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# Bacterial Methylation of Chlorinated Phenols and Guaiacols: Formation of Veratroles from Guaiacols and High-Molecular-Weight Chlorinated Lignin

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Two strains of bacteria, provisionally assigned to the genus *Arthrobacter*, were shown to metabolize mono-, di-, tri-, and tetrachloroguaiacols and pentachlorophenol to the corresponding *O*-methyl compounds. Hydroxylated intermediates were formed only transiently, except for the synthesis by one strain of 3,4,5-trichlorosyringol from 3,4,5-trichloroguaiacol. Two isomeric trichloroveratroles and tetrachloroveratrole were formed by three of the strains from a high-molecular-weight chlorinated lignin isolated from kraft pulp mill bleach plant. The concentrations of methylated metabolites varied widely and did not appear to be correlated with degradation. The possible environmental consequences resulting from synthesis of these highly lipophilic substances are discussed briefly.

It has been demonstrated repeatedly that effluents from pulp and paper mills producing fully bleached chemical pulp contain a wide range of chlorinated organic compounds (1, 5, 10, 15, 17, 18, 29). These include both high- and low-molecular-weight aromatic chloro compounds which are produced during the chlorination stage in the bleach plant, presumably as a result of the complex reactions between chlorine and the lignin contained in the wood pulp.

Some of these compounds, especially chlorinated guaiacols, have been shown to be toxic to juvenile rainbow trout (*Salmo gairdneri*) (16) and to have profound effects on sensitive life cycle stages of zebra fish (*Brachydanio rerio*) (T. Viktor, unpublished data). These effects are displayed at concentrations of 1 mg/liter or less, so the persistence of such compounds in the aquatic environment is a matter of serious concern.

In our laboratory we have been engaged in a study of the biodegradability of such compounds by a range of bacteria isolated by enrichment from areas subject to discharge of bleach plant effluent. The following two aspects have been of particular interest: first, the biodegradability of the toxic polychlorinated guaiacols and second, the possible microbial transformation of polymerized chlorinated lignin into low-molecular-weight components. During these studies, it was observed that incubation of high-molecular-weight chlorinated lignin fractions with these bacteria led to production of metabolites which were seemingly resistant to further transformation. These results are presented here, together with a much more detailed description of the occurrence of analogous reactions during incubation of a variety of monomeric aromatic chlorophenols and chloroguaiacols with bacterial suspensions. Our observations complement several previous studies in which total biodegradation of mono- and dichlorophenols was demonstrated (6, 12, 13, 26).

## MATERIALS AND METHODS

**Growth medium.** A defined mineral base medium (VV2 medium) was used for primary enrichments, for assessing the ability of pure strains to utilize a variety of substrates as sole carbon sources, and for growth of the cells required for the degradative studies. This medium was modified from a previously described medium (27) and contained (in grams per liter): $(NH_4)_2SO_4$, 0.1; $K_2HPO_4$, 0.05; $MgSO_4 \cdot 7\ H_2O$, 0.05; $Ca(NO_3)_2$, 0.01; and $FeNH_4(SO_4)_2$, 0.02. This medium was supplemented with 5 mg of thiamine per liter, 50 μg of biotin per liter, 5 μg of pantothenate per liter, 8 μg of vitamin $B_{12}$ per liter, and 1 ml of trace element solution A5 (14) per liter.

**Isolation of strains.** Isolation of strains was carried out by adding sediment and soil samples to the mineral base medium supplemented with a carbon source. Two strains were obtained by using methane, and a third was obtained by using a bleachery stage C concentrate whose preparation is described below. After successive transfers into fresh medium, strains from the methane enrichments were isolated and purified on defined medium plates containing methanol (1 g/liter) as the sole source of carbon (strain 1395) or on nutrient agar (strain 1487). The strain from the stage C enrichment (strain 1366) was isolated after streaking onto defined medium plates containing 4-hydroxycinnamate as the sole carbon source.

Characterization of the strains was carried out by standard procedures used in our laboratory (21, 22), with the addition of a test for phosphatase activity by hydrolysis of phenolphthalein phosphate (25). All incubations were carried out at 23°C.

**Preparation of substrates. (i) High-molecular-weight chlorinated lignins.** High-molecular-weight chlorinated lignins were prepared by ultrafiltration of chlorination (stage C) and extraction (stage E) liquors obtained from a kraft pulp mill producing fully bleached pulp from pine by a conventional bleaching sequence. This was carried out by using filters which have a molecular weight cutoff of approximately 1,000. The samples were concentrated approximately 40- to 60-fold by this procedure. The product from stage E had a pH of 7.5, which was lowered to 5.0 before ether extraction; the product from stage C had a pH of 2 and was not adjusted. In each case, the liquor was extracted five to seven times successively with equal volumes of ether, thereby completely removing the low-molecular-weight components. During this treatment, the stage E liquor formed a gellike mass which required centrifugation between extractions. The ether extracts were discarded, and the pH of the aqueous phases was adjusted to approximately 7 with 10 M NaOH. During this process, the gel in the stage E liquor dissolved. The remaining ether was removed by passing a rapid stream of nitrogen gas through the stirred solutions for 18 h at room temperature. Both fractions were filtered through glass fiber filters before dilution with equal volumes of twice-concentrated VV2 medium; the pH was adjusted to 6.8, and the solution was sterilized by filtration successively through 1.2- and 0.45-μm membrane filters.

**(ii) Monomeric substrates.** The synthesis of monomeric substrates is described briefly here since although procedures described previously (7, 30) were used, some improvements were incorporated. The purity of each substrate was checked by gas chromatographic analysis (see below) and was ≥98.5%.

**(a) Synthesis of 4,5,6-trichloro- and tetrachloroguaiacols.** Synthesis of 4,5,6-trichloro- and tetrachloroguaiacols was carried out essentially by the method of Fort et al. (7). 4,5,6-Trichloroguaiacol was prepared by passing chlorine gas into a boiling solution of guaiacol in dichloromethane for 10 min. Excess chlorine together with some solvent was immediately removed under a vacuum, the solution was washed three times with water, the dichloromethane extracts were dried ($Na_2SO_4$), the solvent was removed, and the product was recrystallized several times from hexane. Tetrachloroguaiacol was prepared in an analogous fashion by passing chlorine gas into a solution of guaiacol in acetic acid at 2°C for 1 h. The solvent was removed, the residue was dissolved in dichloromethane, and the product was processed as described above for trichloroguaiacol. Tetrachloroguaiacol was recrystallized several times from hexane.

**(b) Synthesis of chlorocatechols, 4-chloroguaiacol, and trichlorosyringol.** Synthesis of chlorocatechols, 4-chloroguaiacol, and trichlorosyringol was carried out by the method of Wilstätter and Müller (30), using sulfuryl chloride in refluxing ether. This proved to be a flexible and controllable method and was successfully used for selective multiple chlorinations by adding 1 mol of the reagent at a time and allowing the reaction to proceed for 1 h between additions. The reactions were followed by gas chromatographic analysis so that the extent of chlorination could be determined unambiguously. Thus, pure 4-chloro-, 4,5-dichloro-, and 3,4,5-trichlorocatechols could readily be prepared; these were recrystallized from toluene. This method was also used for the synthesis of 4-chloroguaiacol from guaiacol and 3,4,5-trichlorosyringol from 2,6-dimethoxyphenol. 4-Chloroguaiacol was a liquid which was purified by chromatography on silica gel, using toluene as the eluant, and chlorosyringol was recrystallized from a mixture of toluene and petroleum ether (40 to 60°C fraction).

**(c) Synthesis of 4,5-dichloro- and 3,4,5-trichloroguaiacols.** Synthesis of 4,5-dichloro- and 3,4,5-trichloroguaiacols was carried out by partial methylation of the catechols, using dimethyl sulfate. The catechols were dissolved in water, and the pH was adjusted to between 8 and 9 under an atmosphere of $N_2$. Dimethyl sulfate (1 mol/mol of catechol) was added, and the mixture was warmed to 50°C. The pH was maintained at 8 to 9 by successive additions of 6 M sodium hydroxide. When the pH had stabilized at about 7.5, the reaction was considered complete. The mixture was acidified to about pH 2 and extracted with toluene twice; then the extracts were dried ($Na_2SO_4$), and the solvent was removed. The residue was redissolved in a small volume of toluene (unreacted catechol was removed by filtration), and the solution was chromatographed on a column of silica gel (Merck Kieselgel 60; 70 to 230 mesh). The column was eluted with toluene, and fractions were collected. 4,5-Dichloroguaiacol and 4,5-dichloroveratrole were eluted together but could readily be separated by extracting the guaiacol into 0.1 M NaOH. Pure 4,5-dichloroveratrole was obtained in this way and was recrystallized from a mixture of methanol and water; the corresponding dichloroguaiacol was recrystallized from petroleum ether (40 to 60°C fraction). From 3,4,5-trichlorocatechol a mixture of the veratrole together with both 3,4,5- and 4,5,6-trichloroguaiacols was obtained, but these compounds were readily separable; the veratrole was eluted first, followed by 4,5,6-trichloroguaiacol accompanied by increasing amounts of 3,4,5-trichloroguaiacol. Fractions were pooled, and the residue after removal of the solvent was recrystallized from hexane to yield pure 3,4,5-trichloroguaiacol totally free from 4,5,6-trichloroguaiacol.

**(d) Synthesis of 3,4,5-trichloroveratrole, tetrachloroveratrole, and chlorinated anisoles.** Synthesis of 3,4,5-trichloroveratrole, tetrachloroveratrole, and chlorinated anisoles was carried out by methylating the corresponding guaiacols and phenols with dimethyl sulfate. Tetrachloroveratrole was recrystallized from a mixture of methanol and water, and purification of 3,4,5-trichloroveratrole was carried out by using preparative plates of silica gel (type $60F_{254}$; 0.25 mm; Merck) and toluene as the ascending phase. The veratrole was extracted with boiling methanol and was obtained as fine needles. Pentachloro- and 2,4,6-trichloroanisoles were prepared similarly from the phenols and were recrystallized from methanol.

**Metabolic experiments.** Metabolic experiments were carried out by using dense cell suspensions prepared from cells grown aerobically at 25°C in the defined medium (VV2 medium) containing 2.5 g of 4-hydroxybenzoate per liter as the sole source of carbon. After 2 to 3 days, the cells were harvested by centrifugation

under aseptic conditions (2,000 × *g*; 15°C; 15 min), washed twice in sterile basal medium, and then suspended in basal medium. Substrates of adequate solubility were dissolved in the basal medium at concentrations of 50 mg/liter, the pH was adjusted to 7.2, and the solutions were sterilized by filtration through 0.22-μm membrane filters. The cell suspension was then added so that the final cell density was about 10 times that of the original culture. Experiments were carried out in cultures having volumes between 125 and 600 ml, depending on the concentration of the metabolite formed. However, experiments in which 4-chloro- and 4,5-dichloroguaiacols and 2,4,6-trichloro- and pentachlorophenols were used were carried out in a totally enclosed system to minimize the loss of the substrates or the metabolites. Sterile solutions of the substrates (15 ml) were added to 60-ml sterile injection bottles, a suitable volume of the cell suspension was added, and the bottles were immediately sealed by using crimp caps with Teflon-coated rubber linings. Pentachlorophenol was not sufficiently soluble in water to allow use of the above-described procedure, and the following method was adopted. A solution of pentachlorophenol in hexane (100 μl; 7 μg/μl) was added to an injection bottle having a nominal volume of 60 ml, the solvent was gently removed by rotating the bottle, and then the cell suspension (15 ml) was added. Bottles were then sealed as described above. For kinetic experiments, 10-ml bottles containing 2 ml of culture were used, and one bottle was sacrificed for each sampling point. For preparative work, the 60-ml bottles were used together with a number of the 10-ml bottles, which were used for periodic assays before harvest of the larger cell volumes. All incubations were carried out in darkness at 23°C with orbital shaking (approximately 100 rpm). Uninoculated controls were included for all substrates, together with controls for viability of the cells. One loopful of the culture was periodically removed and spread onto plates of nutrient agar; these plates were then incubated for 4 days at 23°C.

**Extraction and analysis of substrates and metabolites. (i) Extraction of phenols, catechols, and guaiacols and their conversion into derivatives for gas chromatographic analysis.** Portions of a culture (1 ml) were removed aseptically, acidified with 0.3 ml of 1 M HCl, and extracted with 2-ml portions of toluene containing 2,4,6-tribromophenol and pentachlorobenzene (200 nmol of each) as internal standards. The toluene extract was dried ($Na_2SO_4$), and portions were converted into heptafluorobutyrate esters as follows. A 5-μl portion was mixed with a toluene solution of trimethylamine (25 μl; 0.15 M), heptafluorobutyric anhydride (10 μl) was added, and the reaction was allowed to proceed at room temperature for 10 min. Hexane (1.5 ml) and 2 ml of 1.0 M phosphate buffer (pH 6.0) were added, and the mixture was shaken. The hexane phase was used directly for analysis.

**(ii) Extraction of anisoles and veratroles.** Portions of a culture (1 ml) were removed as described above, made alkaline by adding 0.2 ml of 0.1 M NaOH, and extracted with 2 ml of toluene. The toluene phase was dried ($Na_2SO_4$) and used directly for analysis.

**(iii) Analyses.** Analyses were carried out by using a Varian model 3700 gas chromatograph that was equipped with an electron capture detector ($^{63}Ni$) and an automatic injector (Varian model 8000) and was coupled to a Hewlett-Packard model 3390 A Automation System. A fused silica capillary column (0.22-mm inside diameter by 25 m; type SE-54; Hewlett-Packard) was used, with nitrogen as the carrier gas and a split ratio of 1:30. The temperature of the column was maintained at 170°C; the injector was kept at 250°C, and the detector was kept at 300°C.

Quantification was carried out by using standard solutions of pure reference substances, and retention times are given relative to tetrachloroguaiacol heptafluorobutyrate.

Experiments to eliminate the possibility that substrates or metabolites were bound to cell protein and were therefore not included in the amounts found by direct extraction were carried out as follows. A culture (5 ml) was extracted four times with toluene (5 ml), and the toluene extracts were discarded. The aqueous phase was mixed with 5 ml of 10 M HCl and transferred to a 20-ml injection bottle, which was then sealed with crimp caps fitted with Teflon-coated rubber linings. The sample was then incubated at 60°C for 20 h, cooled, and extracted with toluene (3 ml); the extracts were dried ($Na_2SO_4$), and portions (100 μl) were used for analysis after conversion into suitable derivatives, if necessary. We found in control experiments with pentachlorophenol and 4-chloro-, 4,5-dichloro-, 3,4,5-trichloro-, 4,5,6-trichloro-, and tetrachloroguaiacols that essentially quantitative levels of recovery could be achieved, so no decomposition of the substrates occurred during the digestion procedure.

**Extraction of metabolites and identification. (i) Neutral metabolites.** Extraction of neutral metabolites (anisoles and veratroles) was carried out as follows. A culture (100 ml) was made alkaline by adding 1 M NaOH (2.5 ml) and then extracted three times with toluene (40 ml); for metabolites from 4,5-dichloro- and 3,4,5-trichloroguaiacols, the combined toluene extracts were further extracted twice with 0.1 M NaOH (50 ml) to remove excess guaiacols. The toluene extracts were then dried ($Na_2SO_4$), and the solvent was removed to give final concentrations of metabolites between 100 and 700 ng/μl.

**(ii) Phenolic metabolites.** For phenolic metabolites (catechols and syringol), a culture was acidified to pH <2, extracted as described above with toluene, and concentrated to a suitable degree. Catechol was converted into its pentafluoropropionate derivative, and trichlorosyringol was converted into the acetate derivative before analysis.

**(iii) Identification.** Identification with authentic substances was carried out by gas chromatography-mass spectroscopy, using a Finnigan model 4000 instrument equipped with a NOVA-4 data-processing unit. Operating conditions were as follows: electron energy, 70 eV; emission current, 3.0 mA; electron multiplier voltage, 1.2 kV; ionization temperature, 300°C. A fused silica capillary column (0.32-mm inside diameter by 24 m; type SE-54; Hewlett-Packard) was used with the following temperature program (Grob injection): isothermal for 1 min at 60°C, followed by increasing the temperature at a rate of 10°C/min to 240°C.

## RESULTS

**Characterization of the strains.** Three strains were isolated, although detailed metabolic re-

TABLE 1. Gas chromatographic retention times of guaiacol and catechol heptafluorobutyrates, veratroles, and other neutral metabolites relative to tetrachloroguaiacol heptafluorobutyrate

| Compound | Relative retention time |
|---|---|
| 4-Chlorocatechol | 0.46 |
| 4-Chloroguaiacol | 0.50 |
| 4-Chloroveratrole | 0.64 |
| 4,5-Dichlorocatechol | 0.51 |
| 4,5-Dichloroguaiacol | 0.62 |
| 4,5-Dichloroveratrole | 0.75 |
| 3,4,5-Trichlorocatechol | 0.62 |
| 3,4,5-Trichloroguaiacol | 0.71 |
| 3,4,5-Trichloroveratrole | 1.03 |
| 4,5,6-Trichloroguaiacol | 0.86 |
| 3,5,6-Trichlorocatechol | 0.61 |
| 3,5,6-Trichloroguaiacol | 0.69 |
| 3,5,6-Trichloroveratrole | 0.77 |
| 3,4,5,6-Tetrachlorocatechol | 0.78 |
| 3,4,5,6-Tetrachloroguaiacol | 1.00 |
| 3,4,5,6-Tetrachloroveratrole | 1.29 |
| 3,4,5-Trichlorosyringol acetate | 1.97 |
| 2,4,6-Trichloroanisole | 0.57 |
| Pentachloroanisole | 1.32 |

sults are presented below for only two of them (strains 1395 and 1487). However, we found that the other strain (strain 1366) possessed comparable capability for methylation of 4,5,6-trichloroguaiacol and pentachlorophenol.

All three strains were similar and were positive for the following characteristics: Gram stain; catalase; hydrolysis of urea and DNA; formation of acid in ammonium salts sugar medium from glucose, sucrose, maltose, and arabinose; and growth at the expense of 4-hydroxybenzoate, 3-hydroxybenzoate, vanillate, 4-hydroxycinnamate, 3-phenyllactate, 4-hydroxyphenylacetate, succinate, and pelargonate. The three strains were negative for the following characteristics: oxidase reaction (Kovács); hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside; reduction of nitrate to nitrite; hydrolysis of esculin, gelatin, starch, and Tween 80; decarboxylation (Møller method) of arginine, lysine, and ornithine; and growth with syringate. Variable positive results were observed for the following characteristics: phosphatase (strain 1395); and growth with salicylate (strains 1366 and 1487), guaiacol (strains 1366 and 1395), mandelate (strains 1366 and 1395), methanol (strain 1395), and decanoate (strains 1366 and 1487). All three strains showed variable colony morphologies after growth on nutrient agar, and their cells were rods, cocci, or a mixture of both types of cells. Therefore, with some uncertainty, the strains were assigned to the genus *Arthrobacter*.

**Metabolism of substrates.** Since most of the compounds were relatively resistant to microbial transformation, lengthy incubations were obligatory. Samples were removed periodically, and portions were extracted for determination of the concentrations of the substrates and of possible metabolites. Tests for viability of the cells and for the presence of contaminating organisms were simultaneously carried out.

During the course of the experiments, no loss of cell viability was observed. The appearance of the colonies of the strains used was sufficiently characteristic that the presence of any contaminant could readily be detected; this occurred in one set of experiments, and the results were discarded. We found no evidence that substrates or metabolites were bound to cell proteins and therefore were not extracted by the direct procedure. There was no evidence in uninoculated controls of either loss of the substrate or accumulation of any of the metabolites. In addition, all of the experiments were repeated at least twice with essentially identical results, so that the occurrence of artifacts may reasonably be excluded.

The nature of the metabolites and the determination of their concentrations were initially based on gas chromatographic comparisons with authentic substances. The relative gas chromatography retention times of the substrates and their metabolites are shown in Table 1, and the yields of the metabolites are shown in Table 2. The kinetics of degradation of a selection of the substrates and the synthesis of the metabolites are shown in Fig. 1 through 4.

TABLE 2. Concentrations of metabolites produced by strains 1395 and 1487 from the corresponding guaiacols and phenols

| Strain | Concn (mg/liter) of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 4-Chloroveratrole | 4,5-Dichloroveratrole | 3,4,5-Trichloroveratrole | | 3,4,5,6-Tetrachloroveratrole | 3,4,5-Trichlorosyringol | 2,4,6-Trichloroanisole | Pentachloroanisole |
| | | | From 3,4,5-trichloroguaiacol | From 4,5,6-Trichloroguaiacol | | | | |
| 1395 | 9.0 | 0.5 | 0.5 | 13.0 | 0.9 | 7.0 | ND[a] | 1.0 |
| 1487 | 28.0 | 0.6 | 0.15 | 11.0 | 1.0 | ND | 11 | 0.15 |

[a] ND, Not detected.

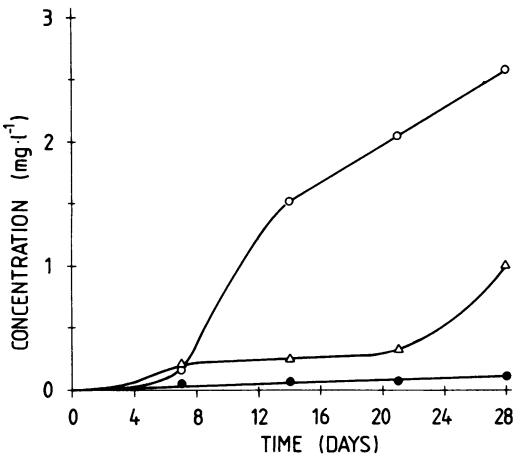


FIG. 1. Kinetics of formation of veratroles from stage E high-molecular-weight chlorinated lignin incubated with strain 1395. Symbols: ○, 3,4,5-trichloroveratrole; ●, 3,5,6-trichloroveratrole; △, tetrachloroveratrole.

All three strains produced the two isomeric trichloroveratroles together with tetrachloroveratrole during incubation with stage E high-molecular-weight (>1,000) chlorinated lignin. These metabolites were also produced from the stage C fraction, although the yields were significantly lower (about 10%).

In view of the complex fragmentation patterns found in the mass spectra of chlorinated compounds due to the presence of the isotopes $^{35}Cl$ and $^{37}Cl$, we felt that it was more appropriate to produce a selection of the most relevant spectra than to attempt interpretation of the spectra.

The mass spectra of the major metabolites produced by strain 1395 from stage E and of authentic substances are shown in Fig. 5 and 6.

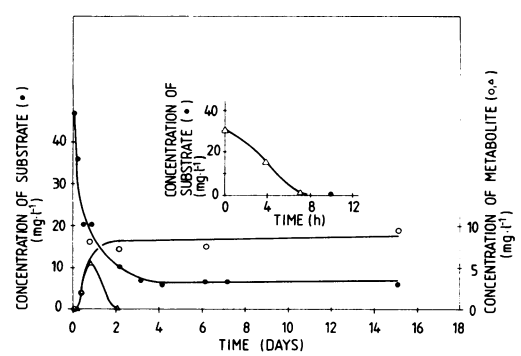


FIG. 2. Kinetics of degradation of 4-chloroguaiacol (●) and synthesis of 4-chlorocatechol (△) and 4-chloroveratrole (○) by strain 1395. The inset shows the degradation of 4-chlorocatechol by strain 1395 in a separate experiment.

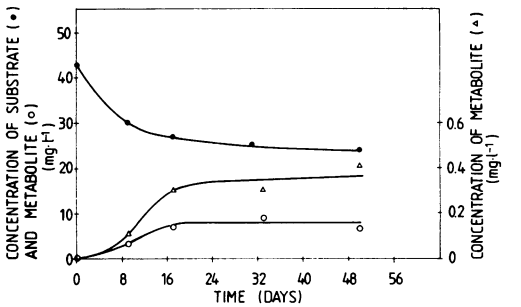


FIG. 3. Kinetics of degradation of 3,4,5-trichloroguaiacol (●) and synthesis of 3,4,5-trichloroveratrole (△) and 3,4,5-trichlorosyringol (○) by strain 1395.

The mass spectra of the 4-chloro-, 4,5-dichloro-, 3,4,5-trichloro-, and tetrachloroveratroles produced by strains 1395 and 1487 from chloroguaiacols were identical to those of the authentic substances. The spectrum of the acetylated metabolite also produced by strain 1395 from 3,4,5-trichloroguaiacol is compared with the spectrum of authentic trichlorosyringol acetate in Fig. 7. The spectra of the chloroanisoles produced from the corresponding chlorophenols are shown in Fig. 8 and 9.

## DISCUSSION

Some general comments should first be made about the strains used and the synthesis of the substrates.

Assignment of the strains to the genus *Arthrobacter* must be regarded as provisional in light of the confused state of coryneform taxonomy (11), and our strains could not be identified with any known species of this genus.

Most of the syntheses were accomplished by standard procedures and presented only minor difficulties; an exception was 3,4,5-trichloroguaiacol. The previously described procedure (19) using direct chlorination followed by partial

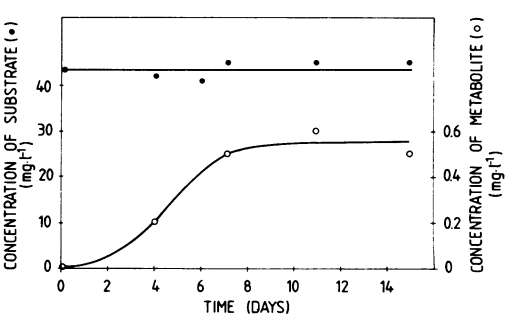


FIG. 4. Kinetics of the synthesis of 4,5-dichloroveratrole (○) by strain 1395. The concentration of 4,5-dichloroguaiacol (●) was virtually unchanged throughout the experiment.

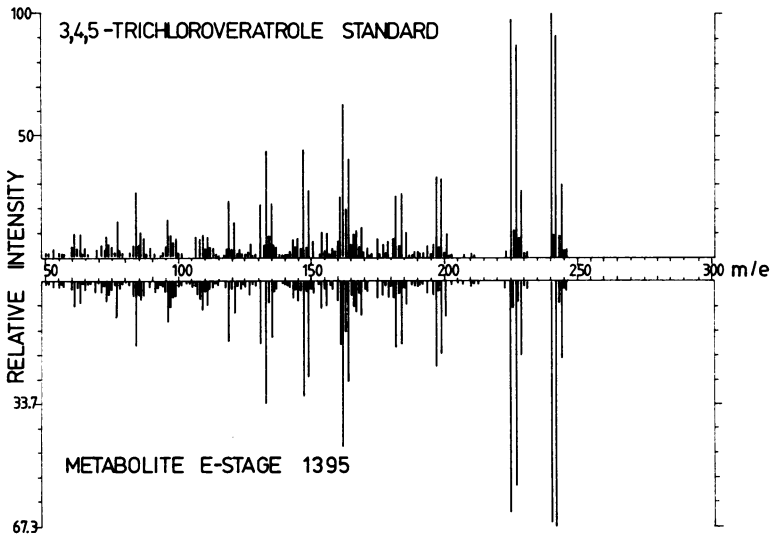


FIG. 5. Mass spectra of authentic 3,4,5-trichloroveratrole and the metabolite from stage E (strain 1395).

methylation of the crude chlorocatechol without purification led in our hands to irreproducible results and negligible yields of the product. By contrast, the use of sulfuryl chloride gave readily reproducible results and resulted in high yields of pure 3,4,5-trichlorocatechol, which could be partially methylated to a readily separable mixture of 3,4,5- and 4,5,6-trichloroguaiacols. Sulfuryl chloride was equally suitable for the other chlorinations, although without the addition of a catalyst it was not sufficiently reactive to bring about complete chlorination of guaiacol.

From the mass spectral data presented above, there can be no reasonable doubt about the identities of the various methylated products. Thus, we can discuss the results obtained. Initial experiments were carried out with high-molecular-weight fractions of chlorinated lignin from stage E and C bleaching. Synthesis of tri- and tetrachloroveratroles was clearly demonstrated. Although authentic 3,5,6-trichloroveratrole was not available, its structure was deduced with a high degree of certainty from the similarity, but not identity, of its mass spectrum to the spectrum of 3,4,5-trichloroveratrole. On the basis of

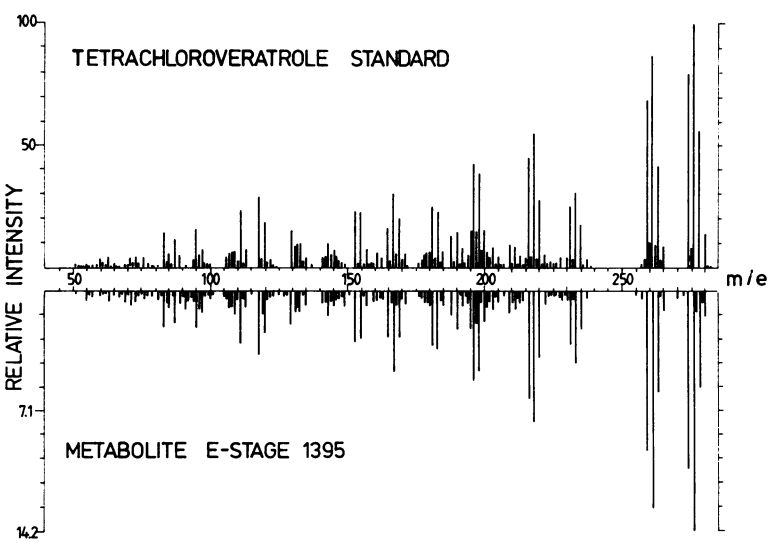


FIG. 6. Mass spectra of authentic tetrachloroveratrole and the metabolite from stage E (strain 1395).

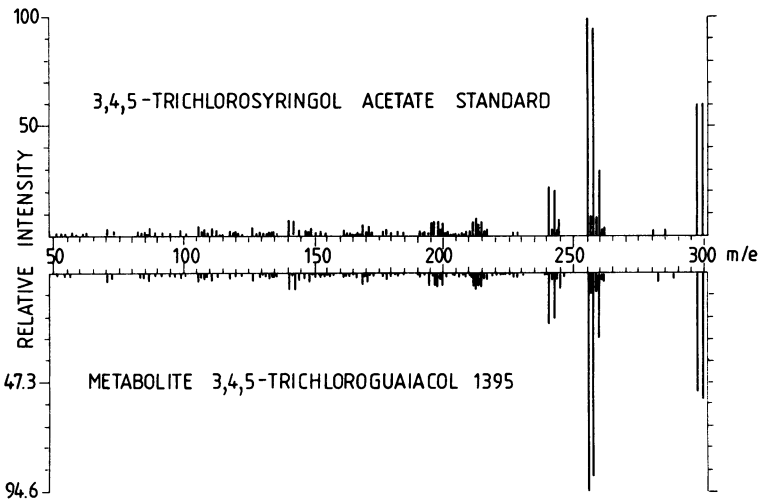


FIG. 7. Mass spectra of authentic 3,4,5-trichlorosyringol acetate and the acetylated metabolite from 3,4,5-trichloroguaiacol (strain 1395).

our experiments, however, we could not unambiguously determine whether the polymer was attacked directly by the bacteria or whether the bacteria methylated low concentrations of guaiacols slowly released from the chemically unstable substrate. Current investigations are aimed at resolving this issue and appear to support the first alternative, at least with respect to the synthesis of 3,4,5-trichloroveratrole.

A more detailed examination was carried out with the monomeric substrates. On the basis of previously published studies on the biodegradation of chlorophenols (6, 12, 13, 26) and methoxybenzoates (4), we expected that metabolism of the chloroguaiacols would proceed by oxidative demethylation to catechols, followed by one of the established modes of ring cleavage (23). Low concentrations of catechol were indeed observed (Fig. 2), but in all cases methylation was the predominant reaction. This generally occurred rapidly at the beginning of the incubation period; the subsequent decrease in the concentration of the substrate then occurred more slowly (Fig. 2 and 3). A more complex situation was encountered during metabolism of 3,4,5-trichloroguaiacol by strain 1395, in which the expected veratrole was formed, albeit at a low concentration, although the major metabolite was 3,4,5-trichlorosyringol (Fig. 3). This occurred presumably by hydroxylation and sub-

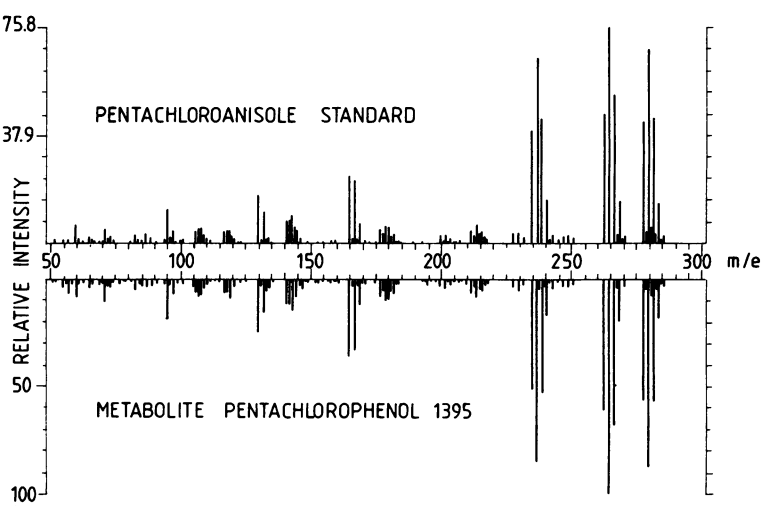


FIG. 8. Mass spectra of authentic pentachloroanisole and the metabolite from pentachlorophenol (strain 1395).

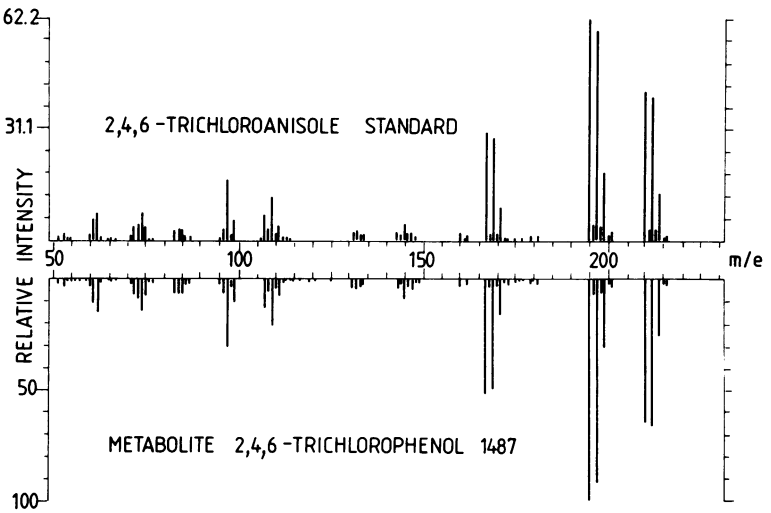


FIG. 9. Mass spectra of authentic 2,4,6-trichloroanisole and the metabolite from 2,4,6-trichlorophenol (strain 1487).

sequent methylation at C-6; the trichlorosyringol appeared to be resistant to further transformation.

Some unexpected findings were the seeming recalcitrance of 4,5-dichloroguaiacol to either degradation or methylation (Fig. 4) and the relatively greater ease with which 4,5,6-trichloroguaiacol was methylated compared with 3,4,5-trichloroguaiacol (Table 2).

Methylation of 2,3,4,6-tetrachlorophenol to the corresponding anisole has been observed in a wide range of fungi (8) and is accompanied by variable degrees of degradation; the corresponding methylation of pentachlorophenol has also been demonstrated with *Trichoderma virgatum* (2). On the other hand, bacterial methylation appears to be a rare phenomenon; methylation of pentachlorophenol to the anisole has been reported in a number of bacteria, although the yields were exceedingly low (0.005 to 0.002%) and were greatly exceeded by the extent of acetylation (24).

The concentrations of the substrates used in this study were probably unrealistically high compared with those which might be expected in natural situations. Nonetheless, in 6 of the 16 experiments, conversion of the substrates to *O*-methylated compounds exceeded 14% and in one case was 50%. Even in the transformations of 3,4,5-trichloroguaiacol and pentachlorophenol, which occurred in much lower yields, conversions were between 0.07 and 0.5% for the two test strains. All of these values considerably exceed the highest value (0.02%) reported for bacterial methylation of pentachlorophenol (24), and the range is no greater than the range observed for fungal metabolism of tetrachlorophenol (8).

In the present study, there did not appear to be any correlation between the relative contributions of methylation and degradation (Fig. 2 and 4). Such transformation may then be viewed as tangential to the normal metabolism of the cell and might function as a detoxification mechanism. At least superficially, this appears to be analogous to secondary metabolism, especially in fungi, but perhaps a closer analogy is with certain mechanisms whereby antibiotic resistance is acquired. For example, in chloramphenicol-resistant strains of bacteria, an inducible acetyl transferase is able to convert the antibiotic into the inactive 3-*O*-acetate (3). Although methylation of catechols is well established in mammalian systems (9), it appears to be much more restricted in the microbial world.

However, catechol *O*-methyl transferase has been demonstrated in some species of *Candida* (20), and the enzyme has been purified (28). It is significant that this enzyme is apparently restricted to monomethylation and dimethoxy products have not been isolated. It is clearly premature to speculate further on the enzymatic basis of our findings.

The present investigations have clearly established a principle, that microbial transformation of a substrate may result in the synthesis of metabolites more resistant to further transformation than the original substrate. Although these metabolites were presumably less toxic to the organisms producing them, in the present case they were highly lipophilic (comparable to dichlorodiphenyltrichloroethane and polychlori-

nated biphenyls and could then plausibly be expected to accumulate in the tissues of higher organisms, such as fish.

The environmental consequences of such transformations are at present unknown, but in order to avoid possible misrepresentation, a number of limitations in the present work and some unresolved issues ought to be pointed out. (i) Methylation of phenols and guaiacols has been demonstrated in three independent strains of bacteria which appear to be taxonomically similar. The frequency of occurrence in the environment of organisms with comparable metabolic activities is the subject of continuing studies; the possible role of other kinds of bacteria, including gram-negative strains, cannot be excluded, even though those microbes isolated hitherto have not displayed such metabolic activity. (ii) The metabolites were formed during incubation with dense cell suspensions, and the synthesis of these compounds during cell growth has not yet been examined. Although such conditions will never be encountered in the environment, they are widely used in respirometric studies of biodegradation. Extrapolation to natural situations clearly requires a systematic examination of the effects of cell density and the concentration of the substrate. (iii) The biodegradation of the metabolites has not yet been systematically examined, although a high degree of persistence might be expected on the basis of the experiments carried out (Fig. 2 and 3). At the same time it should be emphasized that strains capable of synthesizing these metabolites may not be the most appropriate strains for studying their biodegradation. Bacteria have been isolated by enrichment with dimethoxy- and trimethoxybenzoates and may plausibly be more suitable organisms for studying the biodegradability of chlorinated veratroles.

To provide a realistic assessment of the environmental impact, all of these factors must be taken into consideration.

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