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Pathways of 4-Hydroxybenzoate Degradation Among Species of *Bacillus*

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The pathways used by three bacterial strains of the genus *Bacillus* to degrade 4-hydroxybenzoate are delineated. When *B. brevis* strain PHB-2 is grown on 4-hydroxybenzoate, enzymes of the protocatechuate branch of the β-ketoadipate pathway are induced. In contrast, *B. circulans* strain 3 contains high levels of the enzymes of the protocatechuate 2,3-dioxygenase pathway after growth on 4-hydroxybenzoate. *B. laterosporus* strain PHB-7a degrades 4-hydroxybenzoate by a novel reaction sequence. After growth on 4-hydroxybenzoate, strain PHB-7a contains high levels of gentisate oxygenase (EC 1.13.11.4) and maleylpyruvate hydrolase. Whole cells of strain PHB-7a (grown on 4-hydroxybenzoate) accumulate 2,5-dihydroxybenzoate (gentisate) from 4-hydroxybenzoate when incubated in the presence of 1 mM α,α'-dipyridyl. Thus, strain PHB-7a appears to convert 4-hydroxybenzoate to gentisate, which is further degraded by the glutathione-independent gentisic acid pathway. These pathway delineations provide evidence that *Bacillus* species are derived from a diverse evolutionary background.

Bacteria of the genus *Bacillus* (bacilli) include all rod-shaped bacteria capable of aerobically forming refractile endospores that are more resistant than vegetative cells to heat, drying, and other unfavorable environmental conditions (13). Bacilli are among the most ubiquitous of microorganisms, being principal components of the microflora of most soil and water environments (13, 33). It is becoming apparent that *Bacillus* species are probably important as degraders of aromatic compounds in natural environments. An increasing number of reports are appearing in the literature to indicate that bacilli are able to degrade a large variety of low-molecular-weight benzenoid molecules (2-5, 8, 9, 12, 17-20, 28, 31, 32, 36-39; A. J. Willetts and R. B. Cain, Biochem. J. 120:28p, 1970). To date, no definitive study has been reported that examines the pathways *Bacillus* species use to degrade 4-hydroxybenzoic acid. The following summarizes the results of such a study.

MATERIALS AND METHODS

Isolation, identification, and growth of the microorganisms. All *Bacillus* strains were isolated from pasteurized soil by selective enrichment on 4-hydroxybenzoate. The strains were tentatively identified by using the key and procedures of Gordon et al. (13). Stock cultures were maintained on brain heart infusion (Difco) or soil extract agar (13) slants that were stored at 4 C and subcultured biweekly. Microorganisms were grown in the minimal medium previously described (8), except that 4-hydroxybenzoic acid (1.0 g/liter), 3-hydroxybenzoic acid (1.0 g/liter), or succinic acid (2.0 g/liter) replaced 4-hydroxyphenylpropionic acid. A total of 100 ml of medium in a 500-ml flask was inoculated with a loop of cells taken from a stock slant, and the culture was shaken 24 to 48 h at 30 C before being used to inoculate 1 liter of the same medium in a 2-liter flask. The larger culture was shaken at 30 C until cells reached late log phase (18 to 24 h). Cells were collected by centrifugation and washed by resuspension in 0.1 M potassium-sodium phosphate buffer, pH 7.0. This buffer was also used in all reaction mixtures, unless specified otherwise.

Preparation of cell extracts. Washed cell pastes were suspended in 2 to 3 volumes of buffer, and the resulting cell suspensions were passed through a French pressure cell at >15,000 lb/in² (9). Extracted cell suspensions were centrifuged at 26,000 × *g* for 20 min to give clear cell extracts containing 10 to 25 mg of protein per ml as determined by the method of Lowry et al. (26). All procedures were performed at 0 to 5 C.

Enzymatic analyses. Oxygen consumption was measured with an oxygen electrode (Oxygraph, Gilson Medical Electronics, Middleton, Wis.). Dioxygenase assays were performed at 25 C in 1.5 ml of buffer containing 1.0 μmol of substrate and 0.1 to 0.2 mg of cell extract protein. Reactions were initiated by addition of substrate. Oxygenase specific activities were corrected for uptake of oxygen in the absence of substrate.

The following enzymes were assayed by published procedures: maleylpyruvate hydrolase (9), α-hy-

droxymuconic semialdehyde deyhydrogenase (8, 29), and β-carboxy-*cis,cis*-muconate lactonizing enzyme (27). β-Carboxy-*cis,cis*-muconate was generated directly in cuvettes by the action of purified protocatechuate 3,4-dioxygenase on protocatechuate. Crystalline protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* was provided by John Lipscomb, University of Minnesota, Freshwater Biological Institute.

4-Hydroxybenzoate hydroxylase activity in extracts of *B. brevis* strain PHB-2 was assayed spectrophotometrically by observing the initial rate of hydroxylase-catalyzed oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm. The reaction mixture contained in 1.5 ml of buffer: 0.25 μmol of NADH, 0.1 to 0.2 mg of cell extract protein, and 0.2 μmol of 4-hydroxybenzoate. Reactions were initiated by addition of aromatic substrate. Hydroxylase activities were corrected for oxidation of NADH occurring in the absence of substrate.

4-Hydroxybenzoate hydroxylase activity in extracts of *B. circulans* strain 3 was assayed in the oxygen electrode. The reaction mixture contained in 1.5 ml of buffer: 0.2 to 0.3 mg of cell extract protein, 1.0 μmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 1.0 μmol of 4-hydroxybenzoate. Reactions were initiated by addition of the aromatic substrate. Hydroxylase activities were corrected for the additional oxygen consumption that occurred during oxidation of protocatechuate, the product of 4-hydroxybenzoate hydroxylation.

All enzyme specific activities are expressed as micromoles of substrate transformed per minute per milligram of cell extract protein provided.

Spectrophotometric determinations of pyruvate and acetaldehyde were performed using lactate dehydrogenase or alcohol dehydrogenase as previously described (7, 8). Stoichiometry of NADH consumption was determined by observing a decrease in absorbance at 340 nm resulting from NADH oxidation (E = 6,200 for NADH).

Partial purification of *B. brevis* PHB-2 enzymes. Cell-free extract (2.5 ml ≃ 60 mg of protein) prepared from 4-hydroxybenzoate-grown strain PHB-2 was fractioned on a column (55 cm in length by 3 cm in diameter) of agarose beads (Bio-Gel A1.5, Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 0.1 M phosphate buffer (pH 7.0) at a flow rate of 1.2 ml/min. Six-milliliter fractions were collected. Protocatechuate 3,4-dioxygenase activity was located in fractions 22 to 40, and β-carboxy-*cis,cis*-muconate lactonizing activity was located in fractions 28 to 44. Fraction 26 contained appreciable protocatechuate oxygenase activity but no β-carboxymuconate lactonizing activity. By using fraction 26, we were able to accumulate from protocatechuate a compound with spectral properties identical to β-carboxy-*cis,cis*-muconate (27). Fractions containing β-carboxymuconate lactonizing activity did not accumulate absorbing products; thus, a second method of partial purification was attempted.

Cell-free extract (2 ml, diluted with 2 ml of distilled water; 50 mg of protein) prepared from 4-hydroxybenzoate-grown *B. brevis* strain PHB-2 was fractioned on a column (21 cm in length by 2.5 cm in diameter) of diethylaminoethyl-cellulose (Whatman DE52, Whatman Biochemicals Ltd., Springfield Mill, Maidstone, Kent, England) packed in 1 mM phosphate buffer, pH 7.0. The column was washed with the following series of phosphate buffers, all pH 7.0: 25 ml of 1 mM, 25 ml of 10 mM, 25 ml of 50 mM, 25 ml of 100 mM, 25 ml of 150 mM, and 100 ml of 200 mM buffer. The flow rate was 1 ml /min with a fraction size of 5 ml. Protocatechuate 3,4-dioxygenase and β-carboxy-*cis,cis*-muconate lactonizing enzyme eluted together in fractions 32 to 42 (protocatechuate dioxygenase purification being approximately ninefold over the activity in crude extract). Fractions 38 to 40 contained considerable β-carboxymuconate lactonizing activity and converted β-carboxy-*cis,cis*-muconate into a compound with spectral characteristics of β-ketoadipate enol-lactone (λ_{max}, pH 8.0, ≃ 215 nm) (27). This product was degraded very slowly to nonabsorbing products, and no attempt was made to isolate the presumed enol-lactone.

Inhibition of whole cells with α,α′-dipyridyl. Inhibition of gentisate oxidation by whole cells of *B. laterosporus* strain PHB-7a with the Fe^{2+} chelator α,α′-dipyridyl was accomplished as detailed by Hopper and Chapman (21) and Crawford (9).

Materials. Enzymes and cofactors were purchased from the Sigma Chemical Co., St. Louis, Mo. Aromatic compounds were purchased from the Aldrich Chemical Co., Milwaukee, Wis.

RESULTS

Microorganisms. Three physiologically distinct groups (10 strains, total) of *Bacillus* strains were isolated from marsh and agricultural soils by selective enrichment on 4-hydroxybenzoate. Detailed studies of three strains (PHB-2, PHB-7a, and 3) are reported here, each strain being representative of one group of 4-hydroxybenzoate-degrading, aerobic sporeformers. Strain PHB-2 was tentatively identified as a representative of the genus/species *B. brevis*. Isolates PHB-7a and 3 were tentatively identified as strains of *B. laterosporus and B. circulans*, respectively.

4-Hydroxybenzoate degradation by strain PHB-2. Cell-free extracts of 4-hydroxybenzoate-grown strain PHB-2 contain an oxygenase that oxidizes protocatechuate (3,4-dihydroxybenzoate) with consumption of approximately 1 mol of oxygen per mol of substrate (specific activity = 0.18 μmol of oxygen consumed/min per mg of cell extract protein under assay conditions). The ring-fission product formed from protocatechuate exhibits spectral characteristics identical to those of β-carboxy-*cis,cis*-muconate (see above), the ring-fission product of protocatechuate 3,4-dioxygenase (broad λ_{max} at pH 7.0 ≃ 255 nm) (27). In fact, the 3,4-dioxygenase in PHB-2 extracts is easily

assayed by observing changes in absorbance at 270 and 290 nm during oxidation of protocatechuate, as suggested by Stanier and Ingrahm (34). The carboxymuconate formed from protocatechuate is degraded by crude extracts to nonabsorbing products. Extracts prepared from 4-hydroxybenzoate-grown cells contain β-carboxy-*cis,cis*-muconate lactonizing enzyme activity (specific activity = 0.15). When crude extracts of 4-hydroxybenzoate-grown PHB-2 were fractioned on a column of diethylaminoethyl-cellulose, certain column fractions converted β-carboxy-*cis,cis*-muconate into a compound with spectral characteristics of β-ketoadipate enol-lactone (see above). This observation supports the conclusion that the complete protocatechuate branch of the β-ketoadipate pathway (27) is present in 4-hydroxybenzoate-induced strain PHB-2.

Extracts of 4-hydroxybenzoate-grown *B. brevis* strain PHB-2 contain 4-hydroxybenzoate hydroxylase activity (specific activity = 0.25). The hydroxylase is probably a mixed-function oxygenase since it catalyzes consumption of 1 mol of oxygen and 1 mol of NADH per mol of substrate. The product of 4-hydroxybenzoate hydroxylation is apparently protocatechuate, since oxygen consumption increases from 1 to 2 mol of oxygen per mol of substrate when 4-hydroxybenzoate is substituted for protocatechuate and excess NADH is provided (Fig. 1).

Extracts of 4-hydroxybenzoate-grown strain PHB-2 do not oxidize catechol (*o*-dihydroxybenzene) or gentisate (2,5-dihydroxybenzoate).

The levels of enzymes used by strain PHB-2 for degradation of 4-hydroxybenzoate are considerably decreased when cells are grown in the absence of 4-hydroxybenzoate. Extracts prepared from succinate-grown cells contained 1/10 the protocatechuate 3,4-dioxygenase activity (specific activity = 0.03) and 1/4 the β-carboxy-*cis,cis*-muconate lactonizing enzyme activity (specific activity = 0.035) of extracts similarly prepared from 4-hydroxybenzoate-grown cells. Extracts of 3-hydroxybenzoate-grown strain PHB-2 lacked detectable levels of protocatechuate oxygenase or β-carboxy-*cis,cis*-muconate lactonizing enzyme activity.

4-Hydroxybenzoate degradation by *B. circulans* strain 3. Cell-free extracts prepared from 4-hydroxybenzoate-grown *B. circulans* strain 3 do not oxidize catechol or gentisate; however, they do oxidize protocatechuate (specific activity = 0.14 μmol of oxygen consumed/min per mg of cell extract protein under assay conditions). Extracts of 4-hydroxybenzoate-grown cells catalyze consumption of 1 mol of oxygen for each mol of protocatechuate provided. The only detectable ring-fission product formed from protocatechuate shows spectral characteristics identical to those of α-hydroxymuconic semialdehyde, the observed ring-fission product of protocatechuate 2,3-dioxygenase (λ_{max} at pH 7.0 = 375 nm, shifted to 320 nm upon acidification) (1, 8).

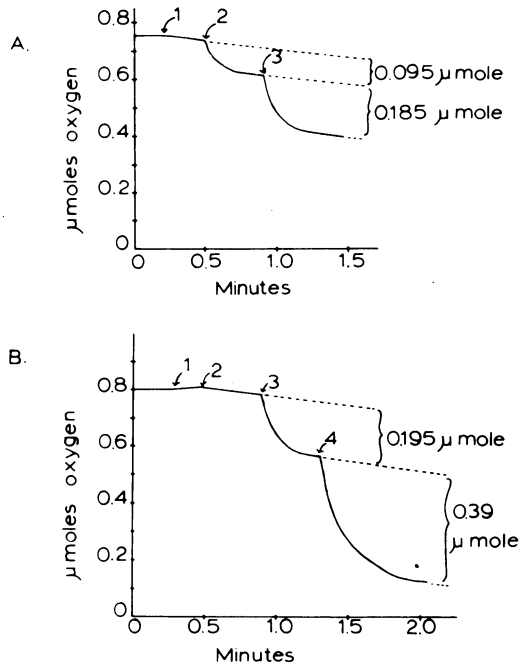

FIG. 1. *Stoichiometry of oxygen consumption during oxidation of protocatechuate and 4-hydroxybenzoate by an extract of 4-hydroxybenzoate-grown B. brevis strain PHB-2. (A) Oxygen consumption was determined with an oxygen electrode. Reactions were performed in a total volume of 1.5 ml. At the arrows, the following additions were made: 1 = 0.5 mg of cell extract protein; 2 = 0.1 μmol of protocatechuate; 3 = 0.2 μmol of protocatechuate. (B) Reaction conditions were as in (A) above. At the arrows, the following additions were made: 1 = 1.0 μmol of NADH; 2 = 0.2 mg of cell extract protein; 3 = 0.1 μmol of 4-hydroxybenzoate; 4 = 0.2 μmol of 4-hydroxybenzoate.*

The yellow ring-fission product is degraded slowly by crude extracts, attributable primarily to α-hydroxymuconic semialdehyde hydrolase activity. Degradation proceeds at a much faster rate when reaction mixtures are supplemented with oxidized nicotinamide adenine dinucleotide (NAD), the known cofactor of α-hydroxymuconic semialdehyde dehydrogenase (29). Extracts of 4-hydroxybenzoate-grown *B. circulans* strain 3 contain α-hydroxymuconic semialdehyde dehydrogenase activity at a specific activity of 0.02.

When cell extracts of 4-hydroxybenzoate-grown strain 3 oxidize protocatechuate in the presence of NAD and 8.3×10^{-5} M ethylenediaminetetraacetic acid essentially as described by Crawford (8), a compound accumulates showing spectral characteristics identical to those of oxalocrotonate (α-hydroxymuconate; λ_{max}'s at pH 7.0 = 238 and 295 nm; λ_{max} at pH 12 = 350 nm) (29). Removal of Mg^{2+} with ethylenediaminetetraacetic acid is known to inactivate α-hydroxymuconate decarboxylase (29). These observations confirm that the product of NAD-dependent α-hydroxymuconic semialdehyde oxidation by *B. circulans* strain 3 is α-hydroxymuconate.

Extracts prepared from 4-hydroxybenzoate-grown strain 3 contain 4-hydroxybenzoate hydroxylase activity (specific activity = 0.021). The product of 4-hydroxybenzoate hydroxylation is protocatechuate, since the reaction results in the formation of a product with spectral characteristics of α-hydroxymuconic semialdehyde. The α-hydroxymuconic semialdehyde is apparently formed by crude extract-catalyzed 2,3-fission of the protocatechuate formed by 3-hydroxylation of 4-hydroxybenzoate. It is not possible to assay the hydroxylase by observing oxidation of reduced pyridine nucleotide at 340 nm, since formation of α-hydroxymuconic semialdehyde (λ_{max} = 375 nm) masks the decrease in absorbance at 340 nm. The enzyme can be assayed in the oxygen electrode (see above). The 4-hydroxybenzoate 3-hydroxylase in crude extracts of strain 3 is approximately four times as active with NADPH as with NADH.

Degradation of protocatechuate by strain 3 is inducible by growth on 4-hydroxybenzoate. Extracts prepared from succinate-grown cells do not oxidize protocatechuate.

4-Hydroxybenzoate degradation by *B. laterosporus* strain PHB-7a. Cell-free extracts of 4-hydroxybenzoate-grown *B. laterosporus* strain PHB-7a oxidize only one of the commonly encountered ring-fission substrates, gentisate (specific activity = 0.42 μmol of oxygen consumed/min per mg of cell extract protein under assay conditions). One mole of oxygen is consumed for each mole of gentisate provided. The ring-fission product formed from gentisate shows spectral properties identical to those of maleylpyruvate, the ring-fission product of gentisate 1,2-dioxygenase (λ_{max} at pH 7.0 $\simeq$ 330 nm, disappearing upon acidification) (10, 24). Maleylpyruvate was further degraded by unsupplemented extracts yielding 1 M equivalent of pyruvate per mol of gentisate provided. Addition of reduced glutathione (GSH) to the reaction mixture did not speed maleylpyruvate degradation (9). The specific activity determined for maleylpyruvate hydrolase in extracts of 4-hydroxybenzoate-grown strain PHB-7a is 0.16 μmol of maleylpyruvate hydrolyzed/min per mg of extract protein under assay conditions.

Extracts of succinate-grown *B. laterosporus* PHB-7a lacked detectable amounts of gentisate dioxygenase.

When whole cells of 4-hydroxybenzoate-grown PHB-7a oxidized 4-hydroxybenzoate in the presence of 1 mM α,α'-dipyridyl (9, 21), gentisate accumulated in the suspending buffer (approximately 30% conversion of 4-hydroxybenzoate to gentisate). Gentisate was unequivocally identified by thin-layer chromatography and gas chromatography/mass spectrometry (m/e = 370 as trimethylsilyl derivative) as previously described (9, 25).

I was unable to demonstrate oxidation of 4-hydroxybenzoate by cell extracts of 4-hydroxybenzoate-grown strain PHB-7a, despite supplementation of reaction mixtures with various combinations of cofactors and enzyme-stabilizing agents. Whole cells grown on 4-hydroxybenzoate immediately oxidized 4-hydroxybenzoate, as determined by measurements with the oxygen electrode.

DISCUSSION

Data summarized above indicate that *B. brevis* strain PHB-2 degrades 4-hydroxybenzoate through the protocatechuate branch of the β-ketoadipate pathway (27), as shown in Fig. 2A. *B. circulans* strain 3 apparently degrades 4-hydroxybenzoate via the recently described protocatechuate 2,3-dioxygenase pathway (8), illustrated in Fig. 2B. *B. laterosporus* strain PHB-7a dissimilates 4-hydroxybenzoate by a novel reaction sequence: 4-hydroxybenzoate is converted to 2,5-dihydroxybenzoate, which is further catabolized by the GSH-independent gentisate pathway (9, 22; Fig. 2C).

These conclusions are in accord with enzyme assays of extracts prepared from 4-hydroxybenzoate-grown cells. Extracts of strain PHB-2 contain high levels of enzymes 1 to 3 of Fig. 2A. Extracts of 4-hydroxybenzoate-grown strain 3 contain high levels of enzymes 6 to 8 of Fig. 2B, whereas similar extracts of strain PHB-7a contain high levels of enzymes 10 and 11 of Fig. 2C.

The presence of α-hydroxymuconate (compound VIII, Fig. 2B) in the pathway of 4-hydroxybenzoate degradation by *B. circulans* strain 3 was demonstrated spectrophotometrically by forcing its accumulation with the Mg^{2+} chelator ethylenediaminetetraacetic acid. In the absence of the chelator, α-hydroxymuconate did not accumulate. These observations im-

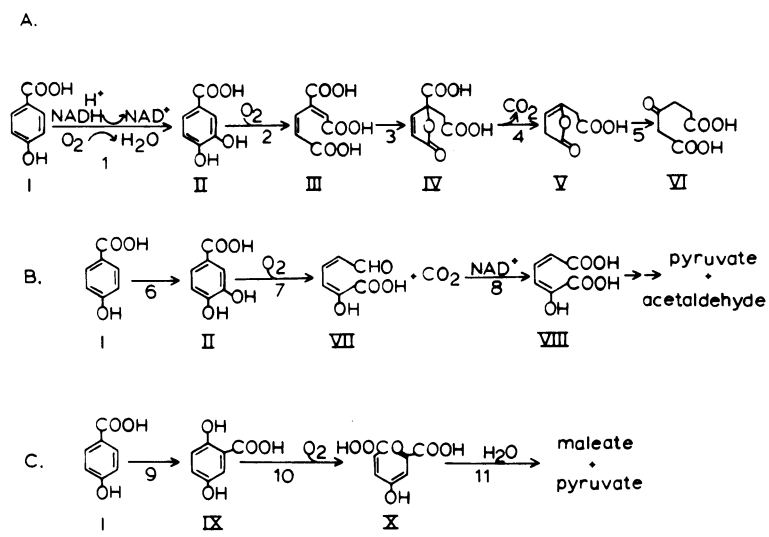

FIG. 2. *Degradation of 4-hydroxybenzoic acid by Bacillus species. (A) Bacillus strain PHB-2; (B) Bacillus strain 3; (C) Bacillus strain PHB-7a. I = 4-hydroxybenzoate; II = protocatechuate; III = β-carboxy-cis,cis-muconate; IV = γ-carboxymuconolactone; V = β-ketoadipate enol-lactone; VI = β-ketoadipate; VII = α-hydroxymuconic semialdehyde; VIII = α-hydroxymuconate; IX = gentisate; X = maleylpyruvate. 1 = 4-hydroxybenzoate 3-hydroxylase; 2 = protocatechuate 3,4-dioxygenase; 3 = β-carboxy-cis,cis-muconate lactonizing enzyme; 4 = γ-carboxymuconolactone decarboxylase; 5 = β-ketoadipate enol-lactone hydrolase; 6 = 4-hydroxybenzoate 3-hydroxylase; 7 = protocatechuate 2,3-dioxygenase; 8 = α-hydroxymuconicsemialdehyde dehydrogenase; 9 = 4-hydroxybenzoate 1-hydroxylase (?); 10 = gentisate 1,2-dioxygenase; 11 = maleylpyruvate hydrolase.*

ply that cell extracts of 4-hydroxybenzoate-grown strain 3 also contain an Mg^{2+}-dependent α-hydroxymuconate decarboxylase, as demonstrated in a benzoate-grown *Azotobacter* (29) and a 4-hydroxyphenylpropionate-grown *Bacillus* (8). Extracts of strain 3 did not accumulate the expected pathway end products, pyruvate and acetaldehyde. This probably results from inactivation of one of the terminal enzymes of the pathway during preparation of extracts and has been observed previously in a strain of *B. circulans* that catabolizes 4-hydroxyphenylpropionate via the protocatechuate 2,3-dioxygenase pathway (8).

Further support for the pathway shown in Fig. 2C are the observations that extracts of 4-hydroxybenzoate-grown *B. laterosporus* strain PHB-7a converted gentisate into an equimolar amount of pyruvate and GSH was not required for maleylpyruvate degradation (9, 21, 22, 24). Also, actual isolation of gentisate as a metabolite of 4-hydroxybenzoate after inhibition of whole cells of 4-hydroxybenzoate-grown strain PHB-7a with the dioxygenase inhibitor α,α'-dipyridyl is direct evidence for the novel conversion of 4-hydroxybenzoate to 2,5-dihydroxybenzoate.

The sequence of Fig. 2A illustrates the protocatechuate branch of the β-ketoadipate pathway (27). Numerous microorganisms, in addition to the *Bacillus* described here, dissimilate 4-hydroxybenzoate via this pathway, including representatives of the following genera: *Pseudomonas* (27), *Acinetobacter* (6), *Nocardia* (D. L. Rann and R. B. Cain, Biochem. J. 114:77p, 1969), *Alcaligenes* (23), and *Azotobacter* (14). It seems likely that *B. brevis* strain PHB-2 uses the complete protocatechuate branch of the β-ketoadipate pathway as delineated by Ornston and Stanier (27), since many years of work have not revealed deviations among bacteria from the reaction sequence first established for species of *Pseudomonas* (35). This is not the first demonstration of the β-ketoadipate pathway among bacilli. Willetts and Cain (38, 39; Biochem. J. 120:28p, 1970) have studied a *Bacillus* that degrades alkylbenzene sulfonate homologues containing an odd-numbered alkyl side chain via the protocatechuate branch of the β-ketoadipate pathway.

The sequence of Fig. 2B illustrates the recently discovered protocatechuate 2,3-dioxygenase pathway. This catabolic pathway has been described thus far only among strains of *B. circulans* (8; R. L. Crawford and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, O3, p. 192). The pathway was first documented in detail with a strain of *B. circulans*

grown on 4-hydroxyphenylpropionate (8).

The conversion of 4-hydroxybenzoate to gentisate, as accomplished by *B. laterosporus* PHB-7a, has not been previously reported. Buswell and Twomey (Proc. Soc. Gen. Microbiol. I:48, 1974) reported in an abstract some evidence to indicate that an unidentified, thermophilic bacterium degraded benzoate by way of gentisate. Since benzoate-grown cells of the thermophile oxidized 4-hydroxybenzoate and 3-hydroxybenzoate, it is conceivable that benzoate was being converted to gentisate via one of these two possible intermediates. Crawford and Chapman (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, O3, p. 192) reported in a preliminary communication that a group of *Bacillus* strains degrades 4-hydroxyphenylpropionate via gentisate and that 4-hydroxybenzoate was a probable intermediate in the conversion of 4-hydroxyphenylpropionate to gentisate.

The mechanism by which strain PHB-7a oxidizes 4-hydroxybenzoate to gentisate has not been determined, since cell-free extracts of 4-hydroxybenzoate-grown cells are not active against the growth substrate. Dehydroxylation to benzoate followed by hydroxylations at C2 and C5 of the ring seems unlikely, but such a sequence cannot yet be ruled out. It seems more reasonable that a hydroxylation at C1 of the 4-hydroxybenzoate ring might occur with concomitant migration of the carboxyl to an *ortho* position on the ring, yielding gentisate. In a somewhat similar situation, *P. acidovorans* produces an enzyme that hydroxylates C1 of the 4-hydroxyphenylacetate ring while simultaneously catalyzing migration of the acetate side chain to ring carbon 2 or 6, forming homogentisate (15).

It is of interest that the gentisate pathway used by *B. laterosporus* strain PHB-7a does not require GSH (24). This is the case with all gentisate-degrading bacilli examined to date (9).

It has been proposed that evolutionary relationships between microorganisms may be revealed by comparing the catabolic routes they use to degrade aromatic molecules (11, 16, 27). Assuming that this is indeed possible, the observations reported here provide added evidence that *Bacillus* species are derived from a diverse evolutionary background. The three pathways described in this paper are chemically very different from one another and probably could not have evolved from a common origin.

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