Demethylation of Veratrole by Cytochrome P-450 in Streptomyces setonii

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The actinomycete Streptomyces setonii 75Vi2 demethylates vanillic acid and guaiacol to protocatechuic acid and catechol, respectively, and then metabolizes the products by the β-ketoadipate pathway. UV spectroscopy showed that this strain could also metabolize veratrole (1,2-dimethoxybenzene). When grown in veratrole-containing media supplemented with 2,2'-dipyridyl to inhibit cleavage of the aromatic ring, S. setonii accumulated catechol, which was detected by both liquid chromatography and gas chromatography. Reduced cell extracts from veratrole-grown cultures, but not sodium succinate-grown cultures, produced a carbon monoxide difference spectrum with a peak at 450 nm that indicated the presence of soluble cytochrome P-450. Addition of veratrole or guaiacol to oxidized cell extracts from veratrole-grown cultures produced difference spectra that indicated that these compounds were substrates for cytochrome P-450. My results suggest that S. setonii produces a cytochrome P-450 that is involved in the demethylation of veratrole and guaiacol to catechol, which is then catabolized by the β-ketoadipate pathway.

Veratrole (1,2-dimethoxybenzene) is a xenobiotic cell depressant that reduces flagellar movement and cytoplasmic streaming in green algae (30) and inhibits glucose oxidation in fat cells (26). Kawakami (16) listed veratrole among the aromatic compounds metabolized by Pseudomonas ovalis; Kaiser and Hanselmann (15) found a mixed culture of anaerobic bacteria (20) that metabolized it to catechol. Veratrole is metabolized by several species of fungi in the genus Fusarium (13) and serves as a substrate for the liginase of the white-rot fungus Phanerochaete chrysosporium (17).

The lignin-degrading actinomycete Streptomyces setonii 75Vi2 metabolizes several aromatic compounds, including benzoic acid, catechol, gentisic acid, guaiacol, m-hydroxybenzoic acid, p-hydroxybenzoic acid, protocatechuic acid, and vanillic acid (21); phenol (1); benzaldehyde, cinnamic acid, p-coumaric acid, ferulic acid, p-hydroxybenzaldehyde, and vanillin (32); and homogentisic acid, p-hydroxyphenylacetic acid, p-hydroxyphenylpyruvic acid, phenylacetic acid, l-phenylalanine, phenylpyruvic acid, and l-tyrosine (30). Demethylation processes are involved in the metabolism of guaiacol (21) and of vanillic acid in cultures that have been grown with ferulic acid (32).

In this paper I report the ability of S. setonii to metabolize veratrole by converting it to guaiacol. Additional experiments were performed to determine whether veratrole was demethylated by an iron-sulfur protein of the putidaramonooxidase type, as in the metabolism of p-anisic acid by Pseudomonas putida (3); a cytochrome P-450, as in the metabolism of isovanillic acid by Nocardia sp. (5) and guaiacol by Moraxella sp. (10); or an H2O2-dependent liginase, as in the metabolism of lignin by Phanerochaete chrysosporium (12, 17).

MATERIALS AND METHODS

Stock cultures of S. setonii 75Vi2 (= ATCC 39116) were maintained on slants of glycerol-asparagine agar (22).

For growth studies, spores from agar slant cultures were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of a liquid medium. This medium contained (per liter of distilled water) 4.5 g of Na2HPO4 · 7H2O, 3.0 g of (NH4)2SO4, 1.0 g of KH2PO4, 500 mg of yeast extract, 500 mg of veratrole (3.6 mM), 200 mg of NaCl, 200 mg of MgSO4 · 7H2O, 50 mg of CaCl2 · 2H2O, and 50 mg of Tween 80 (polyoxyethylene sorbitan monooleate). The pH was adjusted to 7.4. Flasks were inoculated and incubated with reciprocal shaking for 48 h at 37°C. Aromatic compounds in spent culture media were measured by UV spectroscopy, using a Varian Techtron 635 spectrophotometer. Veratrole was omitted in some experiments; in others, it was replaced by 3.6 mM guaiacol or sodium succinate.

To detect intermediate products of veratrole metabolism, the veratrole-containing medium was modified by adding 0.5 mM 2,2'-dipyridyl to inhibit catechol 1,2-dioxigenase (9, 32). Catabolic intermediates were detected by reversed-phase high-performance liquid chromatography with a Varian model 5020 liquid chromatograph. The column was a Varian MicroPak MCH-10 column (30 cm by 4 mm) operated at 26°C. The mobile phase contained 12.5 mM KH2PO4 (adjusted to pH 3.0 with perchloric acid) and acetonitrile (75:25, vol/vol). The flow rate was 1.5 ml/min, and the UV detector was set at 270 nm. Gas chromatography was performed with a Hewlett-Packard model 5840A gas chromatograph fitted with a type DB-5 fused-silica capillary column (30 m; wide bore; film thickness, 0.25 μm) obtained from J & W Scientific. The carrier gas was H2 at a pressure of 34 kPa, and the make-up gas was N2 at a flow rate of 30 ml/min. The injector and flame ionization detector temperatures were 175 and 275°C, respectively.

Induction of cytochrome P-450 was demonstrated in cultures grown in media containing 3.6 mM veratrole, guaiacol, or sodium succinate. Mycelia were harvested by low-speed centrifugation, washed in 114 mM KCl, and disrupted by grinding with alumina (type 305; Sigma Chemical Co.) (18). The extract was suspended in 24 mM Na3HPO4-KH2PO4 buffer (pH 7.4) and centrifuged at 25,000 x g for 20 min. The supernatant was centrifuged again at 105,000 x g for 2 h (29) in a Beckman model L-8-55 ultracentrifuge equipped with a type SW-55TI rotor and then used to measure difference spectra. Protein was determined by the method of Bradford (4).

Difference spectra were obtained with a Hewlett-Packard model 8451A diode array spectrophotometer. Carbon mon-
oxide difference spectra (6, 7) were obtained after sodium dithionite reduction of cell extracts from 48-h cultures grown with veratrole, guaiacol, or sodium succinate. Aerated cell extracts from 48-h veratrole-grown cultures were used to obtain the difference spectra resulting from the addition of 1.35 mM veratrole or guaiacol (10, 28). Difference spectra were also obtained with 0.55 mM metyrapone (2-ethyl-1,2-di-3-pyridyl-1-propanone), an inhibitor of cytochrome P-450 in bacteria (19). Spectral changes were classified as type I or II by the wavelengths of the absorbance peaks and troughs (14).

**RESULTS**

Catabolism of veratrole by *S. setonii* was demonstrated by measuring the UV absorption spectra of inoculated and noninoculated liquid media before and after incubation for 3 days. Noninoculated media containing veratrole had UV absorption peaks at 222 and 272 nm; inoculated media lacked these peaks, producing spectra that were nearly identical to the spectrum of a culture medium containing yeast extract as the only carbon source.

Both veratrole and catechol were identified by high-performance liquid chromatography and gas chromatography in spent culture media after the growth of *S. setonii* on veratrole for 3 days in the presence of 2,2'-dipyridyl. The high-performance liquid chromatography retention times for veratrole and catechol were 12.84 and 3.14 min, respectively; the gas chromatography retention times were 3.66 and 4.06 min, respectively. Guaiacol, the presumed intermediate, was not positively identified in the media.

Cell extracts from cultures grown for 48 h with veratrole, after reduction with sodium dithionite, produced a carbon monoxide difference spectrum with an absorbance peak at 450 nm that indicated the presence of soluble cytochrome P-450 (Fig. 1). Reduced cell extracts from cultures grown with guaiacol produced a CO difference spectrum (data not shown) that also indicated the presence of cytochrome P-450, but cell extracts from cultures grown with sodium succinate produced a spectrum without an absorbance peak at 450 nm (Fig. 1).

The difference spectrum produced by the addition of veratrole to aerated cell cultures of extracts that had been grown on veratrole for 48 h showed a type I interaction with a peak at 394 nm and a trough at 424 nm (Fig. 2). Guaiacol, when added to extracts of veratrole-grown cultures, also produced a difference spectrum showing a type I interaction. The peak was at 390 nm, and the trough was at 418 nm (data not shown). However, the cytochrome P-450 inhibitor metyrapone produced a difference spectrum showing a type II interaction with a peak at 426 nm and a trough at 392 nm (Fig. 2).

**DISCUSSION**

The conversion of veratrole to catechol by *S. setonii*, presumably via guaiacol, makes further metabolism possible via the β-ketoadipate pathway (21). It seems likely that guaiacol is demethylated to catechol as fast as it is produced. Figure 3 shows the steps involved in the transformation of veratrole to the aliphatic product cis,cis-muconate.

Other methoxylated compounds that are O-demethylated by various species of *Streptomyces* include aconitine (2), anisole (27), 10,11-dimethoxyaporphine (25), gauicine (11), papaverine (24), and veratrane (31). Vanillate may be either demethylated or decarboxylated by *S. setonii*, depending on the original growth substrate (21, 32). However, veratrane does not serve as a growth substrate for this organism (21). It is not known whether one or more distinct monoxygenases are involved in demethylation reactions in the streptomycetes; some bacterial O-demethylases are fairly specific for methoxyl groups at particular positions on aromatic rings (11, 23, 25).

Cytochrome P-450 has been reported to be associated with a specific hydroxylase activity in *Streptomyces erythraeus* (8). The type I spectral changes that were observed in the difference spectra of aerated cell extracts of *S. setonii* after the addition of veratrole or guaiacol suggest that both veratrole and guaiacol are substrates for the demethylase activity of cytochrome P-450 (14). The type II spectral change observed after the addition of metyrapone is similar to that observed after the addition of metyrapone to the cytochrome P-450 of *Moraxella* sp. (10).

Thus, in *S. setonii* 75V12, veratrole and guaiacol induce the production of soluble cytochrome P-450, which appears

![FIG. 3. Pathway of veratrole catabolism by *S. setonii*.](http://aem.asm.org/content/52/9/99)
to be involved in the demethylation of these methoxylated aromatic compounds.

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LITERATURE CITED