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Degradation of 3-Hydroxybenzoate by Bacteria of the Genus *Bacillus*

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The pathway whereby certain bacterial strains of the genus *Bacillus* degrade *m*-hydroxybenzoate is delineated. Of 12 strains examined, nine were tentatively classified as representatives of the species *Bacillus brevis*, two of *Bacillus sphaericus* and one of *Bacillus megaterium*. All strains degraded *m*-hydroxybenzoate via the same pathway. *m*-Hydroxybenzoate was hydroxylated to 2,5-dihydroxybenzoate (gentisate), which was oxidized by a gentisate 1,2-dioxygenase yielding maleylpyruvate. Maleylpyruvate was hydrolyzed without prior *cis, cis* to *cis, trans* isomerization yielding pyruvate and maleic acid. Numerous soils were examined by plate-count procedures and found to contain 10^4 to 10^6 aerobic sporeformers able to grow on *m*-hydroxybenzoate per g of dry soil.

Bacteria of the genus *Bacillus* (bacilli) are an important component of the microflora of most soil and water environments and thus are potential agents of biological transformation and degradation of aromatic compounds that enter soil/water ecosystems. Unfortunately, little is known concerning the ability of *Bacillus* species to catabolize aromatic molecules. Certain *Bacillus* strains appear to degrade benzenoid compounds via reaction sequences similar to those described in other bacterial genera (1, 2, 9, 17, 22-25). However, recent investigations indicate that many bacilli may catabolize aromatic molecules via reaction sequences involving novel chemistry (4, 6, 10-12, 20, 21). The following describes my recent investigation into the catabolism of 3-hydroxybenzoate by various species of *Bacillus*. This investigation is part of a continuing project which has a goal of determining the mechanisms whereby bacteria of the genus *Bacillus* degrade aromatic molecules.

MATERIALS AND METHODS

Isolation, identification and growth of the microorganisms. *Bacillus* strains were isolated from pasteurized soil after selective enrichment on various aromatic compounds (Table 1). All strains were identified using the key and procedures of Gordon et al. (7). Stock cultures were maintained on brain heart infusion (Difco) slants that were stored at 4 C and subcultured biweekly. Microorganisms were grown in the minimal medium previously described (4), except that *m*-hydroxybenzoic acid replaced *p*-hydroxyphenylpropionic acid. One liter of medium contained in a 2-liter flask was inoculated with the growth of one stock slant and magnetically stirred at room temperature until cells reached early stationary phase. Cells were collected by centrifugation and washed by resuspension in 0.1 M potassium-sodium phosphate buffer, pH 7.2. This buffer was also used in all reaction mixtures.

Preparation of cell extracts. Washed cell pastes were suspended in 2 to 3 volumes of buffer which contained 25% (by volume) glycerol. The resulting cell suspensions were passed through a French pressure cell (American Instrument Co., Silver Springs, Md.) at >10,000 lb/in^2 applied with a hydraulic press (American Instrument Co.). Extracted cells were centrifuged at 26,000 × *g* for 20 min to give clear cell extracts containing 5 to 15 mg of protein/ml as determined by the method of Gornall et al. (8). All procedures were performed at 0 to 5 C.

Enzyme assays. 2,5-Dihydroxybenzoate 1,2-dioxygenase (EC 1.13.11.4; gentisate 1,2-dioxygenase) was assayed by the procedure of Crawford et al. (5). Maleylpyruvate hydrolase was assayed by observing decrease in absorbance at 334 nm resulting from hydrolysis of maleylpyruvate to pyruvate and maleate (14). The hydrolase assay was performed after the gentisate 1,2-dioxygenase assay in the same reaction mixture while maleylpyruvate concentration was between 3×10^{-5} and 5×10^{-5} M. E_{max} of maleylpyruvate was assumed to be 10,800 (5) and 1 U of hydrolase activity is defined as the amount of protein required to hydrolyze 1 μmol of maleylpyruvate per min. In no instance was there observed an increase in the rate of maleylpyruvate degradation by *Bacillus* extracts on addition of reduced glutathione (GSH). Fumarase (EC 4.2.1.2.) and maleate isomerase activities were assayed essentially as described by Scher and Jakoby (18). Fumarase activity was assayed by observing the decrease in absorb-

TABLE 1. *Enrichment substrate and identification of the various Bacillus strains used during this investigation*

Strain no.	Identification	Compound used for selective enrichment
B5f	*Bacillus brevis*	2-Chlorobenzoate
B9a	*B. brevis*	3-Hydroxybenzoate
B10c	*B. brevis*	4-Hydroxyphenylpropionate
C1a	*B. brevis*	3-Hydroxybenzoate
A2a	*B. brevis*	3-Hydroxybenzoate
C6c	*B. brevis*	4-Hydroxyphenylpropionate
B6f	*B. brevis*	2-Chlorobenzoate
C7c	*B. brevis*	4-Hydroxyphenylpropionate
A3a	*B. brevis*	3-Hydroxybenzoate
B2a	*B. sphaericus*	3-Hydroxybenzoate
B8b	*B. sphaericus*	Phenylacetate
C5f	*B. megaterium*	2-Chlorobenzoate

TABLE 2. *Enzymic analyses of cell-free extracts prepared from m-hydroxybenzoate-grown Bacillus strains*

Strain no.[a]	Sp act[b]		Pyruvate formed per gentisate provided[d]
	Gentisate 1,2-dioxygenase[c]	Maleyl pyruvate hydrolase	
B5f	1.49	0.24	0.84
B9a	2.10	0.30	0.94
B10c	1.19	0.31	0.95
C1a	1.60	0.11	0.91
A2a	3.53	0.56	0.88
C6c	1.98	0.40	1.10
B6f	2.58	0.18	1.23
C7c	2.44	0.24	1.12
A3a	3.16	0.20	0.97
B8b	1.31	0.11	1.05
B2a	1.41	0.42	0.99
C5f	1.25	0.20	0.82

[a] For genus/species designations see Table 1.
[b] Micromoles per minute per milligram of protein.
[c] Corrected for maleylpyruvate hydrolase activity.
[d] Average is 0.98.

ance at 290 nm (A_{290}) resulting from hydration of fumarate to L-malate, whereas maleate isomerase activity was assayed by observing the increase of A_{290} resulting from conversion of maleate to fumarate. Fumarylpyruvate hydrolase was assayed by observing decrease in A_{330} (14, 15) on hydrolysis of fumarylpyruvate to pyruvate and fumarate.

Conversion of gentisate into pyruvate. Spectrophotometric determinations of pyruvate formed enzymatically from gentisate were performed using lactate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) as previously described (3, 4). Values shown in Table 2 are averages of two or more determinations, each initiated with a different concentration of gentisate. The second product formed on hydrolysis of maleylpyruvate is maleate (14; see below). Cell extracts of *m*-hydroxybenzoate-grown *Bacillus* strains did not attack protocatechuate or catechol.

Preparation of fumarylpyruvate. Maleylpyruvate was prepared from gentisate using a purified preparation of gentisate 1,2-dioxygenase. The oxygenase was purified from salicylate-grown *Moraxella osloensis* strain OA3 as previously described (5). Maleylpyruvate was converted to fumarylpyruvate non-enzymatically by means of H_2SO_4 (15).

Counting of bacterial populations. Total viable counts of soil were performed by dilution/plating of soil suspensions onto plate-count agar (Difco). Counts of total aerobic sporeformers were performed on the same medium after pasteurization of soil suspensions at 80 C for 15 min. Counts of total *m*-hydroxybenzoate utilizers and of *m*-hydroxybenzoate-utilizing aerobic sporeformers were performed on minimal medium containing 500 mg of *m*-hydroxybenzoate per liter and 15 g of purified agar (Difco) per liter before and after pasteurization, respectively. Plates were incubated at 25 C.

Thin-layer chromatography (TLC) and gas-chromatography/mass spectrometry. Eastman chromatogram sheets (13181 silica gel; Eastman Kodak Co., Rochester, N.Y.) were used for analytical chromatography of aromatic compounds. Developing solvents were (A) benzene-methanol-acetic acid (45:8:2 by volume) and (B) benzene-ethyl acetate-80% formic acid (9:1:1 by volume). Aromatic compounds on plates were viewed under light of wavelength 253.7 or 375.0 nm. Gentisic acid exhibited a characteristic fluorescence when viewed on chromatograms under the former, but not the latter, wavelength of light. Organic acids were chromatographed on sheets of cellulose (Eastman, 13254 cellulose) using butanol-acetic acid-water (12:3:5 by volume; solvent C) or ethanol-ammonium hydroxide-water (16:1:3 by volume; solvent D) and were located by dipping chromatograms through $AgNO_3$ in acetone followed by alcoholic NaOH (19). In solvent C maleic acid showed a spot of R_f 0.45, whereas fumaric acid showed a spot of R_f 0.80. Corresponding R_fs in solvent D were maleic acid, 0.68, and fumaric acid, 0.25. Analyses using an LKB-9000A gas-chromatograph/mass spectrometer were performed as previously described (16).

Identification of maleate as an enzymic hydrolysis product of maleylpyruvate. The unsaturated, dicarboxylic acid formed on hydrolysis of maleylpyruvate by cell-free extracts of *m*-hydroxybenzoate-grown *Bacillus* sp. was identified as maleate by the following procedures. A solution (total volume, 10 ml) containing 20 μmol of gentisate and approximately 50 mg of cell extract protein (prepared from *m*-hydroxybenzoate-grown *Bacillus* strain A2a or C1a) was incubated at room temperature with stirring for 3 h. The pH of the solution was adjusted to 4.0 and sufficient absolute ethanol was added to give a final concentration of 95%. Precipitated material

was removed by centrifugation and the clear solution was concentrated to 1 to 2 ml by evaporation. Examination of this concentrate by TLC in solvents C and D revealed the presence of pyruvic acid (R_f 0.6 and 0.7, solvent C) (19) and maleic acid (R_f 0.45, solvent C; 0.67, solvent D), but no fumaric acid. When such enzymic reaction solutions were acidified to pH 2.0 and incubated at room temperature overnight prior to work-up, TLC revealed the presence of fumaric acid (R_f 0.8, solvent C; 0.24, solvent D), but no maleic acid. Control experiments indicate that, under the latter procedure, maleate is nonenzymically isomerized to fumarate.

Materials. Enzymes and co-factors were purchased from the Sigma Chemical Co. All compounds listed in Table 1 as well as gentisic acid, organic acids, protocatechuic acid, and catechol were purchased either from the Sigma Chemical Co. or the Aldrich Chemical Co. Commercial compounds were examined for purity by TLC and recrystallized prior to use where necessary.

RESULTS

Soils sampled during this investigation typically yielded 10^8 to 10^{10} bacteria per g of dry soil, as determined by counting on plate-count agar. Of these approximately 10% survived pasteurization and represent the aerobic, spore-producing component of the soil microflora. Of the total countable population of any particular soil, about 1% were able to grow on minimal media containing *m*-hydroxybenzoate as the only source of carbon and energy. Of the aerobic sporeformers in these soils approximately 0.1% were able to germinate and grow on *m*-hydroxybenzoate plates. This represents, as an average for all soils examined, about 0.01% of the total viable count. Though this percentage seems small, it represents 10^4 to 10^6 aerobic sporeformers that are able to utilize *m*-hydroxybenzoate as a carbon/energy source per g of dry soil. Soils examined during this investigation were collected at numerous locations around downtown Albany, N. Y., and served as the source of all microbial strains used in this study.

The *Bacillus* strains used during this investigation are listed in Table 1, along with their genus/species identification and the aromatic compounds upon which they were isolated.

Results of enzymic assays of each strain, after growth on *m*-hydroxybenzoate, are summarized in Table 2. Cell extacts prepared from *m*-hydroxybenzoate-grown bacilli did not attack catechol or protocatechuate and none contained maleylpyruvate isomerase, maleate isomerase, or fumarase activities. Such extracts did readily degrade fumarylpyruvate. Succinate- or glucose-grown cells lacked detectable amounts of aromatic pathway enzymes. The ring-fission product produced by oxidation of gentisate by *Bacillus* extracts showed spectral characteristics expected of maleylpyruvate; a λ_{max} in neutral or basic solution of 334 nm which is abolished upon acidification (5, 15).

It was possible to demonstrate directly the conversion of *m*-hydroxybenzoate to gentisate by using the Fe^{2+} chelator α,α'-dipyridyl as an inhibitor of gentisate oxidation. Whole cells of *Bacillus* strain B9a were harvested after growth on *m*-hydroxybenzoate and allowed to oxidize *m*-hydroxybenzoate in the presence of α,α'-dipyridyl, as described by Hopper and Chapman (13). After a 3-h incubation, cells were removed by centrifugation. The supernatant was acidified to pH 2.0 and an excess of $FeCl_2$ was added to trap α,α'-dipyridyl as its water-soluble iron complex. The cherry-red solution was extracted three times with ethyl acetate. Ethyl acetate extracts were combined, washed once with 0.1 M $FeCl_2$ and twice with water, dried over anhydrous Na_2SO_4, and evaporated to yield a semicrystalline solid. This organic residue was examined by TLC using solvents A and B and shown to contain mostly gentisic acid and some residual *m*-hydroxybenzoic acid. Gentisic acid was unequivocally identified by its retention time and molecular ion (trimethylsilyl derivative, *m/e* = 370) as revealed by gas-chromatography mass spectrometry.

Figure 1 illustrates the spectral changes ob-

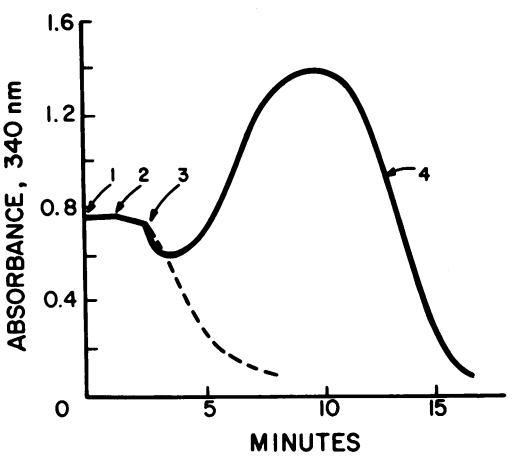

FIG. 1. *Spectral changes observed during oxidation of m-hydroxybenzoate by a cell extract of m-hydroxybenzoate-grown Bacillus strain C5f. The reaction was performed in a total volume of 2.0 ml of potassium-sodium phosphate buffer. The following additions were made as indicated by arrows: 1, NADH, 0.2 mg; 2, cell extract, 0.12 mg of protein; 3, m-hydroxybenzoate, 0.2 μmol; 4, GSH, 0.5 μmol. The dashed line indicates the observed change in absorbance when 0.1 μmol of α,α'-dipyridyl was included in the reaction mixture from zero time.*

served at 340 nm during oxidation of *m*-hydroxybenzoate by a supplemented extract of *m*-hydroxybenzoate-grown *Bacillus* strain C5f. Oxidation was dependent upon addition of reduced pyridine nucleotide to the reaction mixture. No changes in the ultraviolet spectrum of *m*-hydroxybenzoate were observed when NADH was omitted from the reaction mixture. The depicted spectral changes (Fig. 1) were also produced using other extracts prepared from strains chosen at random from the list in Table 1.

The unsaturated, dicarboxylic acid formed on hydrolysis of maleylpyruvate by cell extracts of *m*-hydroxybenzoate-grown *Bacillus* sp. was identified as maleate (cf. Materials and Methods).

DISCUSSION

The data summarized above indicate that all *Bacillus* isolates examined degrade *m*-hydroxybenzoate by the reaction sequence shown in Fig. 2A. This gentisic acid pathway is a modified version of the sequence originally delineated by Lack (15; Fig. 2B) and has been observed previously only in two strains of *Pseudomonas* isolated by Hopper et al. (strains 2,5 and 3,5; references 13, 14 and personal communication).

Spectral changes shown in Fig. 1 are consistent with requirements of the sequence of Fig. 2A. Thus on addition of *m*-hydroxybenzoate to a reaction mixture containing NADH and cell extract prepared from *m*-hydroxybenzoate-induced cells, one observes an initial decrease of A_{340} resulting from oxidation of NADH by an enzymatic hydroxylation of *m*-hydroxybenzoate forming gentisate. Gentisate formed is immediately oxidized by a gentisate 1,2-dioxygenase present in cell extracts, forming maleylpyruvate (λ_{max} = 334 nm) which absorbs strongly at 340 nm. Its formation results in an increase of A_{340}. The final decrease in A_{340} reflects hydrolysis of maleylpyruvate by its hydrolase forming pyruvate and maleate. Hydrolysis of maleylpyruvate is not speeded on addition of GSH. When α,α'-dipyridyl is included in the reaction mixture, gentisate 1,2-dioxygenase is inhibited. Thus NADH oxidation is no longer masked by maleylpyruvate formation and A_{340} continues its initial decrease.

It is conceivable that maleylpyruvate is isomerized to fumarylpyruvate by a GSH-independent isomerase prior to hydrolysis to pyruvate and a 4-carbon, dicarboxylic acid (particularly since cell extracts prepared from *m*-hydroxybenzoate-grown cells readily degrade fumarylpyruvate). This possibility is ruled out by our observation that maleic acid, rather than fumaric acid, accumulates from gentisate when the latter is oxidized by extracts prepared from *m*-hydroxybenzoate-grown *Bacillus* A2a or C1a. Like the nonfluorescent pseudomonad of Hopper et al. (14), the bacterial strains examined here induce an apparently nonfunctional fumarylpyruvate hydrolase when grown on *m*-hydroxybenzoate. Hydrolysis of maleylpyruvate and fumarylpyruvate may be catalyzed by a single

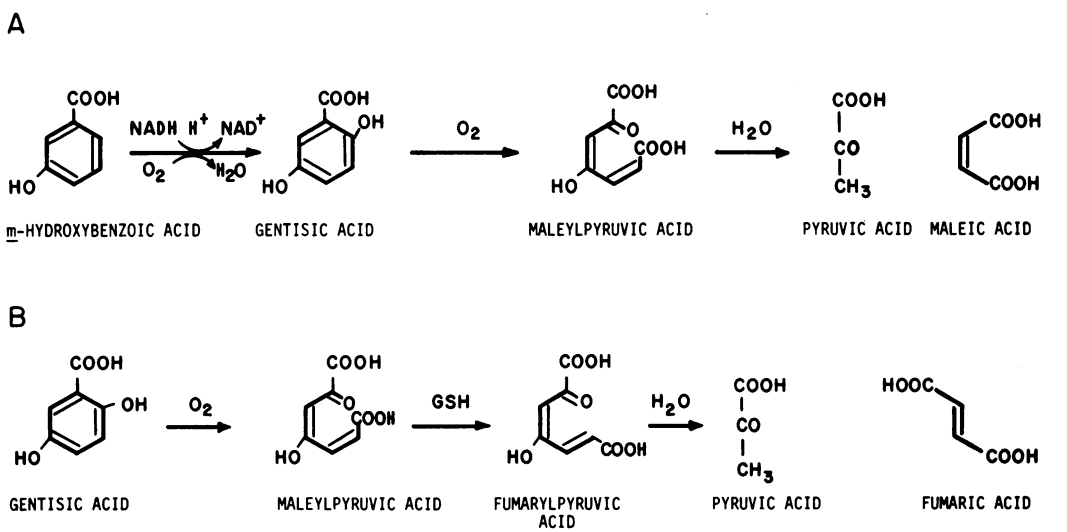

FIG. 2. *The pathways of gentisate degradation. (A) Pathway of degradation of m-hydroxybenzoate by species of Bacillus. (B) Pathway of gentisate degradation as originally delineated by Lack (15).*

enzyme. The answer to this question awaits purification of the hydrolase activities.

The enzymic specific activites and pyruvate yields shown in Table 2 are as expected for the pathway of Fig. 2A. Gentisate pathway enzymes, not present in glucose- or succinate-grown cells, are induced to high levels during growth on *m*-hydroxybenzoate. Also, one molecule of gentisate yields one molecule of pyruvate (Table 2) in a GSH-independent, enzymic reaction.

Actual isolation of gentisic acid as a metabolite of *m*-hydroxybenzoate after inhibition of cells with α,α'-dipyridyl is direct evidence of gentisate participation in the catabolic pathway.

Most of the *Bacillus* strains examined are tentatively classified as isolates of *B. brevis* (nine strains); however, strains of *B. sphaericus* (two strains) and *B. megaterium* (one strain) are also represented. Thus, it seems unlikely that degradation of *m*-hydroxybenzoate via the GSH-independent, gentisate pathway will be of taxonomic value in distinguishing species of *Bacillus*. It may, however, be possible to identify a small group of species with the taxonomically valuable characteristic of ability to grow on *m*-hydroxybenzoate. Our results indicate that the GSH-independent gentisate pathway may be of general occurrence in the genus *Bacillus*, rather than the GSH-dependent pathway. Investigation of many additional strains of *Bacillus* species that utilize gentisate as a catabolic intermediate will be necessary to determine whether or not this is a valid generalization.

As far as I know this is the first demonstration of the presence of a gentisate pathway among bacteria of the genus *Bacillus* (other than a preliminary report by Crawford and Chapman [R. L. Crawford and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, O3, p. 192]) as well as the first report of degradation of *m*-hydroxybenzoate by microorganisms of this classification.

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