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# Biodegradation of Bisphenol A and Other Bisphenols by a Gram-Negative Aerobic Bacterium

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**A novel bacterium designated strain MV1 was isolated from a sludge enrichment taken from the wastewater treatment plant at a plastics manufacturing facility and shown to degrade 2,2-bis(4-hydroxyphenyl)propane (4,4′-isopropylidenediphenol or bisphenol A). Strain MV1 is a gram-negative, aerobic bacillus that grows on bisphenol A as a sole source of carbon and energy. Total carbon analysis for bisphenol A degradation demonstrated that 60% of the carbon was mineralized to $CO_2$, 20% was associated with the bacterial cells, and 20% was converted to soluble organic compounds. Metabolic intermediates detected in the culture medium during growth on bisphenol A were identified as 4-hydroxybenzoic acid, 4-hydroxyacetophenone, 2,2-bis(4-hydroxyphenyl)-1-propanol, and 2,3-bis(4-hydroxyphenyl)-1,2-propanediol. Most of the bisphenol A degraded by strain MV1 is cleaved in some way to form 4-hydroxybenzoic acid and 4-hydroxyacetophenone, which are subsequently mineralized or assimilated into cell carbon. In addition, about 20% of the bisphenol A is hydroxylated to form 2,2-bis(4-hydroxyphenyl)-1-propanol, which is slowly biotransformed to 2,3-bis(4-hydroxyphenyl)-1,2-propanediol. Cells that were grown on bisphenol A degraded a variety of bisphenol alkanes, hydroxylated benzoic acids, and hydroxylated acetophenones during resting-cell assays. Transmission electron microscopy of cells grown on bisphenol A revealed lipid storage granules and intracytoplasmic membranes.**

Bisphenol A, also called 2,2-bis(4-hydroxyphenyl)propane or 4,4′-isopropylidenediphenol, is an industrially important compound used in the production of polycarbonates and other plastics at many chemical manufacturing plants throughout the world. The annual production of bisphenol A exceeds 930 million pounds (1). Manufacturing facilities generate significant quantities of waste containing bisphenol A, some of which is discharged into the terrestrial, aquatic, and marine environments (4).

The health effects from exposure to bisphenol A have been investigated elsewhere (1, 9). One study used mice and guinea pigs as models in an attempt to determine the effects of external exposure to bisphenol A on humans (9). Photoallergic contact dermatitis related to bisphenol A was induced in the mouse model, but there was no response in the guinea pig model. A toxicological study on the developmental toxicity of bisphenol A in rats and mice found that bisphenol A treatment at maternally toxic doses during organogenesis produced fetal toxicity in mice but not in rats. No alteration in the morphologic development of the fetus in either species occurred (10).

In 1988, the ad hoc Bisphenol A Task Group from the Society of Plastics Industry reviewed the data available for the health and ecological effects of bisphenol A with the U.S. Environmental Protection Agency (1). According to standard evaluation procedures published by the U.S. Environmental Protection Agency, bisphenol A was determined to be slightly to moderately toxic for fish and invertebrates. Lethal and effective concentrations of bisphenol A for 50% of the experimental groups were from 1.1 to 10 mg/liter.

Very little is known about the chemical fate of bisphenol A in the environment. A recent study reported that most bisphenol A in a waste stream was degraded rapidly in a chemical plant's biotreatment facility or in surface waters that received a continuous discharge of bisphenol A (4). However, there are no reports on the microorganisms that degrade bisphenol A or the chemistry of bisphenol A biodegradation.

The present investigation reports on the isolation and characterization of a gram-negative, aerobic bacterium (strain MV1) that utilizes bisphenol A as the sole carbon source. The identification of several metabolic intermediates has led to a partial understanding of the biochemical pathway of bisphenol A biodegradation.

## MATERIALS AND METHODS

**Chemicals.** Bisphenol A, bis-(4-hydroxyphenyl)methane, and compounds tested for the ability to support the growth of strain MV1 as the sole carbon source (see Results) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. 2,2-Bis(4-hydroxyphenyl)butane was purchased from TCI American, Inc., Portland, Oreg. 4,4′-Dihydroxystilbene was purchased from Spectrum Chemical Corp., Gardena, Calif. All other bisphenols tested were chemically synthesized at General Electric Corporate Research and Development, Schenectady, N.Y. Bis(trimethylsilyl)trifluoroacetamide was purchased from Supelco, Inc., Bellefonte, Pa. All the components of Luria agar except NaCl were purchased from Difco Laboratories, Detroit, Mich.

**Media and culture conditions.** The PAS mineral salts basal medium used for the isolation and growth of the bisphenol A-degrading microorganism contained the following (in grams per liter of distilled water): $K_2HPO_4$, 4.35; $KH_2PO_4$, 1.7; $NH_4Cl$, 2.1; $MgSO_4$, 0.2; $MnSO_4$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.01; and $CaCl_2 \cdot 2H_2O$, 0.03. The medium was prepared by adding a concentrated solution of phosphates and ammonium salts (PA) to distilled water. PA was autoclaved for 20 min at 121°C and cooled to room temperature before the

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addition of a concentrated, filter-sterilized solution of mineral salts. The pH of the medium was 6.8. Unless specified, bisphenol A (0.5%) was added to PA before it was autoclaved. Since the solubility of bisphenol A is ~1.5 mM in PAS medium, crystals of bisphenol A were present in the medium during growth to maintain the concentration of bisphenol A near saturating levels. Liquid cultures contained 25 ml of PAS medium in 50-ml Erlenmeyer flasks stoppered with polyurethane foam plugs. Unless indicated otherwise, an inoculum (5% [vol/vol]) of strain MV1 grown on bisphenol A was added to each flask and incubated at 30°C in a rotary incubator-shaker at 200 rpm (model G25; New Brunswick Scientific Co., Inc., Edison, N.J.). Cells grown on glucose or 4-hydroxybenzoic acid (4-HBA) were cultured in PAS medium containing 20 mM glucose or 4-HBA. When strain MV1 was grown on bisphenol A in a 1-liter stirred bioreactor, the pH and temperature were maintained at 6.5 and 30°C, respectively. Oxygen was supplied by sparging with air, and the dissolved oxygen was monitored continuously with a dissolved-oxygen probe and analyzer (New Brunswick Scientific).

For solid PAS medium, 16 g of purified agar was added per liter of PA, and this mixture was autoclaved for 20 min and cooled to 50°C before the addition of a concentrated solution of mineral salts. Solid PAS medium containing bisphenol A was prepared by the addition of powdered bisphenol A (2 g/liter) to PAS medium cooled to 50°C and immediately distributed into petri dishes. Cultures grown on solid PAS medium were incubated at 30°C for 1 to 2 weeks.

**Enrichment and isolation.** Ten milliliters of sludge from a wastewater treatment plant at a plastics manufacturing facility was added to a 125-ml foam-stoppered shake flask containing 50 ml of PAS medium and 100 mg of bisphenol A crystals as the carbon source. A subculture was obtained by transferring 2 ml of the grown enrichment into 50 ml of PAS medium containing 100 mg of bisphenol A. Subsequent cultures grown on bisphenol A were used to obtain the pure-culture isolate, bacterial strain MV1.

Strain MV1 was isolated on solid PAS medium containing bisphenol A crystals dispersed throughout the agar. Several colonies that grew on the agar containing bisphenol A were streaked onto Luria agar containing the following (in grams per liter of distilled water): tryptone, 10; yeast extract, 5; NaCl, 5; glucose, 1; and agar, 15. Colonies that grew in 24 to 48 h were restreaked on Luria agar several times to ensure culture purity.

Strain MV1 was submitted to the American Type Culture Collection (Rockville, Md.) for identification. A variety of biochemical and physiological tests routinely used to taxonomically classify microorganisms were performed. The strain was compared with several *Pseudomonas* strains and CDC group Ve (5, 7).

**Growth on other carbon sources.** A culture grown on either bisphenol A or 4-HBA was inoculated into PAS medium containing one of several bisphenols tested (see Fig. 3) or one of the compounds tested for the ability to support the growth of strain MV1 as the sole carbon source (see Results). When the solubilities permitted, the potential substrates were tested at concentrations of 1 and 5 mM. The compounds were filter sterilized and aseptically added to sterile PAS medium. The cultures were incubated at 30°C for up to 5 days. Growth was determined by measuring the increase in turbidity at $A_{600}$ on a Perkin-Elmer Lambda 5 UV-VIS spectrophotometer. High-pressure liquid chromatography (HPLC) analyses of the culture supernatants were performed before and after incubation as described below.

**Inhibition studies.** Inhibition of cell growth by the metabolic intermediates 4-HBA and 4-hydroxyacetophenone (4-HAP) was tested in 20-ml screw-cap tubes containing 4 ml of PAS-glucose medium. Sterile solutions of the metabolites were aseptically added to the tubes to achieve concentrations of 1 to 20 mM. A 5% inoculum of an overnight culture grown on glucose was added to each of the tubes. Growth was measured as described below. The lowest concentration of 4-HBA or 4-HAP that did not produce at least a twofold increase in turbidity at $A_{600}$ was the MIC.

**Growth studies.** Growth rates of strain MV1 on three different carbon sources (bisphenol A, 4-HBA, and glucose) were determined in triplicate 50-ml Erlenmeyer flasks containing 20 ml of PAS medium. Unless indicated otherwise, a 5% inoculum was added and the cultures were agitated at 250 rpm and 30°C. Growth was monitored by measuring turbidity at $A_{600}$ in a 1-cm-wide cuvette on a Perkin-Elmer Lambda 5 UV-VIS spectrophotometer. Turbidity of 1.0 at $A_{600}$ represented $1.6 \times 10^9$ cells per ml, as determined from the number of CFU per milliliter after plating on PAS-glucose agar.

The rate of growth of strain MV1 on bisphenol A was determined in shake flasks containing PAS medium and bisphenol A crystals larger than 2 mm. Cell growth was measured in a 1-cm-wide cuvette as an increase in turbidity at $A_{600}$. The large crystals of bisphenol A settled to the bottom of the flasks before sampling and did not interfere with the cell turbidity measurements. The bisphenol A concentration remained near saturation (~1.5 mM) for the growth period.

The pH optimum was determined by adjusting the pH of PAS medium from 5.0 to 8.0 by altering the relative proportions of mono- and dibasic potassium phosphates. A culture grown at pH 6.5 was inoculated into each flask and incubated at 30°C for 3 days. Either glucose or bisphenol A was added as the sole carbon source, and growth was monitored.

**Carbon balance analysis.** Cultures of strain MV1 were grown in triplicate 120-ml serum bottles crimp sealed with black rubber stoppers. Each bottle contained 19.2 ml of $CO_2$-free PAS medium and the quantity of each substrate indicated in Table 1. The culture used as an inoculum was grown on the same substrate to which it was added. Cells were collected by centrifugation in an Eppendorf microcentrifuge and resuspended in $CO_2$-free PA before being added to bottles. The bottles were pressurized with air to 1.5 atmospheres (ca. 151.9 kPa) and incubated for 48 h at 30°C on a rotary incubator-shaker at 250 rpm.

The inorganic carbon (IC) and total carbon (TC) analyses for the carbon balance were performed on a total organic carbon analyzer (model TOC-500; Shimadzu Corp., Kyoto, Japan). A 1,000-ppm organic carbon standard for TC analysis was prepared by dissolving 2.125 g of reagent-grade potassium hydrogen phthalate in 1 liter of glass-distilled water. Appropriate dilutions were prepared to produce a standard curve from 10 to 200 ppm of organic carbon. A 1,000-ppm IC standard was prepared by dissolving 3.5 g of reagent-grade sodium hydrogen carbonate in 1 liter of glass-distilled water. A standard curve from 10 to 200 ppm of IC was obtained by use of the appropriate dilutions. Gas standards for $CO_2$ gas analysis were prepared by the addition of the appropriate volumes of $N_2$ or $CO_2$ gas to evacuated serum bottles to prepare 10, 20, and 30% $CO_2$ gas standards.

Gas-phase $CO_2$ was determined by use of multiple injections of 50 μl of the gas phase into the IC port of the TOC-500 analyzer. The $CO_2$ dissolved in 20 ml of culture medium was measured directly by use of multiple injections

of 20 μl of culture medium into the IC port. The total $CO_2$ produced from degradation of the substrate was obtained by addition of the liquid- and gas-phase $CO_2$. The air initially added to the bottles contained an insignificant amount of $CO_2$.

The moles of carbon converted to bacterial cells were measured from 1-ml samples removed from the suspended culture medium. The cells were centrifuged at 14,000 rpm for 5 min in an Eppendorf centrifuge (model 5415), resuspended in 5 ml of carbon-free PAS medium, and injected into the TC port of the TOC-500 analyzer. The same procedure was used to determine the amount of carbon added as an inoculum, and the difference is reported as moles of carbon converted to biomass.

The amount of substrate carbon converted to soluble organic carbon was determined after the cells were removed from the culture medium by centrifugation. The cell-free supernatant was injected into both the TC and the IC injection ports on the TOC-500 analyzer. The difference between the TC and the IC values was reported as the soluble organic carbon fraction. Further analysis of the culture supernatant by HPLC was carried out as described below.

**HPLC analysis of culture supernatants.** Water-soluble aromatic compounds were analyzed on a Waters HPLC system consisting of a Waters 490 programmable wavelength detector, a Waters 712 WISP automatic sampler, two Waters model 510 pumps, and a Waters system interface module connected into a Digital model PC380-AA computer. Soluble aromatic compounds were separated on either a Whatman PartiSphere 5 $C_{18}$ reverse-phase column (0.47 by 11 cm) or a Vydac $C_{18}$ reverse-phase column (0.5 by 25 cm; 5 μm; 100 A) by use of a linear gradient of water to acetonitrile as the mobile phase. Both eluants were acidified with trifluoroacetic acid to a concentration of 0.1% (vol/vol). The culture medium was centrifuged to remove the cells and other particulates. The supernatant (25 μl) was injected into the column. Aromatic compounds were monitored with a UV detector at 275 nm.

Several of the metabolites that accumulated in the culture medium were extracted from the cell-free medium with ethyl acetate, concentrated by evaporation, and injected into a Waters HPLC system equipped with a Vydac $C_{18}$ reverse-phase column (1 by 25 cm; 5 μm; 100 A). The eluted compounds were collected, concentrated, and identified by mass spectroscopy and nuclear magnetic resonance (NMR) analysis. All standards used to identify and quantify the various metabolites were prepared from reagent-grade chemicals or from compounds previously purified from the culture medium.

**Identification of metabolites.** Three of the isolated metabolic products, 4-HAP, 2,2-bis(4-hydroxyphenyl)-1-propanol (BHPP), and 2,3-bis(4-hydroxyphenyl)-1,2-propanediol (BHPPD), were identified by mass spectroscopy and $^1H$ NMR and $^{13}C$ NMR analyses. 4-HBA was extracted with diethyl ether from the cell-free culture medium after acidification to pH 2.0 with $H_3PO_4$. The ether extract was concentrated by evaporation, derivatized with bis(trimethylsilyl)trifluoroacetamide, and injected into a gas chromatograph (Hewlett-Packard model 5890 series II) coupled to a mass selective detector (Hewlett-Packard model 5971A). The metabolic products from the biotransformation of 2,2-bis(4-hydroxy-3-methylphenyl)propane were purified by HPLC and identified by mass spectroscopy and $^1H$ NMR analysis. Field desorption and electron impact mass spectroscopies were performed on a Varian MAT 731 apparatus. $^1H$ NMR and $^{13}C$ NMR analyses were done on a Varian XL200 or XL300 or a GE Omega 500 apparatus. Identification of 4-HBA, 4-hydroxy-3-methylbenzoic acid, 4-hydroxy-3-methylacetophenone, and 4-HAP was confirmed by comparison of spectra obtained from isolated metabolites with those of authentic compounds. The mass spectroscopy, $^1H$ NMR, and $^{13}C$ NMR data used for the identification of novel metabolites are summarized below.

**(i) 2,2-Bis(4-hydroxyphenyl)-1-propanol.** The electron impact mass spectrum of the isolated material had a molecular ion ($M^+$) at *m/e* 244, with a dominant fragment at 213 [$(HOC_6H_5)_2C^+CH_3$]. $^1H$ NMR ($CD_3CN$, 500 MHz; reference, tetramethylsilane [TMS]) had the following ppm: 1.57 (s, 3H), 2.62 (t, aliphatic OH, $J$ = 6 Hz), 3.88 (d, 2H, $J$ = 6 Hz), 6.69 (m, 4H), 6.74 (s, 2H, phenolic OH), and 7.03 (m, 4H). $^{13}C$ NMR ($CD_3OD$, 125 MHz; reference, $CD_3OD$) had the following ppm: 24.40 ($CH_3$), 46.48 (C2), 69.85 ($CH_2OH$), 114.04 (*meta*), 128.07 (*ortho*), 138.39 (*ipso*), and 154.63 (*para*).

**(ii) 2,2-Bis(4-hydroxyphenyl)-1,2-propanediol.** The electron impact mass spectrum of the isolated material had a small $M^+$ at *m/e* 260, with the most abundant ion at *m/e* 242 (loss of $H_2O$) and dominant fragments at *m/e* 136 ($HOC_6H_4C_2H_3O^+$) and *m/e* 107 ($HOC_6H_4CH_2^+$). $^1H$ NMR ($CD_3CN$, 200 MHz; reference, TMS) had the following ppm: 2.80 (t, 1H, primary OH), 2.93 (s, 2H, benzylic $CH_2$), 3.07 (s, 1H, tertiary OH), 3.60 (d, 2H, primary alcohol $CH_2$, $J$ = 7.2 Hz), 6.70 and 7.14 (m, 2H each, C2 aryl), 6.56 and 6.79 (m, 2H each, C3 aryl), and 6.62 and 6.75 (broad singlets, phenolic OH). The peaks at 2.80, 3.07, 6.62, and 6.75 all disappeared upon the addition of $D_2O$ and the doublet at 3.60 collapsed to a singlet. $^{13}C$ NMR ($CD_3OD$, 75.6 MHz; reference, $CD_3OD$) had the following ppm: 45.29 (C3), 69.27 (C1), 77.99 (C2), 115.35 and 115.45 (*meta*), 128.35 and 132.78 (*ortho*), 129.22 and 136.37 (*ipso*), and 156.65 and 157.08 (*para*).

**(iii) 2,2-Bis(4-hydroxy-3-methylphenyl)-1-propanol.** The electron impact mass spectrum of the isolated material had $M^+$ at *m/e* 272, with major fragments at *m/e* 241 (loss of $CH_2OH$) and 133 ($C_7H_6OC^+CH_3$). $^1H$ NMR (D-acetone, 200 MHz; reference, TMS) had aliphatic protons at the following ppm: 1.60 (s, 3H), 2.14 (s, 6H), and 3.92 (s, 2H). The aromatic protons were at the following ppm: 6.69 (d, 2H at C5, $J_o$ = 8.5 Hz), 6.88 (dd, 2H at C6, $J_m$ = 2.2 Hz, $J_o$ = 8.1 Hz), and 6.98 (d, 2H at C2, $J_m$ = 8.5).

**Resting-cell assays.** Strain MV1 cells were grown on bisphenol A, 4-HBA, or glucose in PAS medium to a turbidity of about 2.0 at $A_{600}$. The cells were collected by centrifugation, washed in PAS medium, and stored as pellets at −80°C for several days to weeks. Cell pellets were thawed at room temperature and resuspended in PAS medium to an optical density of 2.0 at $A_{600}$. The various bisphenols and other test compounds were introduced as 100 mM solutions in acetone or ethanol to the resuspended cells. The concentrations of the compounds tested in the assays did not exceed 1.2 mM. All assay mixtures were incubated at 30°C on a rotary incubator-shaker. Samples were removed periodically, and the supernatants were analyzed by HPLC as described above. Biodegradation of the various compounds was ascertained on the basis of a decrease in the concentration of the starting compound and the appearance of new aromatic metabolites by HPLC analysis. An assay with 1 mM bisphenol A was run in a parallel with each set of assays as a positive control to ensure that the cells had good metabolic activity. In comparison with freshly grown cells, no significant loss of metabolic activity was observed after storage at −80°C for several weeks. The relative rates

discussed for the degradation of bisphenol A and the metabolites during resting-cell assays were calculated as nanomoles of substrate degraded per minute per milliliter of cell suspension at the time at which degradation was at a maximum.

A colorimetric assay to test for the ring fission mechanism for either catechol or protocatechuic acid in strain MV1 was performed by the method described by Stanier et al. (13). Cells were grown on 4-HBA or bisphenol A in PAS medium for 1 to 3 days, centrifuged, and resuspended to an optical density of 2.0 at $A_{600}$.

**Electron microscopy.** In preparation for electron microscopy, cells were grown for either 2 or 6 days on bisphenol A or for 2 days on glucose or 4-HBA. Cells were harvested in 1.5-ml Eppendorf tubes (Eppendorf model 5415 centrifuge) and fixed in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2) for 2 h. The fixed cells were washed three times in 0.1 M cacodylate, postfixed for 2 h with 1% $OsO_4$ in 0.1 M cacodylate, embedded in 2% agar, and dehydrated in a graded ethanol series from 50 to 100%. The dehydrated cells were embedded in Epox-Araldite (Fullam, Inc., Albany, N.Y.) and polymerized for 24 h at 60°C. The blocks were sectioned on an ultramicrotome, stained in succession with 2% uranyl acetate and Reynolds lead citrate (12), and examined on a Philips CM12 transmission electron microscope.

## RESULTS

**Enrichment and isolation.** Sludge taken from a wastewater treatment facility at a plastics manufacturing plant was enriched on bisphenol A. The enrichment of sludge produced a microbial consortium capable of degrading 0.2% bisphenol A after 1 week of incubation. Strain MV1 was isolated from the enriched consortium on PAS agar medium containing fine crystals of bisphenol A. The growth that resulted after incubation for 1 week did not produce isolated colonies. However, a clearing of the medium was observed around the areas in which confluent cell growth had occurred, due to bisphenol A degradation. Isolated colonies were obtained after streaking of the culture on PAS agar medium containing bisphenol A. Several of these colonies were streaked on Luria agar to ensure that the strain constituted a single bacterial species. However, after growth on the rich medium, strain MV1 grew very poorly when it was returned to PAS agar medium with bisphenol A as the sole carbon source. Only a few patchy growth foci resulted in the regions in which the greatest quantities of cells were applied. After these cells were restreaked on the same medium, much better growth occurred.

**Growth on bisphenol A.** The growth of strain MV1 on bisphenol A was best achieved under aerobic conditions at 30°C in PAS medium at pH 6.5 to 7.0. Under these growth conditions, 10 g of bisphenol A per liter was consumed in 4 days and cell densities of greater than $10^{10}$ cells per ml were achieved. Because of the low solubility of bisphenol A in water, excess crystals of bisphenol A were added to the growth medium to keep the concentration near saturation (~1.5 mM). The nutritional supplement of yeast extract did not stimulate growth. Cell doubling times of about 4 h were observed during growth on bisphenol A; doubling times were 3 h during growth on glucose or 4-HBA.

**Carbon balance for bisphenol A degradation.** The carbon balance for strain MV1 grown on bisphenol A, 4-HBA, and glucose is shown in Table 1. Almost 60% of the carbon in bisphenol A was mineralized to $CO_2$, 20% was associated with the bacterial cells, and the other 20% was soluble organic carbon that remained in the medium. One of the metabolites, BHPPD, accounted for about 45% of the soluble organic carbon fraction. No residual bisphenol A was detected in the culture medium after 48 h of incubation. For comparison, the carbon balance for the metabolism of glucose and 4-HBA is also shown in Table 1. After growth of strain MV1 on 4-HBA or glucose, almost no soluble organic carbon remained. Over 95% of the TC was either mineralized to $CO_2$ or associated with the cells.

TABLE 1. Carbon balance for the biodegradation of bisphenol A, 4-HBA, and glucose by strain MV1[a]

| Substrate | Initial amt (mmol) | Carbon present in the products (mmol): | | | |
|---|---|---|---|---|---|
| | | $CO_2$ | Bacterial cells | Soluble organic carbon | Total |
| Bisphenol A | 2.00 | 1.16 | 0.39 | 0.42 | 1.97 |
| 4-HBA | 3.54 | 2.46 | 0.93 | 0.18 | 3.57 |
| Glucose | 1.25 | 0.59 | 0.67 | 0.03 | 1.29 |

[a] Reported values were converted from millimoles of substrate or product to millimoles of carbon.

**Biodegradation of bisphenol A.** Several compounds were detected in the culture medium when strain MV1 was grown on bisphenol A under conditions in which the concentration of bisphenol A in the culture was maintained at ~1.5 mM. The culture turbidity and accumulation of several metabolites in the medium during growth on bisphenol A are shown in Fig. 1. The first metabolites to accumulate were BHPP

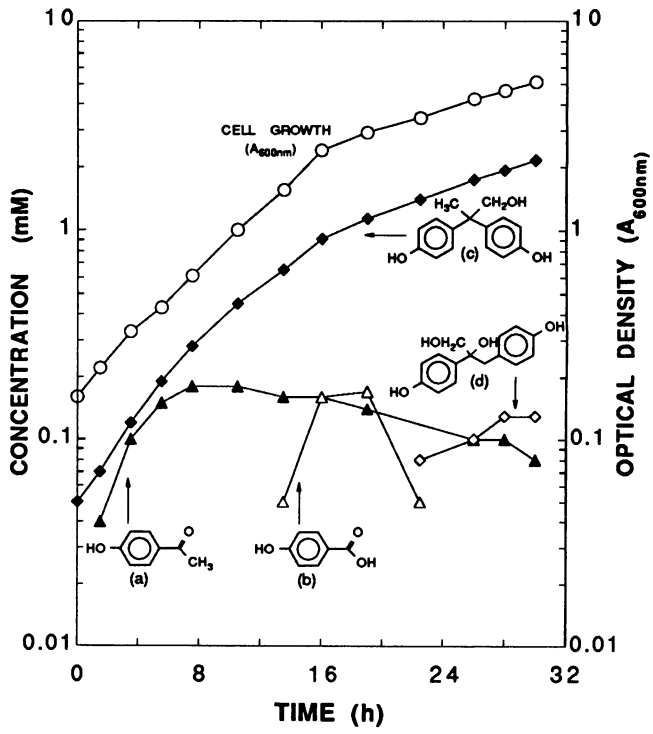


FIG. 1. Cell growth and metabolite formation during the degradation of bisphenol A by strain MV1. The concentration of bisphenol A was 1.0 to 1.5 mM during the growth period shown. Metabolite concentrations of less than 0.03 mM were not quantified. (a) 4-HAP; (b) 4-HBA; (c) BHPP; (d) BHPPD.

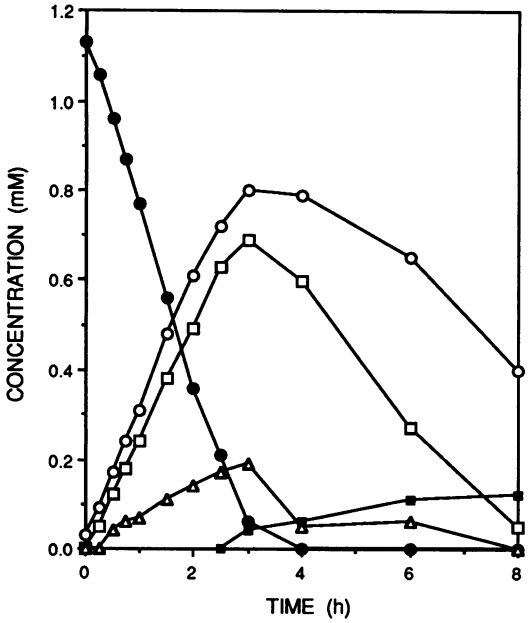


FIG. 2. Bisphenol A degradation and metabolite formation during a resting-cell assay with strain MV1 cells grown on bisphenol A. Symbols: ●, bisphenol A; □, 4-HBA; ○, 4-HAP; △, BHPP; ■, BHPPD.

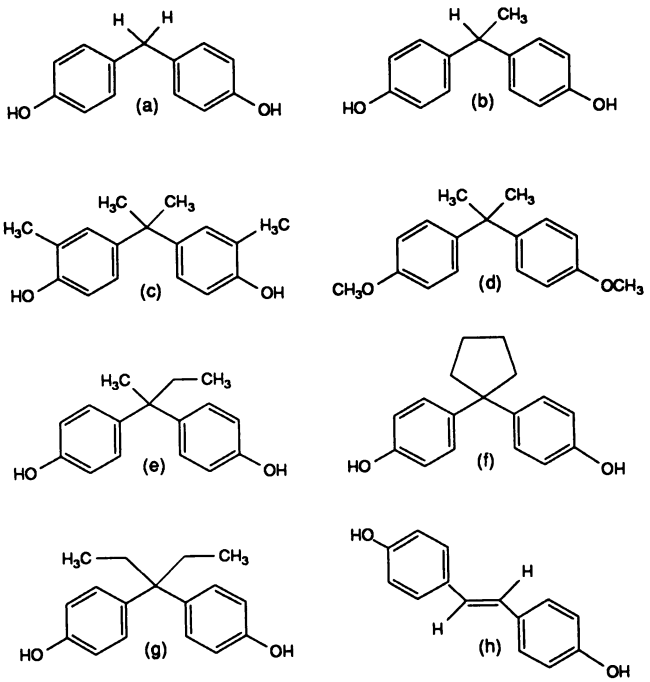


FIG. 3. Bisphenols biotransformed by strain MV1 during resting-cell assays. (a) Bis(4-hydroxyphenyl)methane. (b) Bis(4-hydroxyphenyl)ethane. (c) 2,2-Bis(4-hydroxy-3-methylphenyl)propane. (d) 2,2-Bis(4-methoxyphenyl)propane. (e) 2,2-Bis(4-hydroxyphenyl)butane. (f) 1,1-Bis(4-hydroxyphenyl(cyclopentane. (g) 3,3-Bis(4-hydroxyphenyl)pentane. (h) *trans*-4,4'-Dihydroxystilbene.

and 4-HAP. After 8 h, the accumulation of 4-HAP ceased, and its concentration gradually declined below the levels detectable by HPLC analysis (<0.03 mM). The formation of BHPP was proportional to cell growth, and concentrations of greater than 2 mM were observed in 32 h. The accumulation of 4-HBA in the culture medium was transient, and 4-HBA reached a concentration of only about 0.2 mM before it was consumed. Trace amounts of 4-HBA (<0.05 mM) were detected during exponential growth on bisphenol A. One other metabolite that accumulated in the culture medium was identified as BHPPD. The accumulation of BHPPD in the medium did not occur until after 20 h. Concentrations of BHPPD of greater than 0.1 mM resulted after 30 h of incubation. Both BHPP and BHPPD remained in the culture medium after several days of incubation.

When strain MV1 was grown on bisphenol A under conditions of vigorous aeration, 4-HAP accumulated in the culture medium to inhibitory levels. When the medium in a 1-liter pH-controlled bioreactor was rapidly sparged with air to saturation, concentrations of 4-HAP as high as 2 mM resulted in less than 24 h. However, when the rate of air sparging was reduced to maintain the dissolved oxygen below 5% of air saturation (approximately 0.5 ppm of $O_2$), 4-HAP did not accumulate to inhibitory levels. Also, inhibitory levels of 4-HAP were achieved when strain MV1 was grown in a 50-ml baffled shake flask containing 20 ml of PAS medium at high rates of agitation. The concentration of bisphenol A dissolved in the medium remained near saturation (1.5 mM) in these experiments. In a separate study, concentrations of 4-HAP of greater than 1.5 mM were shown to inhibit the growth of strain MV1.

**Resting-cell assays with bisphenol A and metabolites.** Strain MV1 cells grown on bisphenol A and used in resting-cell assays typically degraded 1 mM bisphenol A in 1 to 3 h. Bisphenol A degradation and metabolite formation during a resting-cell assay are shown in Fig. 2. During the first 3 h, 80 to 90% of the bisphenol A consumed was accounted for by the nearly equimolar amounts of 4-HBA and 4-HAP that accumulated in the medium. BHPP was a minor product formed during the first 3 h and accounted for the balance of the bisphenol A degraded. When the bisphenol A was depleted, 4-HBA and 4-HAP were metabolized further, and most of the BHPP was transformed to BHPPD.

Strain MV1 cells grown on bisphenol A, 4-HBA, or glucose were used in resting-cell assays to determine their relative abilities to degrade bisphenol A, 4-HBA, 4-HAP, and BHPP. Cells grown on bisphenol A degraded bisphenol A six times faster than cells grown on 4-HBA and 20 times faster than cells grown on glucose. Only about half of the bisphenol A was degraded after 30 h by 4-HBA- or glucose-grown cells, compared with complete bisphenol A degradation in less than 4 h by cells grown on bisphenol A. The rate of 4-HBA and 4-HAP degradation was two to three times faster when cells were grown on bisphenol A or 4-HBA rather than on glucose. In addition, cells grown on glucose exhibited a 30- to 45-min lag before any degradation of bisphenol A, 4-HBA, or 4-HAP was observed. The rates at which BHPP was converted to BHPPD were similar for cells grown on any of the three substrates. Regardless of the growth substrate, 4-HBA was always degraded 2.5 to 5.0 times faster than 4-HAP.

**Other aromatic compounds degraded.** Attempts to grow strain MV1 on several of the bisphenols shown in Fig. 3 were unsuccessful. Of all the compounds shown, only *trans*-4,4'-dihydroxystilbene was able to support the growth of strain MV1 as the sole carbon and energy source. In addition to the growth studies, strain MV1 cells grown on bisphenol A were challenged with a variety of compounds structurally similar

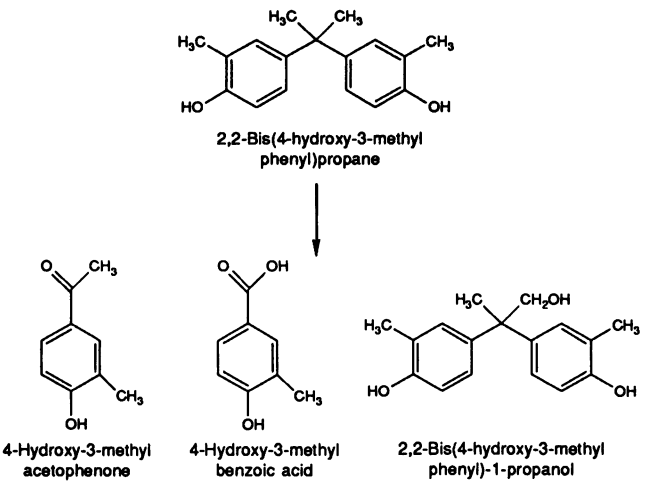


FIG. 4. Metabolites produced from the biodegradation of 2,2-bis(4-hydroxy-3-methylphenyl)propane by strain MV1 during a resting-cell assay.

to bisphenol A in resting-cell assays. The bisphenols that were degraded to some extent by strain MV1 during the resting-cell assays are shown in Fig. 3. The biodegradation products formed in a resting-cell assay containing one of the bisphenols, 2,2-bis(4-hydroxy-3-methylphenyl)propane, are shown in Fig. 4. Three metabolites that accumulated in the medium were 4-hydroxy-3-methylacetophenone, 4-hydroxy-3-methylbenzoic acid, and 2,2-bis(4-hydroxy-3-methylphenyl)-1-propanol; almost equimolar concentrations of 4-hydroxy-3-methylacetophenone and 4-hydroxy-3-methylbenzoic acid were produced during the assay. These products are analogous to three of the compounds found in the culture medium during bisphenol A biodegradation. However, unlike bisphenol A degradation, no further metabolism of these compounds was observed. Many of the metabolites formed during resting-cell assays with the other bisphenols have not been identified.

Several aromatic compounds with structural similarities to the metabolites of bisphenol A biodegradation were examined for the ability to support the growth of strain MV1 as the sole carbon source. The following compounds supported growth: 4-HAP, 4-hydroxybenzaldehyde, 4-HBA, 3-hydroxybenzoic acid, 2-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, and 2,5-dihydroxybenzoic acid. The following compounds did not support growth: 2,6-dimethylphenol, 4-isopropylphenol, 4-*tert*-butylphenol, phenol, catechol, *p*-cresol, *o*-cresol, benzoic acid, 4-aminobenzoic acid, 4-methoxybenzoic acid, 3-nitrobenzoic acid, 4-nitrobenzoic acid, 3-hydroxy-4-nitrobenzoic acid, 4-hydroxy-3-nitrobenzoic acid, 3,5-dimethyl-4-hydroxybenzoic acid, methyl-4-hydroxybenzoic acid, 4-hydroxy-3-methylbenzoic acid, benzaldehyde, 4-hydroxy-3-methylbenzaldehyde, acetophenone, 4-methoxyacetophenone, 4-hydroxy-2-methylacetophenone, 4-hydroxy-3-methylacetophenone, and 4-hydroxyphenylacetic acid.

**Identification of strain MV1.** Extensive biochemical and physiological testing performed at the American Type Culture Collection revealed that strain MV1 most closely resembles aerobic, gram-negative CDC group Ve (5). It shares features of both subgroups Ve-1 and Ve-2, which have recently been named *Chryseomonas luteola* and *Flavimonas oryzihabitans*, respectively (7). However, no definitive taxonomic classification could be assigned. Bacterial strain MV1 has been deposited at the Agricultural Research Service Culture Collection, U.S. Department of Agriculture, Peoria, Ill., and given the strain designation NRRL-B-18737.

**Other biochemical characteristics.** Various biochemical and physiological tests were performed at the American Type Culture Collection. The temperature range for growth was 20 to 37°C, with no growth at 41°C. Strain MV1 displayed monotrichous motility at room temperature. Hydrolysis of starch, esculin, Tween 20, and Tween 80 was observed. Colonies were round, smooth, and dull, with a nondiffusible yellow pigment. The reactions for catalase and phosphatase were positive, and the oxidase reaction was negative. Strain MV1 accumulated polyhydroxybutyrate and grew on DL-hydroxybutyrate as a sole carbon source. The ability of strain MV1 to utilize over 50 different organic compounds was tested. The compounds utilized were arabinose, cellobiose, fructose, glucose, lactose, maltose, rhamnose, xylose, glycerol, ethanol, acetate, butyrate, DL-lactate, malate, propionate, quinate, succinate, valerate, D-alanine, L-histidine, and L-valine. The compounds not utilized were mannitol, ribose, sorbitol, sucrose, trehalose, adonitol, erythritol, *i*-inositol, sebacic acid, acetamide, adipate, methanol, D-gluconate, citraconate, 2-ketogluconate, pelargonate, tartrate, β-alanine, betaine, glycine, D-tryptophan, DL-arginine, benzylamine, butylamine, putrescine, mesoconate, DL-glycerate, and L-tryptophan. No growth occurred under anaerobic conditions, and no nitrate or nitrite reduction was observed.

**Electron microscopy.** Transmission electron micrographs of strain MV1 cells grown on bisphenol A are shown in Fig. 5. Large quantities of lipid storage granules (Fig. 5a and b) consistent with polyhydroxybutyrate production were observed in cells grown on bisphenol A, and the relative amounts of these storage granules increased with prolonged incubation. Intracytoplasmic membranes (Fig. 5b and c) were observed in some cells grown on bisphenol A but were not observed in cells grown on glucose or 4-HBA. The function(s) of these membranes is unclear at this time.

## DISCUSSION

The difficulties encountered in the isolation of strain MV1 from the enrichment consortium may have been due to an inability of the bacterium to tolerate the concentrations of bisphenol A in the medium after it was grown in the absence of bisphenol A or a metabolic deficiency in the enzymes necessary for bisphenol A degradation. For routine growth of strain MV1 on bisphenol A in a liquid culture, excess amounts of bisphenol A crystals were maintained in the medium, apparently keeping the bacterium acclimated to bisphenol A at concentrations close to saturation. Subcultures on bisphenol A were much less successful when the inoculum had depleted bisphenol A from the culture medium.

Carbon balance data revealed that 80% of the carbon in bisphenol A was either mineralized to $CO_2$ or assimilated into the bacterial cells during the biodegradation of bisphenol A by strain MV1. The balance of the carbon was biotransformed into several soluble organic compounds: BHPP, BHPPD, and some compounds yet to be identified. Some of these soluble metabolites were slowly degraded by strain MV1 but did not support cell growth. The only metabolites of bisphenol A that were utilized as substrates for growth were 4-HAP and 4-HBA. The carbon balance for 4-HBA biodegradation demonstrated that very little of the

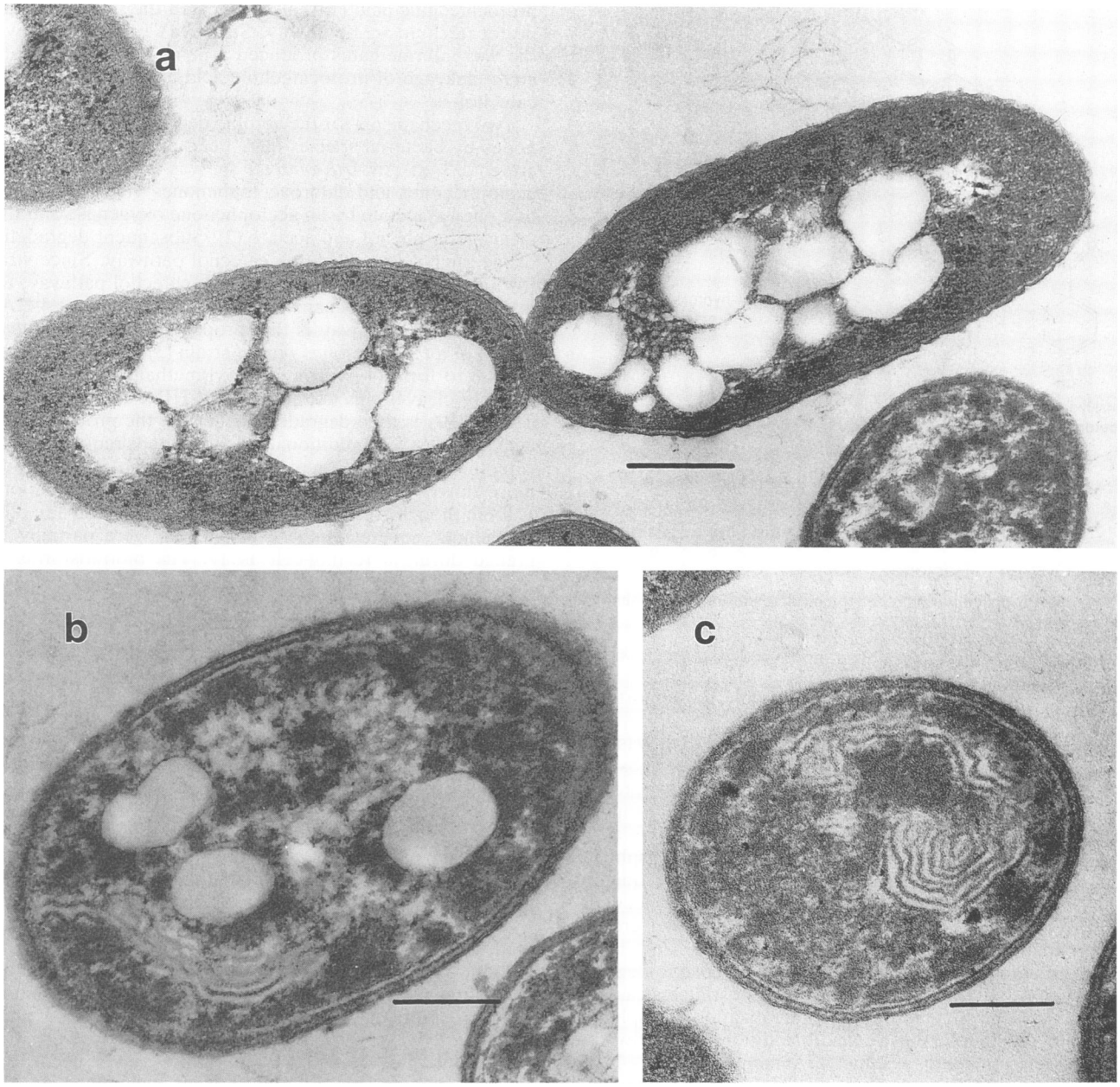


FIG. 5. Transmission electron micrographs of strain MV1 after growth on bisphenol A. Lipid storage granules are the electron-dense (light-colored) inclusion bodies inside the cells in panels a and b. Intracytoplasmic membranes appear to be attached to the cell membrane in panels b and c. Bars, 0.2 μm.

substrate carbon remained in the soluble organic fraction (<5%), since over 95% of the substrate carbon was mineralized to $CO_2$ or associated with the cells. 4-HBA and 4-HAP are metabolic intermediates that lead to mineralization of bisphenol A. BHPP and BHPPD are minor metabolites that account for less than 20% of the bisphenol A degraded. Apparently, these compounds are not on the biochemical pathway that gives rise to the 4-HBA and 4-HAP formed during bisphenol A degradation.

Our current understanding of bisphenol A metabolism is presented in Fig. 6. The primary pathway for the mineralization of bisphenol A occurs via 4-HBA and 4-HAP. These two metabolic intermediates were detected in the culture medium during logarithmic growth but did not persist upon prolonged incubation (except when 4-HAP reached inhibitory concentrations). During resting-cell assays, almost equimolar amounts of 4-HAP and 4-HBA accumulated in the medium during the first 3 h, accounting for 80 to 90% of the bisphenol A degraded. These metabolites were readily degraded after the bisphenol A was depleted. This result indicates that the majority of bisphenol A is cleaved in some way to form 4-HBA and 4-HAP, which are the primary sources of carbon and energy for the cell. A secondary pathway for bisphenol A degradation involves hydroxylation to form BHPP, which is slowly converted to BHPPD. Although BHPPD was very slowly metabolized by strain

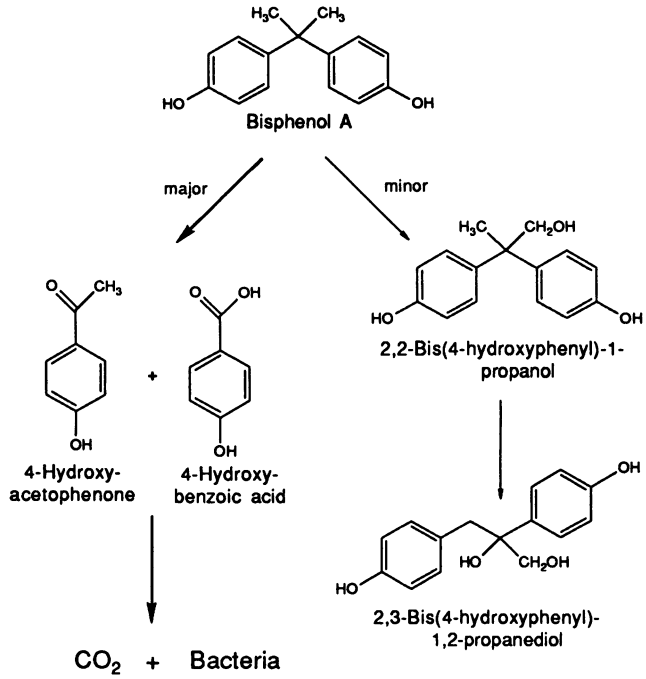


FIG. 6. Proposed pathway for the biodegradation of bisphenol A by strain MV1.

MV1, neither BHPPD nor BHPP was able to support cell growth.

Resting-cell assays demonstrated that 4-HBA was degraded more rapidly than 4-HAP, regardless of the substrate on which the cells were grown. Apparently, during growth on bisphenol A, 4-HBA was also metabolized more rapidly than 4-HAP, especially under conditions of vigorous aeration. When oxygen was in excess, it appears that bisphenol A was cleaved to 4-HBA and 4-HAP much more quickly than 4-HAP was consumed. Consequently, 4-HAP accumulated to inhibitory levels and prevented any further growth or degradation.

The impaired ability of cells grown on glucose to degrade bisphenol A, 4-HBA, or 4-HAP during resting-cell assays suggests that the enzymes for the mineralization of bisphenol A are not abundant in these cells. In addition, cells grown on 4-HBA were not nearly as effective at bisphenol A or 4-HAP degradation as cells grown on bisphenol A. The lag time observed prior to the degradation of bisphenol A or any of its metabolic intermediates for glucose-grown cells suggests that induction of the enzymes responsible for bisphenol A degradation may have occurred.

The aromatic compounds that supported the growth of strain MV1 (see Results) suggest that 4-HBA is catabolized via the protocatechuic acid pathway and not the catechol pathway, which is sometimes used for the oxidation of benzoic acids (6, 14, 15). The lack of growth on benzoic acid, various phenols, cresols, and catechol suggests that the catechol pathway is not active (11). Strain MV1 grew on the hydroxyl-substituted benzoic acids, including protocatechuic acid (see 3,4-dihydroxybenzoic acid). Hydroxybenzoic acids that contained a methyl or a nitro substituent at the 2, 3, or 4 position did not support the growth of strain MV1. The results obtained from an experiment that tested the ring cleavage mechanism of two intermediates central to the metabolism of most aromatic compounds, catechol and protocatechuic acid (13), also indicated the presence of the protocatechuic acid pathway. Strain MV1 cells tested with the two intermediates produced a positive reaction for the *meta* cleavage of protocatechuic acid and no reaction with catechol.

Two mechanisms for the aerobic degradation of acetophenones by bacterial species have been proposed in the literature (2, 3, 8). An *Arthrobacter* sp. was reported to degrade acetophenones and chloroacetophenones via the formation of a phenyl acetate by an acetophenone oxygenase and then cleavage to phenol and acetate (2). Subsequent degradation of the phenol occurs by the catechol pathway. Since strain MV1 does not appear to possess the catechol pathway, this is an improbable mechanism for the degradation of 4-HAP. A more likely pathway is that proposed for an *Alcaligenes* sp. (8) or a *Pseudomonas putida* strain (3) that hydroxylates 4-HAP to form (4-hydroxybenzoyl)methanol and then oxidatively cleaves this compound to 4-HBA and formic acid. The 4-HBA is then degraded further via the protocatechuic acid pathway. The additional oxidative steps required for the catabolism of 4-HAP may explain why 4-HAP is degraded more slowly than 4-HBA during bisphenol A degradation.

Even though strain MV1 was unable to grow on any other bisphenols, several of these bisphenols were partially degraded during a resting-cell assay. The biotransformation products that accumulated in the medium from some of these bisphenols were analogous to those for bisphenol A, indicating that certain enzymes used for bisphenol A degradation may also be reactive with several of the other bisphenols.

All of the metabolites identified during bisphenol A degradation have been isolated from the aqueous cell-free medium. It is not known whether some of these metabolites are formed at the cell surface or are excreted by the cells. Extracellular enzymes do not appear to be present, since no cell-free activity was observed for bisphenol A or any of the metabolites tested. Perhaps the intracytoplasmic membranes that appear to be attached to the inner cell membrane are sites for the oxidation of bisphenol A, permitting the various metabolic intermediates detected in the medium to passively diffuse in and out of the cell matrix.

A taxonomic assignment for strain MV1 could not be made from the current biochemical and physiological information. The final report from the American Type Culture Collection concluded that strain MV1 is a new bacterial isolate that most closely resembles species from CDC group Ve, *C. luteola*, or *F. oryzihabitans* (5, 7). However, strain MV1 could not be placed into either genus because of several characteristic differences from each genus. Further investigations including some genetic analyses may be necessary to determine the proper taxonomic classification for strain MV1.

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