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# UTILIZATION OF METHOXYLATED AROMATIC COMPOUNDS BY THE ACETOGEN *CLOSTRIDIUM THERMOACETICUM*: EXPRESSION AND SPECIFICITY OF THE CO-DEPENDENT O-DEMETHYLATING ACTIVITY

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SUMMARY: The aromatic CO-dependent O-demethylating activity of *Clostridium thermoaceticum* was evaluated. Secondary aromatic substituent groups (-OH, $-CO_2H$, $-CH_2OH$, and $-OCH_3$) were critical to O demethylation. O-demethylating activities and specificities were similar from cells grown at the expense of different methoxylated aromatic compounds; all *O*-methyl-grown cells catalyzed the same sequential O demethylation of multi-methoxylated compounds, suggesting that a broad specificity O demethylase was involved in O demethylation. In cell-fractionation studies, CO-dependent O demethylation was catalyzed by membrane-associated components.


Aromatic compounds are often present as basic components of natural and xenobiotic substances found in the environment. Recent studies with acetogenic bacteria indicate that these anaerobes are involved in the transformation of aromatic compounds in anoxic habitats (1-4). In this regard, *C. thermoaceticum* is competent in the O demethylation of methoxylated aromatic compounds (5), and an inducible, carbon monoxide (CO)-dependent O-demethylating activity that is likely involved in the integration of *O*-methyl groups into the acetyl-CoA (Wood) pathway (6,7) has been demonstrated (8). The purposes of this study were to further (i) resolve the capacity of *C. thermoaceticum* to utilize methoxylated aromatic compounds and (ii) characterize the expression and specificity of the O-demethylating activity.

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## MATERIALS AND METHODS

Organisms and cultivation. *C. thermoaceticum* ATCC 39073 and strain OMD were cultivated at 55°C in an undefined medium in butyl rubber-stoppered aluminum crimp seal culture tubes or serum bottles as previously described (5,8,9). The defined medium was the undefined medium without yeast extract, with nicotinic acid as the sole vitamin, and with sulfide as the sole reducing agent. Unless noted otherwise, stock solutions of methoxylated aromatic compounds were prepared in 80% ethanol, filter sterilized, degassed ($N_2$), and added via syringe at concentrations indicated.

Analytical methods. Substrates and end products were quantitated by UV (210 nm) and refractive index detection with a Hewlett Packard 1090L high performance liquid chromatograph as previously described (8,9). Cell dry weights were determined as previously described (9); an optical density of 1 at 660 nm approximated 0.45 g (cell dry weight)/liter. Growth was monitored at 660 nm with a Bausch and Lomb Spectronic 501. Protein was quantitated by the modified Lowry method (10).

Cell preparation and assay for CO-dependent O-demethylating activity. Cells grown at the expense of a methoxylated aromatic compound were harvested by centrifugation, washed in buffer (70 mM phosphate buffer [pH 7] with [0.025% $Na_2S \cdot 9H_2O$, 0.025% L-cysteine$\cdot$HCl$\cdot H_2O$, 0.016% NaOH]), repelleted, and resuspended in buffer (8). Assays for O-demethylating activity were performed with whole cells, with cells disrupted by a French press, or with cell fractions (see table 4 for cell-fraction preparation). Assays were performed anaerobically (100% CO gas phase) as described previously (8), and all assay manipulations were performed in an anaerobic chamber ($N_2$-$H_2$, 95:5). One unit of activity was defined as one nanomole of methoxylated aromatic compound consumed per min per mg of protein.

## RESULTS AND DISCUSSION

Specificity of *O*-methyl utilization. Of the forty-two methoxylated aromatic compounds tested, twenty supported the growth of *C. thermoaceticum* ATCC 39073 (Table 1). Secondary substituent groups (-OH, -COOH, -$CH_2OH$, and -$OCH_3$) and their position on the aromatic ring were crucial to *O*-methyl utilization and growth. Methoxylated compounds that did not support growth included: *p*-hydroxyanisole; anisole; 3-methoxybenzoic acid; 4-methoxybenzoic acid; 3,4-dimethoxybenzoic acid; 3,4,5-trimethoxybenzoic acid; 3-methoxysalicylic acid; 2-methoxycinnamic acid; 3-methoxycinnamic acid; 2-methoxyacetophenone; *o*-anisamide; *o*-methylanisole; *o*-anisaldehyde; 2-methoxybenzonitrile; *o*-anisidine; 2-methoxybenzenethiol; 2-methoxyphenylacetic acid; 3(2-methoxyphenyl)propionic acid; 3-methoxybenzyl alcohol; 4-methoxybenzyl alcohol; *o*-methoxyphenethyl alcohol; and *o*-vanillin.

Biomass, acetate, and the hydroxylated form of the O-demethylated aromatic compound were the major products of *O*-methyl-dependent growth; the aromatic ring was not degraded (Tables 1 and 2). In addition to O demethylation, secondary substituent groups on vanillic acid, isovanillic acid, and vanillin were also transformed. Vanillic and isovanillic acids were decarboxylated. These observations support earlier

Table 1. Methoxylated aromatic compounds that supported the growth of *C. thermoaceticum* ATCC 39073

| Substrate[a] | Doubling time (h) | Cell yield (dry wt [g per liter]) | Aromatic end product |
|---|---|---|---|
| 1,2-dimethoxybenzene | 11.5 | 0.054 | catechol |
| 1,3-dimethoxybenzene | 20.8 | 0.055 | resorcinol |
| 1,4-dimethoxybenzene | 18.0 | 0.036 | hydroquinone |
| 1,2,3-trimethoxybenzene | 11.0 | 0.095 | 1,2,3-trihydroxybenzene |
| 2-methoxyphenol | 24.5 | 0.052 | catechol |
| 3-methoxyphenol | 27.3 | 0.053 | resorcinol |
| 2,3-dimethoxyphenol | 13.0 | 0.065 | 1,2,3-trihydroxybenzene |
| 2,6-dimethoxyphenol | 21.3 | 0.067 | 1,2,3-trihydroxybenzene |
| 3-methoxycatechol | 15.3 | 0.047 | 1,2,3-trihydroxybenzene |
| 2-methoxybenzoic acid | 21.8 | 0.038 | salicylic acid |
| 2,3-dimethoxybenzoic acid | 20.5 | 0.037 | 3-methoxysalicylic acid |
| Vanillic acid | 11.5 | 0.049 | catechol |
| Isovanillic acid | 7.0 | 0.036 | catechol |
| 5-hydroxyvanillic acid | 25.8 | 0.032 | gallic acid |
| Syringic acid | 20.0 | 0.069 | gallic acid |
| Vanillin | 21.3 | 0.030 | catechol |
| 2-methoxybenzyl alcohol | 19.3 | 0.034 | 2-hydroxybenzyl alcohol |
| 4-hydroxy-3-methoxybenzyl alcohol | 17.3 | 0.022 | -[b] |
| Ferulic acid | 10.0 | 0.043 | caffeic acid |
| Sinapic acid | 15.0 | 0.054 | - |

[a] Final concentration of substrates was 5 mM in the undefined medium.
[b] -, Not determined.

studies in which *C. thermoaceticum* was competent in the transformation of carboxylated aromatic compounds (11) and that an inducible aromatic-dependent decarboxylase was responsible for this activity (12). Vanillin was subject to two transformations, O demethylation and the removal of the aromatic aldehyde group; aldehyde removal likely occurs via sequential oxidation and decarboxylation (13).

With most compounds, growth was generally proportional to the number of utilizable aromatic methoxyl groups (Table 1). However, cell yields often differed between different aromatic compounds with the same number of *O*-methyl residues (e.g., 1,3-dimethoxybenzene and 1,4-dimethoxybenzene). This suggests that (i) the energy conserved from O demethylation differs among methoxylated aromatic compounds, or (ii) some methoxylated aromatic compounds or subsequent products are inhibitory to growth. The molar ratio with vanillate- and syringate-grown cells was relatively the same (Table 2). Interestingly, the molar ratio for methanol-grown cells was similar to that of *O*-methyl-grown cells. In studies with the defined medium, nicotinic acid was the sole vitamin required for *O*-methyl-dependent growth.

Table 2. Acetate production by *C. thermoaceticum* ATCC 39073 grown at the expense of methanol and methoxylated aromatic compounds

| Substrate[a] | Substrate concn (mM)[b] | Methoxyl group consumed (mM) | Acetate produced (mM) | Molar ratio (amount acetate produced per *O*-methyl group) |
|---|---|---|---|---|
| Methanol | 60 | 60 | 35.0 | 0.58 |
| Vanillate | 5 | 5 | 3.2 | 0.64 |
| | 10 | 10 | 4.8 | 0.48 |
| | 15 | 15 | 8.0 | 0.53 |
| Syringate | 5 | 10 | 4.8 | 0.48 |
| | 10 | 20 | 12.8 | 0.64 |
| | 15 | 30 | 19.2 | 0.64 |

[a] NaOH-neutralized solutions of vanillate and syringate were used.
[b] Initial substrate concentrations in the undefined medium. All substrates were used to completion.

In contrast to *C. thermoaceticum* ATCC 39073, *C. thermoaceticum* strain OMD (not capable of autotrophic growth [9]) failed to grow at the expense of either vanillic or syringic acid in the undefined medium. The inability of strain OMD to grow at the expense of methoxylated aromatic compounds is apparently not due to a lack of CO dehydrogenase (14). Three possibilities may explain why strain OMD is not competent in *O*-methyl-dependent growth: (i) a functional O demethylase is absent; (ii) the O demethylation of methoxylated aromatic compounds is not efficiently coupled to energy conservation or anabolic processes; or (iii) the acetyl-CoA pathway is altered in a manner that prevents acetyl-CoA synthesis from *O*-methyl residues.

Expression and specificity of the CO-dependent O-demethylating activity. Syringate-cultivated cells of *C. thermoaceticum* are competent in the O demethylation of a wide variety of growth-supportive methoxylated aromatic substrates (8). However, the exact nature of this catalytic activity remains unresolved.

Cells were grown at the expense of syringic acid, 2,6-dimethoxyphenol, 1,2,3-trimethoxybenzene, or *O*-anisic acid, disrupted by French press, and then assayed for O-demethylating activity using each of these substrates. Disrupted cells were specifically used to eliminate transport-related influences on O-demethylating activities. Significantly, regardless of the growth substrate, disrupted cells (i) were capable of O demethylating all four of the substrates and (ii) displayed essentially the same specific activities for each of the four substrates. In addition, 3,4,5-trimethoxybenzoic acid (not growth supportive) was not O-demethylated by disrupted

Table 3. Capacity of 1,2,3-trimethoxybenzene-grown cells of *C. thermoaceticum* ATCC 39073 to O demethylate 3-methoxycatechol, 2,6-dimethoxyphenol, and 2,3-dimethoxyphenol: Potential intermediates in the sequential O demethylation of 1,2,3-trimethoxybenzene

| Assay no.[a] | Assay substrate(s)[b] | % O demethylation of assay substrate(s)[c] | Aromatic products |
|---|---|---|---|
| 1 | 1,2,3-trimethoxybenzene | 40.5 | 1,2,3-trihydroxybenzene |
| 2 | 3-methoxycatechol | 76.9 | 1,2,3-trihydroxybenzene |
| 3 | 2,6-dimethoxyphenol | 33.5 | 3-methoxycatechol<br>1,2,3-trihydroxybenzene |
| 4 | 2,3-dimethoxyphenol | 48.9 | 3-methoxycatechol<br>1,2,3-trihydroxybenzene |
| 5 | 1,2,3-trimethoxybenzene<br>2,6-dimethoxyphenol | 0<br>60.2 | 3-methoxycatechol<br>1,2,3-trihydroxybenzene |
| 6 | 1,2,3-trimethoxybenzene<br>3-methoxycatechol | 0<br>62.0 | 1,2,3-trihydroxybenzene |
| 7 | 1,2,3-trimethoxybenzene<br>2,3-dimethoxyphenol | 0<br>72.8 | 3-methoxycatechol |
| 8 | 2,3-dimethoxyphenol<br>2,6-dimethoxyphenol | 77.0<br>25.0 | 3-methoxycatechol |

[a] All experiments were conducted with cells grown in the undefined medium in serum bottles with 10 mM 1,2,3-trimethoxybenzene.
[b] Each assay substrate was at a final concentration of 0.5 mM.
[c] Based on an assay with whole cells at a final optical density of 5 at 660 nm and an assay time of 3 min.

cells, indicating that the cell's inability to grow at the expense of a methoxylated aromatic substrate was not due to the inability to transport the aromatic molecule.

When 1,2,3-trimethoxybenzene was present as the sole aromatic substrate in growth studies and in assays for O-demethylating activities, 1,2,3-trihydroxybenzene was the sole aromatic product (Tables 1 and 3). To determine the potential O demethylation sequence of this substrate, 1,2,3-trimethoxybenzene-grown cells were assayed with 1,2,3-trimethoxybenzene and potential intermediates, either alone or in combination (Table 3). Each potential intermediate was O demethylated by 1,2,3-trimethoxybenzene-grown cells. With combinations of 1,2,3-trimethoxybenzene and intermediates, the following order of reactivity (based on O-demethylating activities and end products) was observed: 1,2,3-trimethoxybenzene < 2,6-dimethoxybenzene < 2,3-dimethoxybenzene < 3-methoxycatechol. Thus, the reason no intermediate

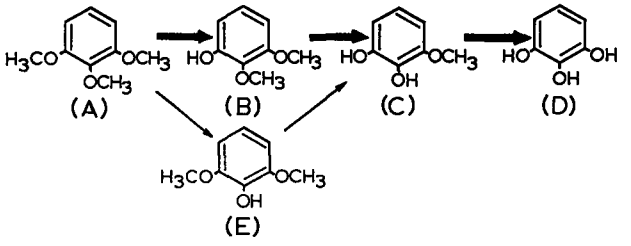


Figure 1. Proposed pathway for the sequential O demethylation of 1,2,3-trimethoxybenzene by *C. thermoaceticum* ATCC 39073. Compounds: A, 1,2,3-trimethoxybenzene; B, 2,3-dimethoxyphenol; C, 3-methoxycatechol; D, 1,2,3-trihydroxybenzene; and E, 2,6-dimethoxyphenol.

was observed during 1,2,3-trimethoxybenzene-dependent growth was because each of the intermediary products was more reactive than the previous compound. In assays (> 5 min) with 1,2,3-trimethoxybenzene and 2,3-dimethoxybenzene, 2,3-dimethoxyphenol was first converted to 3-methoxycatechol and then to 1,2,3-trihydroxybenzene; upon complete consumption of 3-methoxycatechol, 1,2,3-trimethoxybenzene was converted to 1,2,3-trihydroxybenzene. Overall, this information suggested that 1,2,3-trimethoxybenzene was metabolized by the pathway shown in Figure 1 (major pathway indicated by bold arrows).

Localization of the CO-dependent O-demethylating activity. In cell-fractionation experiments (Table 4), O-demethylating activities were higher in fractions containing particulate material (membranes). Since unbroken (whole) cells displayed the highest activity, it can be anticipated that the integrity of the native system is compromised

Table 4. CO-dependent O-demethylating activity by different cell fractions of *C. thermoaceticum* ATCC 39073[a]

| Cell fraction | O-demethylating activity[b] |
|---|---|
| Whole cells | 8.9 |
| Disrupted cells (French press) | 2.4 |
| Disrupted cells at 750 x *g* for 60 min | |
| Resuspended pellet | 5.4 |
| Supernatant (cell-free extract) | 1.3 |
| Supernatant at 48,200 x *g* for 30 min | |
| Resuspended pellet (membranes) | 6.0[c] |

[a] Cells were grown in serum bottles in the undefined medium containing 10 mM syringate (NaOH-neutralized) and were harvested during late-log phase of growth.
[b] Values represent the mean of two experiments.
[c] O-demethylating activity was not observed in the absence of CO.

by cell disruption. These results indicate that membranes or membrane-associated complexes contribute to the overall CO-dependent O-demethylating process.

Conclusion. Collectively, these studies suggest that a single, broad specificity O demethylase was responsible for the O-demethylating activity of *C. thermoaceticum*. This conclusion is supported by the fact that protein profiles, obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis, from cells cultivated at the expense of different methoxylated substrates are identical (5,8, unpublished data). Further studies will be required to determine whether the O demethylase of *C. thermoaceticum* is a membrane-bound or cytoplasmic enzyme and how this activity integrates $C_1$-units into the acetyl-CoA pathway. In this regard, it is interesting to note that certain components of the acetyl-CoA pathway are membrane associated (6,7) and that CO was required for membrane-mediated O demethylation (Table 4).

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