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Expression of an Aromatic-Dependent Decarboxylase Which Provides Growth-Essential CO_2 Equivalents for the Acetogenic (Wood) Pathway of *Clostridium thermoaceticum*

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The acetogen *Clostridium thermoaceticum* generates growth-essential CO_2 equivalents from carboxylated aromatic compounds (e.g., 4-hydroxybenzoate), and these CO_2 equivalents are likely integrated into the acetogenic pathway (T. Hsu, S. L. Daniel, M. F. Lux, and H. L. Drake, J. Bacteriol. 172:212–217, 1990). By using 4-hydroxybenzoate as a model substrate, an assay was developed to study the expression and activity of the decarboxylase involved in the activation of aromatic carboxyl groups. The aromatic-dependent decarboxylase was induced by carboxylated aromatic compounds in the early stages of growth and was not repressed by glucose or other acetogenic substrates; nonutilizable carboxylated aromatic compounds did not induce the decarboxylase. The decarboxylase activity displayed saturation kinetics at both whole-cell and cell extract levels, was sensitive to oxidation, and was not affected by exogenous energy sources. However, at the whole-cell level, metabolic inhibitors decreased the decarboxylase activity. Supplemental biotin or avidin did not significantly affect decarboxylation. The aromatic-dependent decarboxylase was specific for benzoates with a hydroxyl group in the *para* position of the aromatic ring; the *meta* position could be occupied by various substituent groups (-H, -OH, -OCH_3, -Cl, or -F). The carboxyl carbon from [carboxyl-^{14}C]vanillate went primarily to $^{14}CO_2$ in short-term decarboxylase assays. During growth, the aromatic carboxyl group went primarily to CO_2 under CO_2-enriched conditions. However, under CO_2-limited conditions, the aromatic carboxyl carbon went nearly totally to acetate, with equal distribution between the carboxyl and methyl carbons, thus demonstrating that acetate could be totally synthesized from aromatic carboxyl groups. In contrast, when cocultivated (i.e., supplemented) with CO under CO_2-limited conditions, the aromatic carboxyl group went primarily to the methyl carbon of acetate.

The acetyl coenzyme A (acetyl-CoA) or Wood pathway is an autotrophic process fundamental to acetogenic bacteria (12, 24, 39), a group of organisms that play important environmental roles in anaerobic habitats, including various gastrointestinal tract ecosystems (5, 23). However, since true autotrophic environments (i.e., environments that are totally free of heterotrophic substrates) seldom if ever exist in nature, acetogens are likely fused to environmental food chains by virtue of other diverse metabolic activities which are not strictly autotrophic. In this regard, recent findings suggest that acetogens are metabolically integrated in nature to the anaerobic biotransformation of lignin-derived aromatic compounds (3, 9, 42). Studies to date indicate that acetogens contribute to this process by integrating the following aromatic substituents into the flow of both carbon and energy during acetogenesis: methoxyl groups (2, 6–8, 11, 21), acrylate groups (14, 37), aldehydes (28), and carboxyl groups (16).

Although the anaerobic degradation of aromatic compounds is not well defined, recent studies have shown that carboxylation (13, 18, 19, 36) and decarboxylation (22, 32) reactions may play important initiating roles in the usage of aromatic compounds under anaerobic conditions. Given the potential for the acetogen *Clostridium thermoaceticum* to derive growth-essential CO_2 equivalents from carboxylated aromatic compounds, it has been proposed that some acetogens may initiate the anaerobic biotransformation of carboxylated aromatic compounds (16). To better understand the catalytic events central to the use of aromatic carboxyl groups by *C. thermoaceticum*, in this study, we evaluated the expression and activity of an aromatic-dependent decarboxylase that is specific for carboxyl groups derived from the structure shown in Fig. 1 (where R is a variable substituent group). In addition, the fate of this carboxyl group in acetogenesis was resolved by ^{14}C tracer studies.

MATERIALS AND METHODS

Cultivation of *C. thermoaceticum*. *C. thermoaceticum* ATCC 39073 (10) was cultivated in undefined medium at 55°C in crimp-sealed culture tubes (18 by 150 mm) (Bellco series 2048; Bellco Glass Inc., Vineland, N.J.). The undefined medium contained (in milligrams per liter) $NaHCO_3$ (5,000), KH_2PO_4 (500), NaCl (400), NH_4Cl (400), $MgCl \cdot 6H_2O$ (330), $CaCl_2 \cdot 2H_2O$ (50), resazurin (1), yeast extract (1,000), nicotinic acid (0.25), cyanocobalamin (0.25), *p*-aminobenzoic acid (0.25), calcium D-pantothenate (0.25), thiamine · HCl (0.25), riboflavin (0.25), lipoic acid (0.15), folic acid (0.1), biotin (0.1), pyridoxal · HCl (0.05), sodium nitrilotriacetate (7.5), $MnSO_4 \cdot H_2O$ (2.5), $FeSO_4 \cdot 7H_2O$ (0.5), $Co(NO_3)_2 \cdot 6H_2O$ (0.5), $ZnCl_2$ (0.5), $NiCl_2 \cdot 6H_2O$ (0.25), $CuSO_4 \cdot 5H_2O$ (0.05), $AlK(SO_4)_2 \cdot 12H_2O$ (0.05), H_3BO_3 (0.05), and $Na_2MoO_4 \cdot 2H_2O$ (0.05). Sodium sulfide-cysteine reducer (34) was added after the medium was boiled and cooled under CO_2; the medium was subsequently dispensed (7 ml per tube) under CO_2, and the tubes were crimp sealed and autoclaved. Glucose, methanol, and sodium formate were added at concentrations of 10, 60, and 100 mM, respectively, before autoclaving. For cultivation at the expense of CO or H_2, culture tubes were pressurized at room

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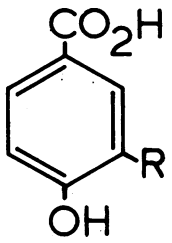

FIG. 1. Structure of the carboxylated aromatic compound (with R as the variable substituent group in the *meta* position) for use in decarboxylase assays.

temperature to 70 kPa (10 lb/in^2) over the atmospheric pressure (final total pressure, 171.3 kPa) with 100% CO or H_2 prior to incubation. After the medium was autoclaved, aromatic compounds were added (to an initial concentration of approximately 5 mM) by anaerobic injection. Growth was initiated by injecting 0.5 ml of inoculum per culture tube. The total volume of the culture tubes approximated 28 ml, and the ratio of the gas phase to the liquid phase approximated 3:1. When larger quantities of cells were required, the above protocol was scaled up to 160-ml serum bottles. Stock solutions of aromatic compounds were prepared as previously described (16). The aromatic compounds studied were stable under the conditions utilized; transformations were not observed in the absence of cells. In this study, no distinction was made between the acid and dissociated ionic forms of aromatic carboxylic acids.

For CO_2-limited media (see Tables 5 and 6), $NaHCO_3$ was replaced with Na_2HPO_4 (5.68 g/liter) and NaH_2PO_4 (7.2 g/liter). Unless otherwise indicated, the initial gas phase of the CO_2-limited medium was 100% N_2. The initial pH of all media at the time of inoculation was 6.4 to 6.5.

Harvesting of cells and preparation of cell extracts. Cells were harvested by centrifugation under anaerobic conditions. Cell pellets were washed once in 50 mM sodium phosphate buffer (pH 6.0) plus 0.1% sodium thioglycolate as a reducer to minimize loss of activity due to oxidation, repelleted by centrifugation, and resuspended in the same buffer.

Cell extracts were prepared by lysozyme digestion, as previously outlined (26), or by French press followed by centrifugation (25,000 × *g* for 30 min) to remove cell debris. Both extracts yielded similar decarboxylase activities.

Assays for the aromatic-dependent decarboxylase. Decarboxylase assays were performed immediately with freshly harvested cells or freshly prepared cell extracts. Assays were performed anaerobically at 55°C in 25-ml crimp-sealed vials, and all cell and cell extract preparations or other manipulations required for the assays were performed in a Coy anaerobic chamber (N_2-H_2, 95:5). The standard assay volume was 2 ml, and the assay buffer was 50 mM sodium phosphate, pH 6.0, with 0.1% sodium thioglycolate. For a standard assay of whole cells, the cell concentration in the assay buffer was adjusted to an optical density at 660 nm of approximately 4. The reaction mixture was preincubated for 5 min at 55°C, and the reaction was initiated by injecting 25 μl of a stock solution of the aromatic compound to be assayed; unless otherwise indicated, the standard aromatic compound utilized in the assay was 4-hydroxybenzoate at an initial concentration of 0.5 mM. The standard assay time was 2 min, and aromatic-dependent decarboxylase activity was quantitated by measuring the amount of decarboxylation by high-pressure liquid chromatographic analysis (e.g., conversion of 4-hydroxybenzoate to phenol). For assays with whole cells, the specific activity was defined as nanomoles of 4-hydroxybenzoate (or an alternative aromatic compound) decarboxylated per minute per milligram (dry weight) of cells. For assays with cell extracts, the specific activity was defined as nanomoles of 4-hydroxybenzoate decarboxylated per minute per milligram of protein. Ethanol, used to solubilize aromatic compounds for stock solutions, had no significant effect on decarboxylase activity over the range of 0 to 8% (well under the final ethanol concentration in standard assays).

^{14}C distribution profiles after growth and decarboxylase assays. Cells were cultivated in medium containing 5 mM [carboxyl-^{14}C]vanillate (approximately 2.7 × 10^4 dpm/μmol) (see Tables 5 and 6). Following growth, a sample of culture medium was removed for analysis of ^{14}C in acetate and for its biomass. For analysis of $^{14}CO_2$, the culture tube was connected to triplicate 1 N KOH traps for CO_2 and then acidified with 0.75 ml of 2 N H_2SO_4. The headspace gas of the culture tube was flushed through the traps for 5 min with a steady stream of N_2, and the trapped $^{14}CO_2$ was quantitated by liquid scintillation in Safety Solve (Research Products International Corp., Mount Prospect, Ill.) with an LS 6800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). ^{14}C-labeled cells were harvested by microfiltration, washed four times, and analyzed for ^{14}C by liquid scintillation, as described previously for the analysis of ^{63}Ni-labeled cells (41).

After cells were removed by centrifugation, [^{14}C]acetate (acetic acid) was purified from the cultivation medium by Dowex 1 column chromatography (29, 35); column performance was confirmed by high-pressure liquid chromatographic analysis and by chromatography of authentic [^{14}C]acetate. Purified [^{14}C]acetate was degraded by the azide decarboxylation procedure; the validity of the degradation was confirmed with authentic [1-^{14}C]acetate and [2-^{14}C]acetate (29, 31).

In decarboxylase assays, [carboxyl-^{14}C]vanillate was added to an approximate concentration of 3.2 × 10^5 dpm/μmol of vanillate (see Table 3). When decarboxylation was nearly complete (25 min), the assay bottle was acidified with 0.1 ml of 0.4 N H_2SO_4 and the $^{14}CO_2$ was trapped and quantitated as described above.

Analytical methods. Aromatic compounds were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Aldrich Chemical Co., Inc. (Milwaukee, Wis.). [Carboxyl-^{14}C]vanillate and [2-^{14}C]acetate were from Research Products International Corporation (Mount Prospect, Ill.). [1-^{14}C]acetate was obtained from ICN Chemical and Radioisotope Division (Costa Mesa, Calif.). Growth was monitored at 600 nm with a Spectronic 501 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.), and cell dry weights were determined as previously described (33). An optical density of 1.0 at 600 nm was equivalent to 0.66 mg (dry weight) of cells per ml. None of the compounds studied influenced the turbidity (A_{660}) of culture media, and a linear relationship was shown to exist between biomass and absorbance. Aromatic compounds were analyzed with a 1090L high-performance liquid chromatograph (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a fermentation monitoring column (Bio-Rad Laboratories, Richmond, Calif.) and a 4290 integrator (Spectra Physics, Bedford, Mass.). The column temperature was 60°C, the mobile phase was 0.01 N H_2SO_4 at 0.8 ml/min, the sample size was 10 μl, and the detector was at 200 nm. Cells were removed by microcentrifugation and microfiltration prior to chromatographic

TABLE 1. Expression of the aromatic decarboxylase[a]

Growth-supportive substrate	Decarboxylase sp act	
	Without 4-hydroxybenzoate	With 4-hydroxybenzoate
Glucose	0.13	10.40
Methanol	0.65	9.94
Formate	0	1.46[b]
1,2,3-Trimethoxybenzene	0.18	1.34[b]
CO	0.01	11.17[c]
H_2	0.16	25.00
Syringate	0	ND[d]
Vanillate	10.29	ND

[a] Assayed with intact cells harvested at late log or early stationary phase of growth. As indicated, 4-hydroxybenzoate (5 mM) was or was not added to growth medium.

[b] Poor growth; 4-hydroxybenzoate was inhibitory to formate- and trimethoxybenzene-dependent growth.

[c] No detectable activity was observed when 4-hydroxybenzoate was replaced with benzoate, 2-hydroxybenzoate, or 3-hydroxybenzoate.

[d] Not determined.

analysis of fermentation broths. Protein was estimated by the Bradford procedure (4).

RESULTS

Expression of the aromatic-dependent decarboxylase. Previous studies established the potential of *C. thermoaceticum* to utilize the carboxyl groups of various aromatic compounds, including 4-hydroxybenzoate, when cultivated at the expense of CO (16). By using 4-hydroxybenzoate as the substrate, an assay (see Materials and Methods) was developed to assess the expression and activity of the aromatic-dependent decarboxylase. In initial studies, both washed cells and extracts of cells cultivated in CO medium supplemented with 4-hydroxybenzoate contained high levels of decarboxylase activity. When other cultures were evaluated, only cells cultivated in the presence of 4-hydroxybenzoate (or other utilizable carboxylated aromatic compounds, e.g., vanillate) contained significant levels of decarboxylase activity (Table 1). Although the initial concentration of 4-hydroxybenzoate in growth media was routinely kept at 5 mM, 0.5 mM 4-hydroxybenzoate induced similar levels of decarboxylase activity (data not shown).

H_2-cultivated cells, which have an absolute requirement for supplemental CO_2, yielded higher levels of activity than the other cultures evaluated. For reasons unknown at this time, 4-hydroxybenzoate was inhibitory to formate- and trimethoxybenzene-dependent growth; this inhibitory effect on growth was likely responsible for the relatively low levels of activity observed in formate and trimethoxybenzene cultures.

Syringate is not subject to decarboxylation by *C. thermoaceticum* (16). Significantly, syringate-cultivated cells did not display any decarboxylase activity (Table 1); neither could 4-hydroxybenzoate-induced cells decarboxylate syringate (see below and Table 3). In addition, CO-dependent *O*-demethylase (40) was not detected in 4-hydroxybenzoate-induced cells (i.e., in cells not cultivated with a utilizable aromatic methoxyl group [data not shown]).

Fate of the aromatic carboxyl group during decarboxylase assays. The above findings demonstrated that the aromatic ring was decarboxylated during decarboxylase assays, suggesting that, as previously postulated (16), the aromatic carboxyl group is the origin of growth-essential CO_2 equivalents. Consistent with this hypothesis, when [carboxyl-^{14}C]vanillate was used as the substrate, $^{14}CO_2$ constituted the main ^{14}C-labeled product recovered from decarboxylase assays (Table 2).

TABLE 2. Production of $^{14}CO_2$ from [carboxyl-^{14}C]vanillate during the decarboxylase assay[a]

Assay and fraction	dpm	Recovery of dpm (%)
Without cells		100.0
[^{14}C]vanillate[b]	315,000	
$^{14}CO_2$	2,000	
With cells		99.6
[^{14}C]vanillate[b]	40,000	
$^{14}CO_2$	277,000	

[a] Cells were cultivated at the expense of 10 mM vanillate in undefined CO_2-enriched media. Disintegrations per minute values are rounded to the nearest thousand. The decarboxylase assay period was 25 min (near completion).

[b] Unreacted soluble fraction after removal of CO_2.

Effects of the assay condition and culture age on decarboxylase activity. Under standard assay conditions with induced cells, the pH and optimum temperature of decarboxylase activity approximated 6.0 and 55°C, respectively; autoclaved cells contained no detectable activity (data not shown). Decarboxylase activity was linear over the assay period and was expressed maximally during early growth (Fig. 2A). The activity declined subsequent to depletion of the substrate (i.e., decarboxylation of 4-hydroxybenzoate), indicating a rapid turnover of the enzyme in the absence of inducer. The reason for the short-term plateau in activity during the initial stages of substrate depletion (Fig. 2A) was not resolved, although its appearance was observed in replicate experiments. During the period of maximum decarboxylation, the overall specific activity observed during growth approximated 40 nmol of 4-hydroxybenzoate decarboxylated per min per mg (dry weight) of cells. To maximize activity levels, subsequent studies were performed with cells harvested during early growth (approximately 30 h postinoculation). Induced intact cells displayed saturation kinetics (Fig. 2B); the apparent K_m and V_{max} for the decarboxylase activity of intact cells approximated 0.67 mM 4-hydroxybenzoate and 110 nmol of 4-hydroxybenzoate decarboxylated per min per mg (dry weight) of cells, respectively. The decarboxylase activity of cell extracts also displayed substrate-saturable kinetics up to 2.5 mM 4-hydroxybenzoate; at higher substrate concentrations, the activity was inhibited. On the basis of double-reciprocal plots over the range of 0 to 2.5 mM 4-hydroxybenzoate, the apparent K_m and V_{max} for the decarboxylase activity of cell extracts approximated 3.33 mM 4-hydroxybenzoate and 217 nmol of 4-hydroxybenzoate decarboxylated per min per mg of protein, respectively.

Specificity. When 4-hydroxybenzoate-induced cells were tested with a variety of carboxylated aromatic compounds, only benzoates with a *para* hydroxyl group were reactive (Table 3). The *meta* position could be occupied by various substituents, including halogens. No other combination of the substituent groups tested yielded a reactive carboxyl group. Furthermore, when cells were cultivated at the expense of CO and supplemented with benzoate, 2-hydroxybenzoate, 3-hydroxybenzoate, or 4-hydroxybenzoate, only the 4-hydroxybenzoate-supplemented cultures decarboxylated the aromatic ring during growth. In addition, the aromatic-dependent decarboxylase was induced only in the

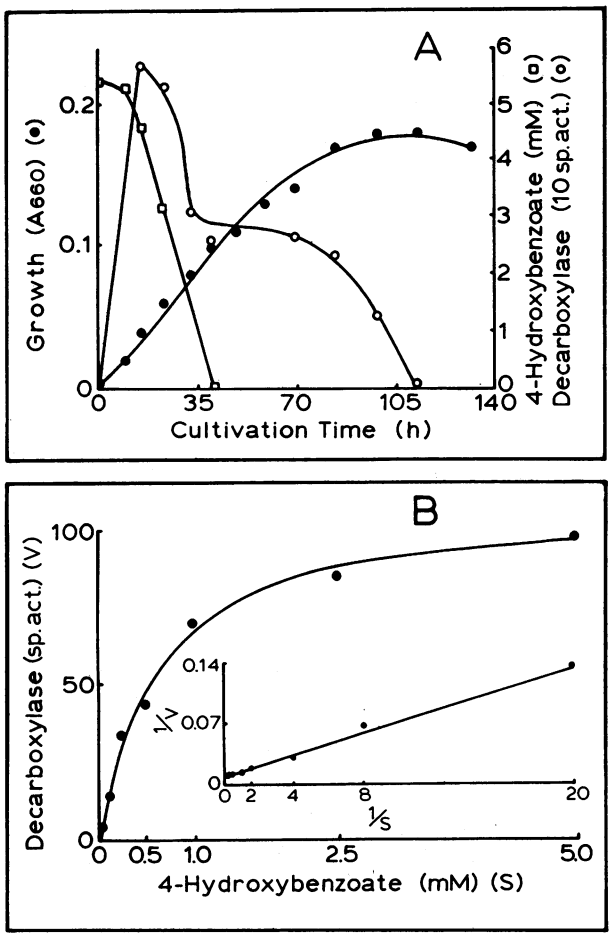

FIG. 2. Effects of culture age (A) and substrate concentration (B) on aromatic-dependent decarboxylase activity. (A) Cells were cultivated at the expense of CO plus 4-hydroxybenzoate (monitored during growth as indicated), harvested at the indicated times, and assayed for decarboxylase; the inoculum was from a CO culture which contained essentially no detectable decarboxylase (Table 1). (B) A kinetic study was performed with cells cultivated with CO plus 4-hydroxybenzoate and harvested midgrowth.

4-hydroxybenzoate cultures; none of the other benzoate-supplemented cultures contained detectable levels of the decarboxylase (Table 1). All of the substrates which were reactive with induced cells were also decarboxylated by cell extracts of induced cells (data not shown). In all of the decarboxylase assays with 4-hydroxybenzoate-induced cells, the aromatic ring was always recovered in the decarboxylated form.

Effects of metabolic inhibitors, external energy sources, and avidin on decarboxylase activity. Certain metabolic inhibitors were inhibitory to decarboxylase activity at the whole-cell level; O_2 was likewise inhibitory (Table 4). Although these findings suggested that the energy state of the intact cell was important to the decarboxylase activity, external energy sources (i.e., acetogenic substrates) did not greatly influence activity (Table 4). This is in contrast to the aromatic-dependent *O*-demethylase which requires either CO or pyruvate as the "CO" donor (40). Avidin, an inhibitor of biotin-dependent carboxylases, had very little effect on activity at either the whole-cell or cell extract level, and the putative Na^+/H^+ antiporter inhibitors amiloride and harmaline (1, 20) did not significantly affect decarboxylase activity (Table 4).

TABLE 3. Specificity of aromatic-dependent decarboxylase activity[a]

Substrate	Product	Sp act
Reactive		
4-Hydroxybenzoate	Phenol	55.5
3,4-Dihydroxybenzoate (P)	2-Hydroxyphenol (C)	10.9
3-Methoxy-4-hydroxybenzoate (V)	2-Methoxyphenol (G)	17.0
3-Chloro-4-hydroxybenzoate	2-Chlorophenol	30.2
3-Fluoro-4-hydroxybenzoate	2-Fluorophenol	28.8
Unreactive		
Benzoate	2-Hydroxybenzoate	
3-Hydroxybenzoate	2,3-Dihydroxybenzoate	
2,4-Dihydroxybenzoate	2,5-Dihydroxybenzoate	
2,6-Dihydroxybenzoate	3,5-Dihydroxybenzoate	
3,5-Dimethoxy-4-hydroxybenzoate (S)	3,4,5-Trihydroxybenzoate (GA)	
3,4-Dimethoxybenzoate	3,4,5-Trimethoxybenzoate	
4-Methoxybenzoate (A)	Toluene	
6-Hydroxynicotinic acid[b]		

[a] Cells were cultivated with CO plus 5 mM 4-hydroxybenzoate and assayed with the indicated substrate. Abbreviations: P, protocatechuate; C, catechol; V, vanillate; G, guaiacol; S, syringate; GA, gallate; A, *p*-anisate.
[b] Not decarboxylated during CO-dependent growth.

Integration of the carboxyl group into the acetyl-CoA pathway: total synthesis of acetate from aromatic carboxyl groups. The findings described above demonstrate that an inducible aromatic-dependent decarboxylase yields CO_2 equivalents in short-term assays. To ascertain the fate of this CO_2 equivalent relative to growth and acetogenesis, cells were cultivated at the expense of [carboxyl-^{14}C]vanillate (Table 5). Under CO_2-enriched conditions, the aromatic carboxyl group went primarily to CO_2 (cultures A and B), whereas while under CO_2-limited conditions, the carboxyl group was found primarily in acetate (cultures C and D). However, when CO_2-limited medium was enriched with CO, the aromatic carboxyl group again yielded significant levels of CO_2 (cultures E and F). In addition, a greater percentage of the aromatic carboxyl group went to biomass synthesis under CO-enriched, CO_2-limited conditions than under the other conditions evaluated. The fluctuations in percent recoveries may have been due to leakage of gas ($^{14}CO_2$) during growth and to incomplete trapping of CO_2 in the KOH traps.

These cultivation environments also affected the distribution of aromatic carboxyl group-derived carbon (i.e., ^{14}C) in acetate (Table 6). Significantly, under CO_2-limited conditions, the aromatic carboxyl carbon was equally distributed between the carboxyl and methyl carbons (cultures C and D), thus indicating the total synthesis of acetate from aromatic carboxyl groups. In contrast, when cocultivated (i.e., supplemented) with CO under the same conditions, the aromatic carboxyl group went primarily to the methyl carbon of acetate (cultures E and F).

DISCUSSION

Unlike the CO-dependent *O*-demethylase (40), the aromatic-dependent decarboxylase was not repressed by glucose or other external energy sources. In addition, the *O*-demethylase and decarboxylase were not necessarily induced by the same aromatic compound. For example, syringate induced only the *O*-demethylase and was not subject to decarboxylation, while vanillate induced both the *O*-demethylase and decarboxylase. These findings indicate that the regulatory

TABLE 4. Effects of metabolic inhibitors and external energy sources on decarboxylase activity[a]

Effector	Concn or amt	Sp act (%)[b]
Metabolic inhibitor		
TBT	0	58.0
	100 μM[c]	13.2 (23)
CCCP	0	53.9
	1,000 μM[c]	6.4 (12)
Monensin	0	44.6
	10 μM[c]	1.5 (3)
Amiloride	0	57.0
	200 μM[c]	51.5 (90)
Harmaline	0	51.8
	500 μM	51.5 (99)
Triton X-100	0	49.0
	0.5%	1.6 (3)
Energy source		
N_2 (control)	100%[d]	44.4
H_2	100%[d]	50.7 (114)
CO	100%[d]	38.3 (86)
Glucose	10 mM	41.2 (93)
O_2	100%[d]	0.0 (0)
Other		
Biotin	0.4 mg	50.8 (115)
Avidin	0.5 U[e]	51.4 (117)
Biotin	0.4 mg	127.7[f] (121)
Avidin	0.5 U	96.3[f] (91)

[a] Cells were cultivated at the expense of CO plus 5 mM 4-hydroxybenzoate and preincubated for 5 min with effector prior to the assay. In all assays, phenol was the end product detected. Unless otherwise indicated, assays were with intact cells. Abbreviations: TBT, tributyltin chloride; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.
[b] Percentage of control.
[c] Growth-inhibitory concentration.
[d] One hundred percent of gas phase.
[e] One unit of avidin binds 1 μg of biotin.
[f] Assay performed with cell extract.

mechanisms (i.e., operons) which control the expression of these two catalysts are likely dissimilar.

As determined by this and previous studies (16, 40), *C. thermoaceticum* metabolizes vanillate to catechol by two separate pathways (Fig. 3). This conclusion follows from the facts that both vanillate and protocatechuate are substrates of the decarboxylase and that both vanillate and guaiacol are substrates for the CO-dependent *O*-demethylase. During growth, whether decarboxylation preceeds O demethylation may depend on the availability of exogenous CO_2. In the absence of CO_2, the cell is unable to grow at the sole expense of methoxyl groups because CO_2 is essential to the flow of methoxyl-derived reductant (see below). Under such conditions, decarboxylation may be prerequisite to O demethylation. If, on the other hand, the cell is not limited by its supply of CO_2, O demethylation may occur prior to decarboxylation. In support of this possibility, protocatechuate is a transient product of vanillate in CO_2-enriched cultures (16). In addition to the use of methoxyl groups as methyl donors via the *O*-demethylase-dependent synthesis of acetyl-CoA (40), the oxidation of methoxyl groups must also occur during methoxyl-dependent growth. Since the nature and specificity of the oxidoreductase systems involved have not been resolved, the overall fate of aromatic methoxyl groups cannot be stated.

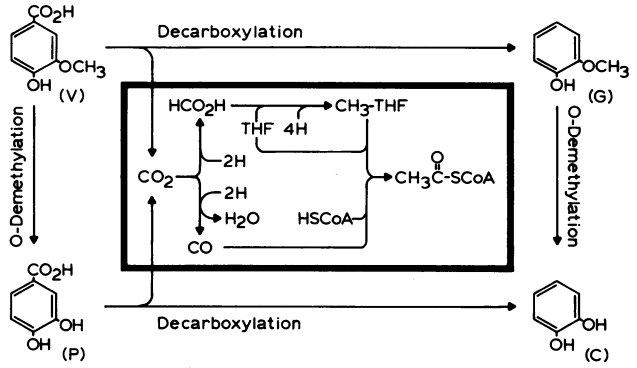

FIG. 3. Bidirectional pathway for the biotransformation of vanillate and total synthesis of acetyl-CoA from aromatic carboxyl groups by *C. thermoaceticum*. Abbreviations: CoA, coenzyme A; V, vanillate; P, protocatechuate; G, guaiacol; C, catechol.

Carbon dioxide is the terminal electron acceptor in aceto-

TABLE 5. Distribution of ^{14}C from [carboxyl-^{14}C]vanillate after vanillate-dependent growth by *C. thermoaceticum*[a]

Growth condition	Initial vanillate (dpm)	Distribution of ^{14}C (dpm)			% dpm recovered
		CO_2	Biomass	Spent medium[b]	
CO_2 enriched					
A	1,351,000	728,000	17,000	262,000	85.5
B	1,622,000	941,000	13,000	471,000	87.9
Avg % distribution[c]		69.2	1.3	29.6	
CO_2 limited					
C	1,193,000	27,000	43,000	1,006,000	95.6
D	1,527,000	28,000	37,000	1,343,000	92.3
Avg % distribution[c]		2.3	3.3	94.4	
CO_2 limited + CO					
E	2,604,000	752,000	142,000	1,155,000	78.7
F	1,588,000	455,000	82,000	696,000	77.6
Avg % distribution[c]		36.8	6.8	56.5	

[a] Disintegrations per minute values are rounded to the nearest thousand.
[b] Primarily [^{14}C]acetate. Uninoculated controls yielded essentially no ^{14}C in the CO_2 fraction. In addition, the spent medium (supernatant fluid) of uninoculated controls contained unreacted vanillate as the sole ^{14}C-labeled compound, and the percentage of disintegrations per minute recovered from uninoculated controls approximated 100. No residual vanillate was detected in inoculated cultures at the time of harvest (see reference 16 for a typical vanillate-dependent growth curve and substrate-product profile).
[c] Percentage of total disintegrations per minute recovered in CO_2, biomass, or spent medium (supernatant) fractions.

genesis (24) and is essential or stimulatory to the growth of *C. thermoaceticum* when growth is at the expense of an autotrophic substrate (H_2 or CO), methanol, methoxylated aromatic compounds, or glucose (16 and unpublished data). Carbon dioxide also plays a fundamental role in the CO-dependent growth of *Clostridium thermoautotrophicum* (34), an acetogen closely related to *C. thermoaceticum* (38). Since CO_2 plays such a key role in the bioenergetics of acetogenesis, and since carboxylated aromatic compounds can serve as (i.e., yield) CO_2 equivalents, it follows that aromatic carboxyl groups may contribute to the bioenergetics of growth. Indeed, the decarboxylation of protocatechuate is coincident with increased cell yields (16). Furthermore, under conditions of CO_2 limitation, the aromatic carboxyl group was incorporated with equal frequency into both carbons of acetate. To the best of our knowledge, this is the first evidence verifying the total synthesis of acetate from aromatic carboxyl groups. However, how tightly aromatic-derived CO_2 equivalents are coupled to the acetyl-CoA pathway and cell energetics may be dependent upon growth conditions.

Previous growth studies demonstrated that $^{14}CO_2$ goes preferentially (62.9% of the total ^{14}C recovered in acetate) to the carboxyl group of acetate under CO_2-enriched conditions (29). That the aromatic carboxyl group gave rise to CO_2 in short-term assays (Table 3) and went preferentially (62.5% of the total ^{14}C recovered in acetate) to the carboxyl group of acetate under CO_2-enriched conditions (Table 5) argues strongly in favor of the aromatic carboxyl group being released as free CO_2 and then reutilized. However, when CO_2 was limited, a condition under which the aromatic carboxyl group must theoretically act as (i.e., replace) CO_2 in acetogenesis, the aromatic carboxyl group did not behave like free CO_2 in that it went with equal frequency to both carbons of acetate. Furthermore, very little of the carboxyl group was recovered as unused CO_2 under these conditions. Thus, aromatic carboxyl groups may be more tightly coupled to some phase of the acetyl-CoA pathway under conditions of CO_2 limitation.

When CO, which is an immediate precursor of the carbonyl group in acetyl-CoA synthesis (17), was available, the aromatic carboxyl group went preferentially to the methyl carbon of acetate (Tables 5 and 6). Under these conditions, the aromatic carboxyl group appears equivalent to formate, which is the preferred origin of the methyl carbon of acetate and the immediate precursor for the tetrahydrofolate pathway (24, 29). It is therefore likely that, in this case, the carboxyl group is reduced initially to the level of formate via CO-derived reductant. It is interesting that cells reduced aromatic carboxyl groups to the methyl level even when both preformed methyl and carbonyl groups were available for the synthesis of acetyl-CoA (Table 5, cultures E and F). This fact indicates that, even under conditions that allow the total synthesis of acetate from preexisting methyl- and carbonyl-level precursors (40), cells are still obligately dependent upon electron transport-dependent phosphorylation coupled to the reduction of CO_2 (which, in this case, is derived from aromatic carboxyl groups) via the tetrahydrofolate pathway (25, 30). Although these findings indicate that environmental conditions influence the use of aromatic carboxyl groups in acetogenesis, decarboxylation per se apparently is not obligately integrated into acetogenesis, since the aromatic carboxyl group went to free CO_2 in short-term assays (Table 3).

Even though external energy sources did not greatly influence decarboxylation at the whole-cell level, the capacity to utilize (decarboxylate) aromatic carboxyl groups may depend on the energy state of a cell, since metabolic inhibitors influenced decarboxylation. The inhibition observed with metabolic inhibitors may be due to energy-dependent transport processes. The energy-dependent transport of nickel ($NiCl_2$) by acetogens can be coupled to the use of internal energy sources (15, 27, 41). Thus, energy-dependent transport of aromatic compounds may also be linked to internal energy reserves, in which case transport and decarboxylation may initially occur independent of external sources of energy.

Not all acetogens have the capacity to utilize aromatic carboxyl groups (16). For acetogens that have this potential, aromatic carboxyl groups can (i) be growth essential under conditions of CO_2 limitation, (ii) induce an aromatic-dependent decarboxylase, and (iii) be utilized as CO_2 equivalents in the total synthesis of acetate.

TABLE 6. Distribution of ^{14}C between the methyl and carboxyl carbons of acetate[a]

Growth condition	Acetate degraded (dpm)	Distribution of ^{14}C (dpm) (%)[b]		% dpm recovered
		Methyl	Carboxyl	
CO_2 enriched				
A	203,000	73,000 (38)	117,000 (62)	93.6
B	236,000	67,000 (37)	114,000 (63)	76.6
Avg % distribution[c]		37.5	62.5	
CO_2 limited				
C	781,000	269,000 (46)	313,000 (54)	74.6
D	741,000	332,000 (52)	310,000 (48)	86.6
Avg % distribution		49.0	51.0	
CO_2 limited + CO				
E	587,000	513,000 (90)	56,000 (10)	96.9
F	387,000	282,000 (78)	78,000 (22)	93.2
Avg % distribution		84.0	16.0	

[a] [^{14}C]acetate was purified from the supernatant fraction obtained in the experiment described in Table 5. Disintegrations per minute values are rounded to the nearest thousand.

[b] Percentage of total disintegrations per minute recovered in both the methyl and carboxyl carbons.

[c] Percentage of total disintegrations per minute recovered in the methyl or carboxyl fraction.

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