a stream of nitrogen. The residue was derivatized with 150 μl of a 20% concentration of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (Pierce Chemicals, Rockford, Ill.) in acetonitrile.

Analysis of the labelled phenylalanine was performed by concentrating 70 ml of culture medium to 10 ml with a rotary evaporator. A 1.5-ml aliquot was taken, 1.5 ml of 12 M HCl was added to it, and the solution was heated in a sealed vial at 110°C for 64 h. A volume of 100 μl of a 740-ppm solution of ornithine was added as an internal standard, and a 1-ml aliquot was applied to an ion-exchange SCX column (Analytichem International, Harbor City, Calif.). The amino acids were eluted with 5 ml of a 20% (vol/vol) solution of NH_4OH in methanol, the solvent was evaporated in vacuo, and the residue was derivatized with 100 μl of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Pierce Chemicals) at 70°C for 30 min.

Labelled compounds. [3,5- ^2H]phenol, [1- ^{13}C]benzoic acid, and [U- ^{13}C]sodium acetate were purchased from CDN Isotopes (Pointe-Claire, Québec, Canada). [U- ^2H]phenol was purchased from Aldrich (Milwaukee, Wis.). [U- ^{14}C]phenol was from Sigma Chemical Co. (St. Louis, Mo.). [5,6,7,8,9- ^2H]phenylalanine was from Isotech (Miamisburg, Ohio). [2,2,3,3- ^2H]PPA was synthesized by hydrogenation of a 3-phenylpropynoic acid (Aldrich) ether solution with deuterium gas (CDN) over a 10% palladium-in-charcoal catalyst. [5,6,7,8,9- ^2H]PPA was synthesized by treatment of [U- ^2H]benzene with ethyl 3-chloropropionate (Aldrich) in the presence of AlCl_3 . The mass spectrum of the labelled ethyl 3-phenylpropionate showed that 50% of the compound contained five deuterium atoms, while the remaining 50% contained four deuterium atoms. The ethyl ester function was then removed with sodium hydroxide in methanol to produce the desired compound with the same deuterium enrichment as that of the ethyl ester.

Radiolabelling analysis. The radioactivity measurements were performed on 1-ml aliquots to which 4 ml of a scintillation cocktail (Ultima Gold XR; Packard Instruments, Meriden, Conn.) was added, and the radioactivity was analyzed with a Tracor Analytic Delta 300 scintillation counter.

A volume of 200 μl of a 6.5 mM aqueous solution of [U- ^{14}C]phenol (50 $\mu\text{Ci/ml}$) was added to 80 ml of the culture medium containing 1.6 mM phenol.

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FIG. 1. Concentrations of various metabolites produced by the degradation of phenol by the methanogenic consortium. Dotted lines refer to the right axis. Symbols: $\blacktriangle$, phenol; $\triangle$, benzoic acid; $\bullet$, phenylacetic acid; $\circ$, phenylpropionic acid.

After 14 days of incubation, a duplicate sample was analyzed by GC-MS, and no residual phenol, benzoic acid, 3-PPA, or phenylacetic acid was observed. The culture medium was made alkaline ($\text{pH} > 14$) with concentrated sodium hydroxide to keep the CO_2 produced in solution, and the radioactivity of this solution was measured. A 1-ml aliquot of the alkaline medium was acidified ($\text{pH} < 2$) with concentrated HCl and degassed for 15 min by bubbling with nitrogen to remove CO_2 , and the radioactivity was measured. The same procedure was repeated with another 1 ml of alkaline medium, but the aliquot was extracted twice with 5 ml of ether to remove any radioactive hydrophobic intermediates before the radioactivity was analyzed. This series of experiments was performed in duplicate.

RESULTS

PPA. The phenol-degrading consortium was inoculated into culture medium containing phenol, and the metabolites were analyzed by GC-MS at various time intervals (Fig. 1). In addition to benzoic acid, two other metabolites, PPA and phenylacetic acid, were identified by comparison of their mass spectra with those of an MS data bank (National Institute of Standards and Technology, Gaithersburg, Md.) and by comparison of their retention times with authentic standards.

The experiment was repeated with $[3,5\text{-}^2\text{H}]$ phenol (1.6 mM). The mass spectra of the benzoic acid presented a mass shift of two (data not shown), indicating that all of the benzoic acid produced contained the two deuterium labels. The mass spectrum of unlabelled PPA (Fig. 2a) presents ions at m/z 222, 207, 104, and 91. The two former ions correspond to the molecular ion of its trimethylsilyl derivative and to the loss of a methyl group (from the trimethylsilyl group). The m/z 91 ion corresponds to a fragment containing the phenyl ring with one methylene group. The spectrum of the PPA obtained with $[3,5\text{-}^2\text{H}]$ phenol as the substrate (Fig. 2b) shows the same ions as those in Fig. 2a in addition to ions at m/z 224, 209, 106, and 93. These ions confirm the incorporation of two deuterium atoms in the molecule, probably on the aromatic ring, as indicated by the m/z 93 ion. In this experiment, PPA was 68% labelled, as calculated by the abundances of the m/z 224, 209, 106, and 93 ions divided by the sum of the abundances of these $M+2$ ions and those of the m/z 222, 207, 104, and 91 ions.

The spectrum of the PPA peak from the consortium fed $[1\text{-}^{13}\text{C}]$ benzoic acid (1.6 mM) in addition to unlabelled phenol is presented in Fig. 2c. The incorporation of one ^{13}C in the molecule was 47%. The presence of an m/z 92 ion also indicates that the ^{13}C is probably located at the 3 position of PPA, adjacent to the aromatic ring.

Upon feeding $[\text{U-}^{13}\text{C}]$ sodium acetate (0.1%, wt/vol) to the consortium, the spectrum of PPA revealed enrichment with two ^{13}C 's, as demonstrated by the m/z 209 and 224 ions in Fig.

2d. The m/z 104 ion in unlabelled PPA (Fig. 2a) corresponds to the loss of the derivatized acid function with the additional loss of one hydrogen. In the spectrum of Fig. 2d, this ion shows only a $M+1$ ion, indicating that only one of the ^{13}C 's is retained in this ion. This along with the fact that the m/z 91 ion does not show any enrichment indicates that the two ^{13}C 's are located at the 1 and 2 positions of PPA.

The consortium was inoculated in the usual phenol-containing medium to which 0.1% (wt/vol) of $[5,6,7,8,9\text{-}^2\text{H}]$ phenylalanine was added. Figure 3a presents the concentrations of the various metabolites detected. PPA (Fig. 2e) was 100% enriched with five deuterium atoms. Phenylacetic acid (data not shown) was also enriched with five deuterium atoms. Another compound, identified as 3-phenyllactic acid, was also observed and found to be labelled with five deuterium atoms on its aromatic ring (data not shown). Benzoic acid also contained trace amounts of labels.

When phenylpyruvic acid was added (1.6 mM) to the usual phenol-containing medium, large concentrations of phenylacetic acid (Fig. 3b) along with some 3-phenyllactic acid were observed. When cinnamic acid (1.6 mM) was also added to the culture medium in which phenol was replaced by the same concentration of $[\text{U-}^2\text{H}]$ phenol, large concentrations of PPA were observed and its mass spectrum did not show any deuterium enrichment.

Mineralization of phenol. The two cultures, after being made alkaline, retained 37 and 39% of the radioactivity in the aqueous phase. This indicates that an average of 62% of the radioactive carbon of phenol was transformed into a water-insoluble gas, most probably methane. After acidification and degassing, they retained 13 and 7% of the initial radioactivity, suggesting that phenol was, on the average, 28% transformed into CO_2 . After extraction with ether, the average residual radioactivity in the aqueous phase was 4% (5 and 3% for the two cultures, respectively). A control experiment performed with a 14-day-old bacterial culture to which was added the same amount of radioactive phenol as that in the previous experiment showed a 99.8% removal of radioactivity after the same ether extraction procedure.

GC-MS analysis of phenylalanine. $[2,2,3,3\text{-}^2\text{H}]$ PPA (0.26 mM) was added to the inoculated culture medium containing phenol. After 14 days, the labelled PPA was completely degraded, on the basis of GC-MS, and the phenylalanine present in the medium, either in free form or incorporated into proteins, was analyzed by GC-MS after acid hydrolysis and showed no deuterium enrichment. The mass spectrum of unlabelled phenylalanine derivatized with two *tert*-butyldimethylsilyl functions, one on the amino function and one on the carboxy function, is presented in Fig. 4a. The molecular ion at m/z 393 is not apparent. The m/z 336 ion corresponds to the loss of a *tert*-butyl function, and the m/z 306 ion is the additional loss of CO. The ion at m/z 302 corresponds to the loss of the aromatic ring and its adjacent methylene group. $[5,6,7,8,9\text{-}^2\text{H}]$ PPA (0.26 mM) was fed to the consortium, and a section of the mass spectrum of phenylalanine is illustrated in Fig. 4b. The m/z 308 and 336 ions showed $M+4$ and $M+5$, indicating that the molecule was labelled. The fact that the m/z 302 ion was not labelled confirmed that the labels were on the aromatic ring of phenylalanine. The extent of the labelling was about 2% on average for all the scans of the phenylalanine peak. When $[\text{U-}^2\text{H}]$ phenol (1.6 mM) was fed to the consortium, phenylalanine was 10% labelled. Although these enrichments are small, especially for the former experiment, they are significant, since no interfering ions are observed at m/z 312 and 340 for phenylalanine.

FIG. 2. Mass spectra of trimethylsilyl derivative (TMS) of unlabelled PPA (a) and PPA from a culture fed either [U-²H]phenol (b), [1-¹³C]benzoic acid (c), [U-¹³C]sodium acetate (d), or [5,6,7,8,9-²H]phenylalanine (e).

DISCUSSION

The metabolic transformation of phenol into phenylalanine by this methanogenic consortium, as deduced from the preceding experiments, is illustrated in Fig. 5. The first step of phenol transformation is carboxylation, to produce *para*-hydroxybenzoic acid, followed by dehydroxylation to give benzoic acid. These two steps, as performed by this consortium and with others, have been described previously (6, 12, 16, 24).

Under methanogenic conditions, the degradation pathway of benzoic acid generally encountered in the literature is reduction and cleavage of the aromatic ring, to produce six- or seven-carbon fatty acids which are further degraded into acetic acid and ultimately into methane and CO₂ (9). The results obtained in this experiment with radioactive phenol confirm that most of the radioactivity is released in the form of gas (62%) and CO₂ (29%). There is, however, 4% of radioactivity that remains in the medium as a water-soluble species. Although this value is small, it is significant compared with the control, which showed a 99.8% removal of the radioactivity after the same extraction procedures. This is an indication that

there is another, although minor, metabolic pathway in which phenol is transformed into a polar, water-soluble species. The metabolic pathway presented in Fig. 5 could account for the transformation of phenol into a water-soluble compound like phenylalanine, with PPA as the key intermediate.

PPA was also often identified as a product of phenylalanine catabolism (10, 21). In our experiments, the concentrations of PPA observed were much higher when a phenylalanine-enriched medium was used (Fig. 3a), indicating that phenylalanine probably was also a precursor of PPA. This was further confirmed when labelled phenylalanine was added to the medium and the PPA produced was found to be completely labelled (Fig. 2e). The fact that no unlabelled PPA was observed could be due to the relatively large amounts of phenylalanine used in this experiment (0.1%) in comparison with those present in the usual 0.05% Proteose Peptone culture medium. This could shift the equilibrium between PPA and phenylpyruvic acid because of the large amounts of phenylpyruvic acid produced by deamination of phenylalanine.

Phenylacetic acid was also consistently found in every cul-

FIG. 3. Concentrations of various metabolites produced by the methanogenic consortium in a phenol-containing medium containing either [5,6,7,8,9- ^2H]phenylalanine (a) or phenylpyruvic acid (b). Dotted lines refer to the right axis. Symbols: ▲, phenol; △, benzoic acid; ●, phenylacetic acid; ○, phenylpropionic acid.

ture. It is also reported in the literature to be a product of phenylalanine degradation by anaerobic bacteria (20, 22). Phenylacetic acid is generally considered to be the decarboxylation product of phenylpyruvic acid, which is produced by deamination of phenylalanine (20). When the consortium was incubated in a labelled phenylalanine-enriched medium, the concentrations of phenylacetic acid were much higher (Fig. 3a) and phenylacetic acid, like PPA, was almost quantitatively labelled. 3-Phenyllactic acid was also found in this phenylalanine-rich medium and could be produced by reduction of the ketone group of phenylpyruvic acid. When phenylpyruvic acid was added to the culture medium, high concentrations of phenylacetic acid were rapidly produced in the few minutes between the time the compound was added to the medium and the time the extraction was performed. This explains the high concentrations of phenylacetic acid shown on day 0 in Fig. 3b. 3-Phenyllactic acid was also observed in the course of the experiment. The detection of these two compounds indicates that phenylpyruvic acid is indeed involved in phenylalanine catabolism with this consortium. All of this information suggests that with our consortium, the phenylalanine present in the Proteose Peptone is rapidly transformed into phenylpyruvic acid, which is the precursor of phenylacetic acid, 3-phenyllactic acid, and PPA. This could account for part of the PPA observed.

There are other indications that there is another precursor to PPA. When labelled phenol was used, part of the PPA was found to be labelled, most probably on the aromatic ring (Fig. 2b). When [1- ^{13}C]benzoic acid was used along with unlabelled phenol, part of the PPA was found to be labelled at the 3 position (Fig. 2c). This suggests that benzoic acid is transformed into PPA by the addition of two carbon atoms to its

carboxyl group. Acetic acid was found to be the source of these two carbon atoms, as demonstrated by the [U- ^{13}C]acetate experiment in which the two labels were found at the 1 and 2 positions of PPA (Fig. 2d). No intermediates between benzoic acid and PPA could be observed in any of these experiments. Cinnamic acid, which was described as a precursor of PPA in the degradation of phenylalanine by various clostridia (13, 21), was rapidly degraded by the consortium and rapidly produced high concentrations of PPA. However, because this compound was not observed in the normal culture medium, it is difficult to prove that it is the intermediate between benzoic acid and PPA.

Two experiments were performed with labelled PPA to prove that this compound can be converted back into phenylalanine. The first experiment used [2,2,3,3- ^2H]PPA, and no labelling could be found in phenylalanine. The second experiment performed under identical conditions but with [5,6,7,8,9- ^2H]PPA showed labelling of the aromatic ring of phenylalanine. The latter experiment proves that PPA is indeed a precursor of phenylalanine. The former indicates that this transformation is likely to proceed through phenylpyruvic acid as the intermediate, since the loss of the four deuterium atoms in phenylalanine can be explained by the loss of two deuterium atoms at the 2 position by the formation of the ketone function and by the loss at the 3 position of the two other atoms by the facile keto-enol tautomerism of phenylpyruvic acid (Fig. 5).

The metabolic pathway described in Fig. 5 can be seen as a series of reversible reactions starting with benzoic acid being transformed into PPA, which is transformed into phenylpyruvic acid and into phenylalanine. With this consortium and under these conditions, this pathway is relatively minor compared with the transformation of benzoic acid into methane

FIG. 4. (a) Mass spectrum of the unlabelled *tert*-butyldimethylsilyl (TBDMS) derivative of phenylalanine; (b) enlarged portion of the mass spectrum of phenylalanine from a culture fed labelled PPA.

FIG. 5. Proposed metabolic pathway of phenol and benzoic acid by the methanogenic consortium.

and CO₂. The low incorporation of deuterium in phenylalanine with [5,6,7,8,9-²H]PPA is probably due to the low speed at which this compound is used by the bacteria. With labelled phenol, the incorporation of deuterium was much higher, probably because the benzoic acid was used more rapidly than PPA by the bacteria that metabolized it. This can be deduced from the long time required (14 days) to degrade the low concentration (0.26 mM) of exogenously applied PPA. In both cases, the relatively low incorporation of benzoic acid into phenylalanine can be caused by the fact that the consortium is inoculated in a medium containing Proteose Peptone, a good source of readily available amino acids for the synthesis of proteins, while benzoic acid is produced later by transformation of phenol. Benzoic acid is also efficiently removed from the culture through methanogenic degradation. The transformation of benzoic acid into PPA could present an alternative to the bacteria for the synthesis of phenylalanine when the medium becomes depleted with easily accessible sources of phenylalanine. Attempts to increase the transformation of benzoic acid into PPA by use of a culture medium poorer in nutrients failed, since there is a strict requirement of the consortium for a minimal concentration of amino acid or Proteose Peptone to obtain phenol or benzoic acid degradation.

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