Degradation of 3-Hydroxybenzoate by Bacteria of the Genus Bacillus

RONALD L. CRAWFORD

Freshwater Biological Institute, College of Biological Sciences, University of Minnesota, Navarre, Minnesota 55392

Received for publication 22 April 1975

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 10⁴ to 10⁶ 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-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/in² 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 was assayed by the procedure of Crawford et al. (5). Maleylpyruvate hydrolyase was assayed by observing decrease in absorbance at 334 nm resulting from hydrolysis of maleylpyruvate to pyruvate and maleate (14). The hydrolyase 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 × 10⁻⁴ M. E₉₅ of maleylpyruvate was assumed to be 10,800 (5) and 1 U of hydrolyase 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 absorbance at 386 nm in the reaction mixture containing 0.02 M potassium-phosphate buffer, pH 7.2, 0.01 M fumaric acid, 0.01 M glyoxylate, and 500 units of fumarase. A decrease of 1% per min was considered to be 1 U of activity. Maleate isomerase activity was assayed by observing the increase in absorbance at 386 nm in the reaction mixture containing 0.02 M potassium-phosphate buffer, pH 7.2, 0.01 M maleic acid, 0.01 M glyoxylate, and 500 units of fumarase. A decrease of 1% per min was considered to be 1 U of activity.

439
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 $A_{346}$ resulting from conversion of maleate to fumarate. Fumarylpyruvate hydrolyase was assayed by observing decrease in $A_{346}$ (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 protocatechuic 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$_2$SO$_4$ (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利用者 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 (45:3:2 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 $R_f$ in solvent D were maleic acid, 0.88, 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 (Rf 0.6 and 0.7, solvent C) (19) and maleic acid (Rf 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 (Rf 0.5, 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 10⁸ to 10⁹ 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 10⁴ to 10⁵ 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 extracts 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 λₘₐₓ 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 Fe²⁺ chelator a,a'-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 a,a'-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 FeCl₂ was added to trap a,a'-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 FeCl₃ and twice with water, dried over anhydrous Na₂SO₄, 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-
observed 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 A₃₄₀ 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 (λₘₐₓ = 334 nm) which absorbs strongly at 340 nm. Its formation results in an increase of A₃₄₀. The final decrease in A₃₄₀ reflects hydrolysis of maleylpyruvate by its hydrolyase 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 A₃₄₀ continues its initial decrease.

It is conceivable that maleylpyruvate is isomerized to fumarylpalpyruvate 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 hydroxylase 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).](image-url)
enzyme. The answer to this question awaits purification of the hydroxylase activities.

The enzymic specific activities 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, 03, p. 192]) as well as the first report of degradation of m-hydroxybenzoate by microorganisms of this classification.

ACKNOWLEDGMENTS

I thank Patricia Pike for technical assistance, the New York State Department of Health for financial support, and Peter Chapman for valuable discussions. This work was performed while I was a Research Scientist with the New York State Health Department, Division of Laboratories and Research.

LITERATURE CITED

1. Bouknight, R. R., and H. L. Sadoff. 1975. Tryptophan catabolism in Bacillus megaterium. J. Bacteriol. 121:70-76.
2. Buswell, J. A. 1974. The meta-cleavage of catechol by a thermophilic Bacillus species. Biochem. Biophys. Res. Commun. 60:934-941.
3. Collinsworth, W. L., P. J. Chapman, and S. Dayley. 1973. Stereosepecific enzymes in the degradation of aromatic compounds by Pseudomonas putida. J. Bacteriol. 113:922-931.
4. Crawford, R. L. 1975. Novel pathway for degradation of protocatechuic acid in Bacillus species. J. Bacteriol. 121:531-536.
5. Crawford, R. L., S. W. Hutton, and P. J. Chapman. 1975. Purification and properties of gentisate 1,2-dioxxygenase from Moraxella osloensis. J. Bacteriol. 121:794-799.
6. Ensign, J. C., and S. C. Rittenberg. 1964. The pathway of nicotinic acid oxidation by a Bacillus species. J. Biol. Chem. 239:2285-2291.
7. Gordon, R. E., W. C. Haynes, and C. H.-N. Pang. 1973. The genus Bacillus. Agriculture handbook no. 427. U.S. Government Printing Office, Washington, D.C.
8. Gornall, A. G., C. J. Bardwill, and M. M. David. 1949. Determination of serum proteins by means of the biuret method. J. Biol. Chem. 177:715-726.
9. Hegeman, G. D., Y. E. Rosenberg, and G. L. Kenyon. 1970. Mandelic acid racemase from Pseudomonas putida. Purification and properties of the enzyme. Biochemistry 9:4029-4036.
10. Hirschberg, R., and J. C. Ensign. 1971. Oxidation of nicotinic acid by a Bacillus species. I. Purification and properties of nicotinic acid and 6-hydroxynicotinic acid hydroxylases. J. Bacteriol. 108:751-756.
11. Hirschberg, R., and J. C. Ensign. 1971. Oxidation of nicotinic acid by a Bacillus species. Source of oxygen atoms for the hydroxylation of nicotinic acid and 6-hydroxynicotinic acid. J. Bacteriol. 108:751-759.
12. Hirschberg, R., and J. C. Ensign. 1972. Oxidation of nicotinic acid by a Bacillus species. Regulation of nicotinic acid and 6-hydroxynicotinic acid hydroxylases. J. Bacteriol. 112:392-397.
13. Hopper, D. J., and P. J. Chapman. 1970. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of m-cresol, 3,5-xylene and 2,5-xylene. Biochem. J. 122:19-26.
14. Hopper, D. J., P. J. Chapman, and S. Dayley. 1968. Enzymic formation of D-malate. Biochem. J. 110:798-800.
15. Lack, L. 1959. The enzymic oxidation of gentisic acid. Biochem. Biophys. Acta 34:117-123.
16. Leung, P.-T., P. J. Chapman, and S. Dayley. 1974. Purification and properties of 4-hydroxy-2-ketopimelate aldolase from Acinetobacter. J. Bacteriol. 120:168-172.
17. Prasad, C., and V. R. Srinivasan. 1970. Tryptophan catabolism during sporulation in Bacillus cereus. Biochem. J. 119:343-349.
18. Scher, W., and W. B. Jakoby. 1969. Maleate isomerase. J. Biol. Chem. 244:1878-1882.
19. Smith, I. 1960. Chromatographic and electrophoretic techniques, p. 272-290, vol. 1. William Heinemann Medical Books Ltd., London.
20. Spokes, J. R., and N. Walker. 1974. Chlorophenol and chlorobenzoic acid co-metabolism by different genera of soil bacteria. Arch. Microbiol. 96:125-134.
21. Wallnoefer, P., and G. Engelhardt. 1971. Degradation of phenylamides by Bacillus sphaericus. Arch. Microbiol. 98:315-323.
22. Willetts, A. J. 1974. Microbial metabolism of alkylbenzenesulphonates. The oxidation of key aromatic compounds by a Bacillus. Antonie van Leeuwenhoek J. Microbiol. Serot. 49:547-559.
23. Willetts, A. J., and R. B. Cain. 1970. Microbial metabolism of alkylbenzenesulphonates. Biochem. J. 121:70-76.
24. Willetts, A. J., and R. B. Cain. 1972. Microbial metabolism of alkylbenzenesulphonates. Enzyme systems of a *Bacillus* species responsible for β-oxidation of the alkyl side chain of alkylbenzenesulphonates. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 38:534–555.

25. Willetts, A. J., and R. B. Cain. 1972. Microbial metabolism of alkylbenzenesulphonates. Bacterial metabolism of undecyl-benzene-p-sulphonate and dodecylbenzene-p-sulphonate. *Biochem. J.* 129:389–402.