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Characterization of a CO-Dependent O-Demethylating Enzyme System from the Acetogen *Clostridium thermoaceticum*

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An inducible O-demethylating enzyme system was characterized from *Clostridium thermoaceticum* cultivated at the expense of syringate. Glucose and methanol, but not CO, partially repressed its expression. Induced whole cells catalyzed the carbon monoxide (CO)-dependent O demethylation of methoxylated aromatic compounds with the concomitant formation of acetate. Pyruvate and, to a lesser extent, H_2-CO_2 could replace CO in these reactions. KCN inhibited pyruvate-dependent activity but not the CO-dependent activity. The ATPase inhibitor *N,N'*-dicyclohexylcarbodiimide, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone, and methyl viologen did not appreciably inhibit O demethylation by induced cells, whereas Triton X-100 was inhibitory. The enzyme system appeared to convert syringate sequentially to 5-hydroxyvanillate and gallate. The proposed overall reaction stoichiometry was as follows: syringate + 2CO + $2H_2O$ → gallate + 2 acetates. Growth-supportive methoxylated aromatic compounds were O demethylated by syringate-cultivated cells and inhibitory to syringate O demethylation.

Clostridium thermoaceticum, originally isolated from horse manure (8) and a likely inhabitant of anaerobic soils and sediments, has been used to elucidate the acetogenic (Wood) pathway of autotrophic metabolism (11, 16, 21). Given the origin of the original isolate, it seemed likely that this acetogen is capable of utilizing lignin-derived compounds for energy and growth. In this regard, we recently demonstrated that *C. thermoaceticum* is competent in growth and acetogenesis from methoxylated aromatic compounds, and that such compounds may exert regulatory effects on protein expression (3a).

Acetogenesis from methoxylated aromatic compounds would require the formation of two C_1 precursors for acetate formation. One C_1 precursor would be required at the CO level, and the other would be required at the methyl level (origins of the carboxyl and methyl positions of acetate, respectively). Since the methyl carbon of acetate is essentially preformed when methoxylated aromatic compounds are available, acetogenesis under these conditions might only require an O-demethylation-transfer reaction in concert with carbonylation by CO and the formation of acetyl coenzyme A by acetogenic acetyl coenzyme A synthetase (CO dehydrogenase) (21). Enzymological studies from heterotrophically grown *C. thermoaceticum* have shown that pyruvate, CO, and H_2-CO_2 can generate the CO-level carbon requisite to acetogenesis (22), and pyruvate and CO have been found to stimulate O demethylation of vanillate by extracts of *Acetobacterium woodii* (A. C. Frazer and L. Y. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K186, p. 233; A. C. Frazer, personal communication).

Utilizing CO as the CO donor, this study outlines the characterization of an O-demethylating enzyme system of *C. thermoaceticum* which (i) is induced by methoxylated aromatic compounds, (ii) is responsible for the O demethylation of syringate according to the reaction outlined in Fig. 1, and (iii) is likely involved in acetogenesis from a variety of growth supportive methoxylated aromatic compounds.

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MATERIALS AND METHODS

Organism and cultivation. *C. thermoaceticum* ATCC 39073 was cultivated in undefined medium as previously outlined (3a). Unless otherwise indicated, cells were cultivated in undefined medium with 10 mM syringic acid and a 100% CO_2 gas phase (at atmospheric pressure). Additional subtrates used for cultivation were glucose (55 mM), methanol (250 mM), and CO or H_2 (103 kPa [10 lb/in^2] over atmospheric pressure).

O-demethylase assay. Assays were performed anaerobically at 55°C in 25-ml crimp-sealed vials, and all cell preparations were performed in a Coy anaerobic chamber (N_2-H_2, 95:5). The standard assay volume was 2 ml, and the assay buffer contained 75 mM sodium phosphate buffer (pH 7.0) and reducer (0.025% $Na_2S \cdot 9H_2O$, 0.025% L-cysteine hydrochloride [monohydrate], 0.016% NaOH; reducer was shown to not be essential to activity but was added to minimize effects of oxidation). Cells (which had been harvested by centrifugation, stored frozen at −20°C, and thawed before the assay; activity was not appreciably affected by this treatment) were suspended in assay buffer and dispensed into assay vials (10 mg [dry weight] of cells per assay, equivalent to a final optical density of approximately 11 at 660 nm), which were subsequently crimp sealed and flushed with 100% CO (or other gas phase as indicated). The final concentration of CO approximated 940 μmol per assay (approximately 1.2 μmol of dissolved CO at 55°C [15]). Assays were initiated by injection of syringic acid (or alternative methoxylated aromatic compound) to a final concentration of 0.58 mM (unless otherwise indicated). Activity was measured by quantitating the O demethylation (consumption) of syringic acid (or alternative methoxylated aromatic compound). The standard assay time was 5 min, and one unit of activity is equivalent to one nanomole of substrate consumed per minute per milligram (dry weight) of cells.

Analytical methods. Methoxylated aromatic compounds were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Aldrich Chemical Co., Inc. (Milwaukee, Wis.), and stock solutions were prepared in either 80% ethanol or distilled, deionized water (NaOH neutralized [3a]). Growth

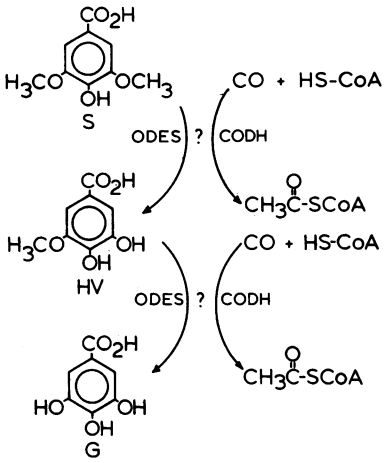

FIG. 1. Proposed reaction sequence for the conversion of syringate (S) to gallate (G). HV, 5-Hydroxyvanillate; HS-CoA, coenzyme A; CODH, CO dehydrogenase (acetyl coenzyme A synthetase); ODES, O-demethylating enzyme system.

was monitored at 660 nm with a Spectronic 501 (Bausch & Lomb, Inc., Rochester, N.Y.) spectrophotometer, and cell dry weights were determined as previously described (19). Aromatic compounds were analyzed by high-performance liquid chromatography with a Hewlett-Packard Co. (Palo Alto, Calif.) 1090L high-performance liquid chromatograph equipped with a Bio-Rad Laboratories (Richmond, Calif.) Fermentation monitoring column and a Spectra-Physics (Bedford, Mass.) 4290 integrator. High-performance liquid chromatography was at 60°C; the mobile phase was 10% acetonitrile at 0.5 ml/min. The sample size was 1 μl, and the mobile phase was monitored at 210 nm. In addition, aromatic products observed by chromatography were qualitatively verified by UV-absorption spectral analysis with a Gilford Systems (Oberlin, Ohio) 2600 recording spectrophotometer. Acetate was quantitated by high-performance liquid chromatography as previously outlined (3). Protein was estimated by the Bradford procedure (2). Slab sodium dodecyl sulfate-polyacrylamide gel electrophoresis was with the Laemmli buffer system (14), and cell extracts for electrophoretic analysis were prepared by lysozyme digestion as previously described (17).

RESULTS

Expression of O-demethylating enzyme system. Cells cultivated at the expense of methoxylated aromatic compounds contained a predominant protein (molecular weight approximating 22,000; data not shown), which was negligible from cells cultivated at the expense of other substrates (Fig. 2). Cells cultivated at the expense of syringate catalyzed the rapid CO-dependent O demethylation of syringate, whereas cells cultivated at the expense of other substrates displayed lower activity (Table 1). Glucose and methanol appeared to repress the levels of activity in glucose-syringate and methanol-syringate cultures, respectively.

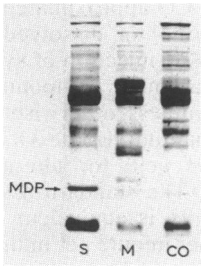

FIG. 2. Comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles. Cells were cultivated on syringate (S) (similar to gel profiles from cells cultivated with other methoxylated aromatic compounds), methanol (M), and CO (CO) (similar to profile from glucose cells). MDP, Methoxylated aromatic compound-dependent protein. Each lane contained 10 μg of protein.

TABLE 1. Expression of the O-demethylating enzyme system

Growth substrate	U
Glucose	0.0
Methanol	2.6
CO	2.1
Syringate	11.6
Glucose-syringate	1.4
Methanol-syringate	3.5
CO-syringate	10.3
CO-gallate[a]	1.6

[a] Gallate alone does not support growth.

Optimum conditions for *O*-demethylase activity. Under standard assay conditions, the pH and temperature optima approximated 6.4 and 80°C, respectively (data not shown). However, to more closely approximate the standard growth conditions of *C. thermoaceticum*, pH 7 and 55°C were utilized to further characterize the whole-cell activity. The activity was linear until approximately 80% of the syringate was consumed (8 to 10 min) and, over the range of 0.2 to 2.5 mM syringate (with a 100% CO gas phase), followed Michaelis-Menten kinetics (apparent K_m and V_{max} values approximated 0.5 mM syringate and 28.6 nmol of syringate consumed per min per mg [dry weight] of cells, respectively). Specific activities increased approximately twofold with crude cell extracts derived from lysozyme-digested cells.

CO requirement. Either CO or pyruvate was essential to activity; marginal activity was observed with H_2-CO_2 (Table 2). This finding is consistent with the concept that a suitable CO donor is required for O demethylation.

Effect of KCN and other inhibitors. CO dehydrogenase is inhibited by KCN but is protected from KCN inhibition by CO (5, 18). KCN did not appreciably inhibit CO-dependent activity but nearly totally inhibited the pyruvate-dependent reaction (Table 3). Other metabolic inhibitors and uncou-

TABLE 2. Substrate effects on O demethylation of syringate

Assay system[a]	Units
Syringate-CO (control)	11.6
Syringate-N_2	0.5
Syringate-H_2	<0.2
Syringate-CO_2	0.5
Syringate-H_2-CO_2[b]	1.4
Syringate-CO[c]	4.2
Syringate-pyruvate (10 mM)[d]	13.5
Syringate-glucose (10 mM)[d]	0
Syringate-formate (10 mM)[d]	0

[a] Unless otherwise indicated, gas phases were 100% at atmospheric pressure.

[b] One hundred percent N_2 at atmospheric pressure with an additional 62 kPa (6 lb/in^2) of H_2 and 62 kPa of CO_2 overpressure.

[c] Cells were exposed to air for 5 min before the assay.

[d] Assay gas phase was 100% N_2.

TABLE 3. Effects of inhibitors on O demethylation of syringate

Assay system	U
Syringate-CO (control)	13.0
Syringate-CO-KCN (0.5 mM)	11.1
Syringate-pyruvate (10 mM)	12.3
Syringate-pyruvate-KCN (0.5 mM)	0.7
Syringate-CO-DCCD[a] (0.8 mM)[b]	15.5
Syringate-CO-CCCP[c] (0.8 mM)[b]	13.4
Syringate-CO-MeV[d] (0.05 mM)[b]	12.8
Syringate-CO-$CuCl_2$ (0.05 mM)[b]	14.0
Syringate–CO–Triton X-100 (1%)	0.2
Syringate–CO–Triton X-100 (0.5%)	1.2

[a] DCCD, *N,N'*-Dicyclohexylcarbodiimide.
[b] Growth-inhibitory concentration.
[c] CCCP, Carbonyl cyanide *m*-chlorophenylhydrazone.
[d] MeV, Methyl viologen.

plers were not inhibitory. However, Triton X-100 strongly inhibited O demethylation.

Syringate-derived products during growth and *O*-demethylase assays. When *C. thermoaceticum* was cultivated at the expense of syringate, gallate was the major aromatic end product; only minor levels of 5-hydroxyvanillate were detected during growth (Fig. 3). The overall specific activity for syringate consumption during growth approximated 11.5 nmol of syringate consumed per min per mg (dry weight) of cells.

In standard enzyme assays with syringate-cultivated cells, gallate was essentially the sole aromatic product detected; however, at pH 4.8 and 8 (nonoptimal pHs), 5-hydroxyvanillate was the major aromatic product detected (data not shown). To study the syringate-derived aromatic product profile in more detail, the syringate concentration was increased fivefold and the velocity of the enzyme system was impaired by lowering the assay temperature to 37°C. Under these conditions, significant levels of 5-hydroxyvanillate were formed at pH 7; at nonoptimal pHs, the sole product detected (after 28 h) from syringate was 5-hydroxyvanillate (data not shown).

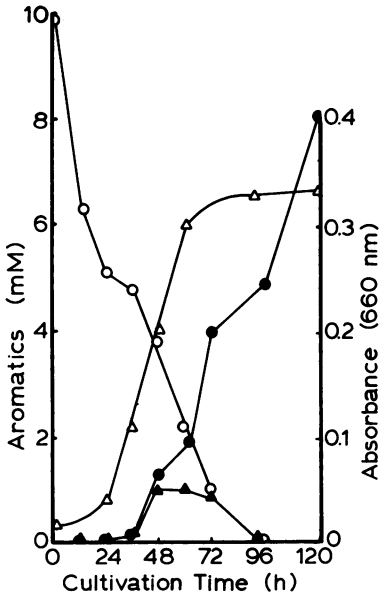

FIG. 3. Growth and product profiles of syringate-cultivated *C. thermoaceticum*. Symbols: △, growth; ○, syringate; ●, gallate; ▲, 5-hydroxyvanillate.

TABLE 4. Capacity of syringate-cultivated cells to O demethylate various O-methoxylated aromatic compounds

O-Methoxylated compound[a]	O demethylation (% of control)
Syringate[b] (control)	100
Vanillate[b]	179
5-Hydroxyvanillate[b]	43
2-Methoxybenzoate[b]	46
1,2-Dimethoxybenzene[b]	38
1,3-Dimethoxybenzene[b]	189
1,4-Dimethoxybenzene[b]	124
1,2,3-Trimethoxybenzene[b]	89
2-Methoxyphenol[b]	100
3,4-Dimethoxybenzoate[c]	0
3,4,5-Trimethoxybenzoate[c]	0

[a] Gallate was nonreactive in assays and is not growth supportive.
[b] Growth supportive.
[c] Not growth supportive.

The syringate concentration was increased 10-fold (5 mM) to yield levels of acetate that could be quantitated. Under these conditions, 28.5 mM acetate was detected after syringate had been consumed (1 to 2 h), yielding a final acetate formed/*O*-methyl consumed ratio of 2.85. Since the theoretical ratio with CO as the CO donor is 1 (aromatic OCH_3 + CO + H_2O → aromatic OH + CH_3CO_2H), the excess acetate most likely was produced from CO-dependent acetogenesis ($4CO + 2H_2O \rightarrow CH_3CO_2H + 2CO_2$). When 10 mM pyruvate was used instead of CO as the CO donor in such assays, the acetate formed/*O*-methyl consumed ratio approximated 1.85, and the acetate formed/syringate consumed ratio approximated 3.7, values very close to the theoretical values of 2 and 4, respectively, for the pyruvate-coupled reaction (syringate + 2 pyruvates + $2H_2O$ → gallate + 4 acetates).

Specificity. Methoxylated aromatic compounds which support the growth of *C. thermoaceticum* were O demethylated by syringate-cultivated cells in standard *O*-demethylase assays (Table 4). In addition, when O demethylation of syringate was assessed in the presence of these compounds, the extent of O demethylation of syringate decreased. For example, with 0.58 mM each of syringate and 5-hydroxyvanillate, syringate O demethylation decreased by approximately 38%; however, the amount of gallate formed (0.37 mM) was the same as with syringate alone.

DISCUSSION

The proposed overall O-demethylating acetogenic reaction for syringate-dependent activity is illustrated in Fig. 1. A methoxy group from a suitable methoxylated aromatic compound serves as the methyl donor in the formation of acetyl coenzyme A, which would subsequently be converted to either biomass or acetate. O demethylation requires a CO donor; CO, pyruvate, and, to a lesser extent, H_2-CO_2 can serve in this capacity. Acetyl coenzyme A synthetase is likely involved in the overall reaction sequence since (i) CO is required, (ii) acetate is formed, and (iii) KCN selectively inhibits the pyruvate reaction (but not the CO reaction) (5, 18). When cells are cultivated at the sole expense of methoxylated aromatic compounds (i.e., in the absence of CO), the CO donor would likely be derived via reduction of CO_2.

Under optimal conditions, 5-hydroxyvanillate was not detected in appreciable quantities. This finding raises the question as to whether 5-hydroxyvanillate is released from the O-demethylating enzyme system before O demethylation

of the second methoxy group of syringate. Although a greater affinity for 5-hydroxyvanillate would possibly explain the observed results, 5-hydroxyvanillate did not appear to have any greater capacity to react than did syringate. Differential permeabilities of the cell to these compounds may also be responsible for these observations. In contrast, syringate-dependent growth of *Clostridium formicoaceticum* yields high levels of 5-hydroxyvanillate before the formation of gallate (M. F. Bryson and H. L. Drake, unpublished data).

Numerous acetogenic bacteria have been shown to utilize methoxylated aromatic compounds. These include *Acetobacterium carbinolicum* (6), *Acetobacterium malicum* (20), *A. woodii* (1), *Acetobacterium* sp. (7), *C. formicoaceticum* (M. F. Bryson and H. L. Drake, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, I107, p. 198), *C. thermoaceticum* (3a), *Clostridium thermoautotrophicum* (3a), *Eubacterium limosum* (12), *Syntrophococcus sucromutans* (13), TH-001 (9), and isolates (*Sporomusa termitida*) from the termite gut (2a). With strain TH-001 grown at the expense of *O*-[*methyl*-^{14}C]vanillate, the methoxy carbon of vanillate was preferentially incorporated into the methyl position of acetate (10). This observation is consistent with the proposed reaction sequence in Fig. 1. In addition, experiments with 3[*methoxy*-^{18}O]anisate (3-methoxybenzoate) demonstrated that *E. limosum* and *A. woodii* utilize (cleave) the methyl rather than methoxy group from methoxylated aromatic compounds (4). As in the case of *C. thermoaceticum*, the capacity of *E. limosum*, *A. woodii*, and TH-001 to utilize these compounds is inducible (4, 22), and the enzyme system(s) of *E. limosum* and *A. woodii* responsible for 3-anisate O demethylation displays broad specificity (4).

O demethylation was not affected by metabolic inhibitors, suggesting that O demethylation may not be strictly dependent upon growth or the initial energy state of the cell. Determining how the O-demethylating enzyme system is integrated to the Wood pathway of acetogenesis will require further analysis and purification of the enzyme system.

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