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Methane Fermentation of Ferulate and Benzoate: Anaerobic Degradation Pathways

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The anaerobic biodegradation of ferulate and benzoate in stabilized methanogenic consortia was examined in detail. Up to 99% of the ferulate and 98% of the benzoate were converted to carbon dioxide and methane. Methanogenesis was inhibited with 2-bromoethanesulfonic acid, which reduced the gas production and enhanced the buildup of intermediates. Use of high-performance liquid chromatography and two gas chromatographic procedures yielded identification of the following compounds: caffeate, *p*-hydroxycinnamate, cinnamate, phenylpropionate, phenylacetate, benzoate, and toluene during ferulate degradation; and benzene, cyclohexane, methylcyclohexane, cyclohexanecarboxylate, cyclohexanone, 1-methylcyclohexanone, pimelate, adipate, succinate, lactate, heptanoate, caproate, isocaproate, valerate, butyrate, isobutyrate, propionate, and acetate during the degradation of either benzoate or ferulate. Based on the identification of the above compounds, more complete reductive pathways for ferulate and benzoate are proposed.

Benzoate has been used as the model compound for a number of studies on the anaerobic degradation of aromatic compounds. Methanogenic benzoate degradation was studied by several different laboratories (2, 9, 17, 19, 24), and pathways for ring reduction and fission were described. The information was reviewed and the pathways were summarized by Evans (7). More complex aromatic substrates have also been investigated. Methanogenic fermentation of phenylpropionate and phenylacetate was examined by Balba and Evans (3), and a few related aromatic compounds were considered during the investigations on benzoate (6, 26). Kaiser and Hanselmann (16) studied the anaerobic degradation of methoxyl and hydroxyl trisubstituted benzenoids by methanogenic consortia enriched from freshwater anaerobic sediments. Bryant and co-workers (S. Barik and M. P. Bryant, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I72, p. 133) investigated the degradation of benzoate, phenylacetate, and phenol by methanogenic enrichments and by syntrophic associations of hydrogen-producing and hydrogen-utilizing bacteria. Two pure cultures were recently described which catalyze the anaerobic ring fission of trihydroxylated benzenoids (20, 23).

Previous investigations in our laboratory (11, 12) examined methanogenic degradation of a range of 11 monoaromatic compounds with varying ring substituents and oxidation states, including ferulate, the most complex of the tested compounds (13). By inhibiting methanogenesis with 2-bromoethanesulfonic acid (BESA), cinnamate, phenylpropionate, phenylacetate, benzoate, cyclohexanecarboxylate, pimelate, adipate, butyrate, propionate, and acetate were observed as metabolites. A reductive catabolic pathway was described which merges with that for benzoate, suggesting that for a portion of the pathway a common set of reactions may take place during the degradation of this class of compounds (13).

In this study, benzoate and ferulate degradation to methane and carbon dioxide is detailed by gas chromatographic (GC), capillary gas chromatographic (CGC), and high-performance liquid chromatographic (HPLC) procedures. Compounds which we believe are being reported for the first time are toluene, *p*-hydroxycinnamate, and caffeate for ferulate catabolism and 1-methylcyclohexanone, cyclohexanone, methylcyclohexane, cyclohexane, and benzene for both ferulate and benzoate catabolism.

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MATERIALS AND METHODS

Culture procedures. Stable methanogenic consortia metabolizing ferulate and benzoate were initiated with 10% inocula from a 6-liter anaerobic digester fed with sewage sludge. The enrichments were maintained by transferring 50% of the culture volume to fresh aromatic defined media every 10 days. After several transfers, the stabilized consortia were refed 300 mg of the aromatic substrate per liter every 20 days. The cultures were incubated anaerobically with 30% CO_2–70% N_2 gas atmosphere at 35°C in the dark. A serum bottle variation of the Hungate technique (18) was adapted for the maintenance of the cultures. The prereduced defined media and the syringe techniques used with the cultures were described previously (11–13). The study was carried out on 33 ferulate- and 20 benzoate-metabolizing consortia, which had been maintained for 6 months to 3 years. Gas production results were obtained by subtracting the gas produced by replicate background controls (fed no substrate) from the gas produced by the cultures.

After the consortia were well established, inhibition of methane formation was undertaken on randomly selected cultures, six ferulate and four benzoate, with 0.1 or 1 mM BESA, an analog of coenzyme M specific for methyl transfer reactions (25). Enhanced levels of intermediates were thus detectable.

Microscopy. Microscopic studies were carried out on 6-month-old ferulate or benzoate consortia. Phase-contrast and epifluorescence (with acridine orange) techniques were applied with an Olympus Vanox model microscope.

Analytical procedures. (i) HPLC. Caffeate, *p*-hydroxycinnamate, cinnamate, phenylpropionate, phenylacetate, benzoate, toluene, benzene, cyclohexanone, and 1-methylcyclohexanone were identified and quantitated by HPLC with a Spectra-Physics SP 3500B system. A 10-μl portion of supernatant from centrifuged samples was in-

jected onto a reverse-phase column (250 mm; Spherisorb ODS, 10 μm). The mobile phase consisted of acetonitrile and 0.01 N perchloric acid in a linear gradient of 10 to 50% acetonitrile. The flow rate was 1.2 ml/min. A variable-wavelength UV detector was set at six different wavelengths (190, 210, 222, 254, 284, and 310 nm) for determination of the different intermediates. Calibration curves for standards of each compound were determined at all six wavelengths. A minimum of six replicate injections were made for each sample taken. Except for cyclohexanone and 1-methylcyclohexanone, all of the compounds examined by HPLC could be detected at more than one wavelength. The lower sensitivity limit of the procedure was 0.1 ppm (0.1 mg/liter) for each compound.

(ii) CGC. Determinations of cyclohexane and methylcyclohexane and confirmation of toluene, benzene, cyclohexanone, and methylcyclohexanone, which were independently detected by HPLC, were undertaken with CGC (Carlo Erba Fractovap 2900 with a flame ionization detector). Samples were prepared by centrifuging the culture fluid and then extracting a 1.5-ml sample of the supernatant with 0.3 ml of toluene; 3 μl of the extract was injected splitless (30 s) onto a glass capillary column (50 m; UCON LB 60086, Jaeggi Laboratory, Trogen, Switzerland) for low-boiling compounds. Column temperature was programmed for 5 min at 30°C up to 150°C (at 5°C/min) for 39 min. The carrier gas was hydrogen, set at a flow rate of 0.25 kg/min. For detection of toluene and benzene, ether was used as the solvent, with other procedures remaining the same. Cyclohexanecarboxylate could also be detected with CGC after the samples were first methylated with DIAZALD (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide; Aldrich Chemical Co., Inc., Milwaukee, Wis.), a diazomethane-generating kit (13).

(iii) GC. Volatile fatty acids were determined and quantitated by packed-column GC (13). Nonvolatile acids were qualitatively determined under the same operating conditions. Samples were prepared according to the method of Holdeman and Moore (14). Gas composition was determined with a Fisher-Hamilton gas partitioner, model 25V.

RESULTS

Morphological characteristics of the consortia. The morphological types reported for ferulate-degrading consortia (13) were again found in both ferulate and benzoate cultures: short rods (2 by 0.6 μm), curved rods (15 by 0.6 μm), and long chains of large rods (25 to 80 by 1 μm). In addition to these, two new forms were detected: packets of large coccoidal cells (to 2.5 μm in diameter) and short fat sporogenic rods (1 by 2 to 3 μm). The curved rods, chains of large rods, and packets of cocci were diluted out upon subculturing with BESA. They were presumptively identified as *Methanospirillum* (4, 8), *Methanothrix* (15), and *Methanosarcina* (4) species, based on their morphological features.

Carbon balances. Acclimated and stabilized consortia readily used added substrate without a lag. Ferulate was metabolized in 6 days (Fig. 1A), and benzoate was metabolized in 4 days (Fig. 2A). Gas production leveled off shortly thereafter. The amount of total gas produced was 2.32 ± 0.60 mmol of C for ferulate and 2.55 ± 0.87 mmol of C for benzoate (values are expressed as mean ± standard error), which agrees well with the amount of substrate added, 2.52 and 2.58 mmol of C, respectively. During ferulate decomposition two early metabolites, cinnamate and phenylpropionate, appeared in detectable quantities (0.4 to 0.5 mM C) in these uninhibited cultures (Fig. 1A).

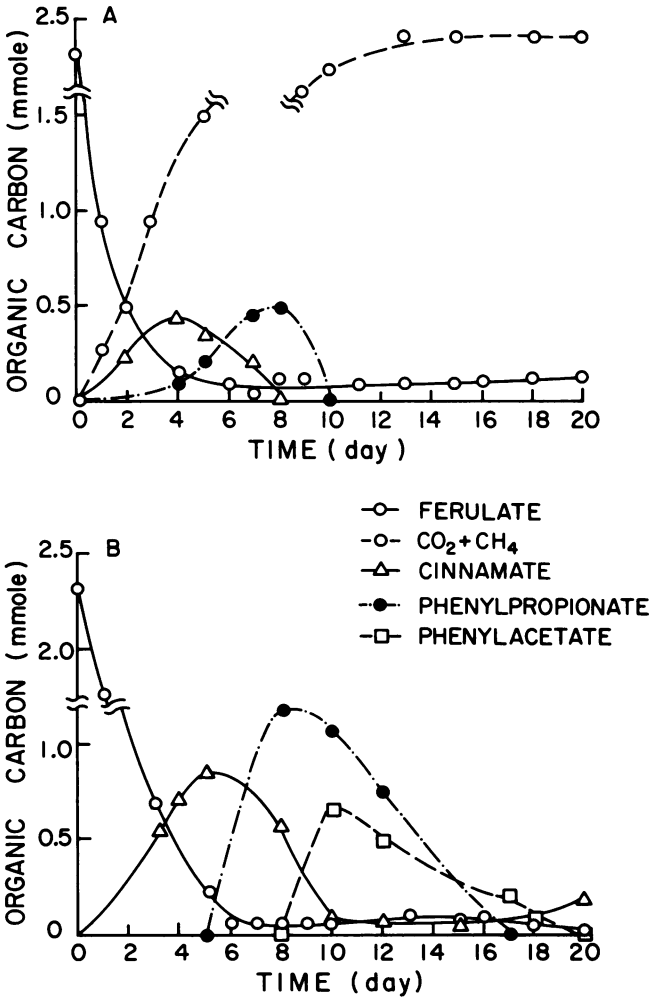

FIG. 1. Ferulate metabolism by (A) enriched methanogenic consortia and (B) enriched methanogenic consortia amended with 0.1 mM BESA. (A) Data illustrate ferulate utilization, cumulative gas production, and the transient appearance of the intermediates cinnamate and phenylpropionate. Results are the average of six replicate consortia. (B) Data were collected during the second month of continuous inhibition and illustrate the transient appearance of the intermediates cinnamate, phenylpropionate, and phenylacetate. Results are the average of six replicate consortia.

Aromatic, cyclic, and ring fission intermediates. After addition of BESA, other metabolites became detectable. The extent of substrate utilization remained the same as that in the uninhibited cultures (Fig. 1, 2, and 3), whereas the total gas produced was reduced to less than 15% of that in the uninhibited cultures. Ten metabolites of ferulate catabolism previoulsy reported by Healy et al. (13) were observed, and 16 additional compounds were detected: caffeate, *p*-hydroxycinnamate, and toluene for ferulate degradation, and benzene, methylcyclohexane, cyclohexane, 1-methylcyclohexanone, cyclohexanone, succinate, lactate, heptanoate, isocaproate, caproate, isovalerate, valerate, and isobutyrate for ferulate or benzoate degradation. Cinnamate and phenylpropionate appeared in the inhibited cultures in concentrations two to three times higher than in the uninhibited cultures (Fig. 1). An additional metabolite, phenylacetate, is also shown in Fig. 1B. It is important to note the sequence of appearance of these three intermedi-

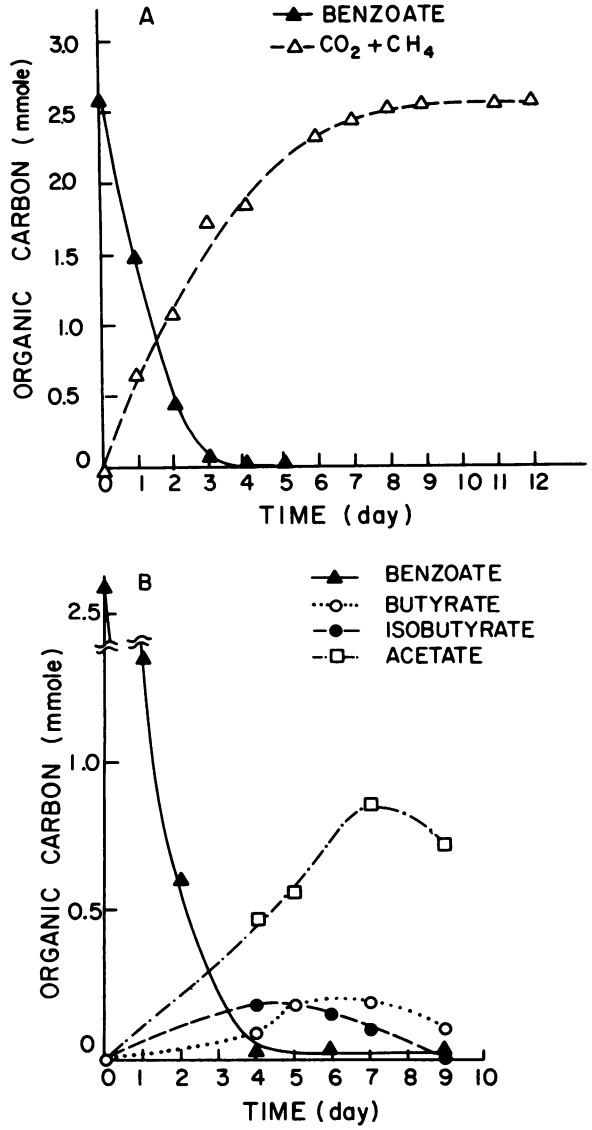

FIG. 2. Benzoate metabolism by (A) enriched methanogenic consortia and (B) enriched methanogenic consortia inhibited with 1 mM BESA. (A) Data illustrate benzoate utilization and cumulative gas production. Results are the average of four replicate consortia. (B) Data were collected during the third month of continuous inhibition and illustrate the appearance of the late ring fission products butyrate, isobutyrate, and acetate. Results are the average of four replicate consortia.

ates: phenylpropionate was detected after cinnamate and was followed by phenylacetate. Figure 3 illustrates the time course of events for three other compounds, benzoate, cyclohexane, and acetate. Benzoate and cyclohexane increased to levels reflecting 35 and 30%, respectively, of the organic carbon added as ferulate before being further metabolized. After benzoate and cyclohexane began to decrease, acetate concentrations rose substantially and accounted for 83% of the total organic carbon. Similar sequential results were obtained in benzoate cultures amended with BESA. Butyrate and isobutyrate, two of a number of late aliphatic metabolites, appeared only transiently (Fig. 2B); acetate accumulation, however, remained high.

Figure 4 illustrates the formation of the newly detected metabolites cyclohexanone, 1-methylcyclohexanone, toluene, and benzene in BESA-amended cultures degrading ferulate. The transient appearance of cyclohexanone and 1-methylcyclohexanone is consistent with their role as ring fission precursors. Toluene and benzene (detected with both HPLC and CGC) were metabolized during the incubation period in the cultures which had been inhibited for 3 months (Fig. 4). However, after 1 year of inhibition, concentrations of both of these hydrocarbons accumulated and remained (data not shown). This indicates that they were not true metabolic intermediates. As related to these findings, it is important to emphasize that the addition of BESA might have affected not only methanogens, but also other members of the mixed cultures and consequently changed the electron flow and major catabolic routes.

Figures 5 and 6 summarize the carbon balance during the metabolism of ferulate and benzoate by acclimated consortia amended with BESA. Nonvolatile organic acids are not included since they were not quantitated. A total of 94 to 102% carbon was recovered during ferulate metabolism (Fig. 5), and 80 to 91% was recovered during benzoate transformation (Fig. 6). High initial concentrations of the aromatic intermediates in ferulate-metabolizing cultures, and of alicyclic compounds in benzoate-metabolizing cultures, decreased almost completely towards the end of incubation. On the contrary, volatile fatty acids (most of which was acetate accounting for up to 82.6% of the total carbon) increased, indicating the ring fission. The calculated hydrogen recovery data for ferulate-degrading consortia amended with BESA for 2 months were 100.2% on day 5 of incubation, 99.9% on day 10, and 88.6% on day 20. In benzoate-degrading consortia amended with BESA, hydrogen recovery was 92% on day 2, 106.3% on day 5, and 90.2% on day 12 of incubation.

DISCUSSION

Since BESA-amended systems continued to metabolize the added aromatic carbon, it can be suggested that their

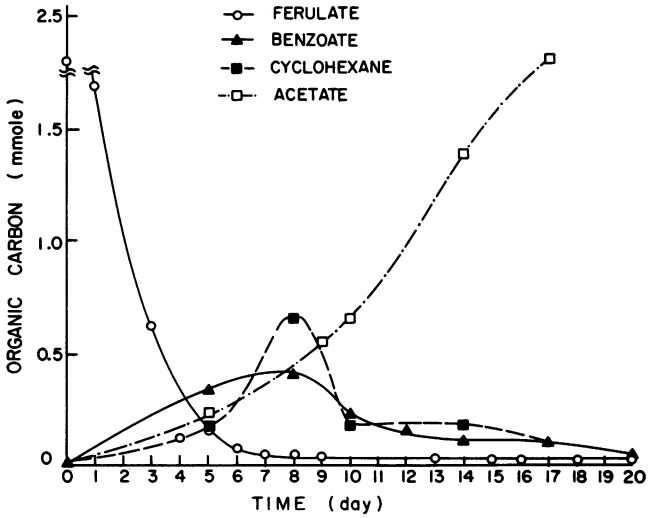

FIG. 3. Ferulate metabolism by enriched methanogenic consortia inhibited with 0.1 mM BESA. Data were collected during the third month of continuous inhibition and illustrate the accumulation of acetate and the transient appearance of benzoate and cyclohexane. Results are the average of four replicate consortia.

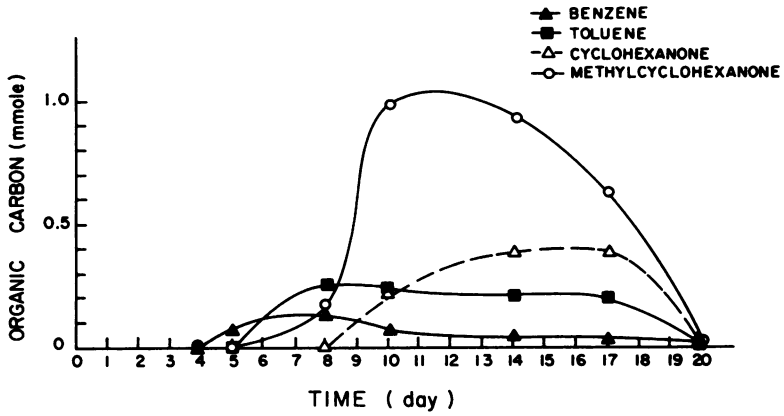

FIG. 4. Transient appearance of toluene, benzene, cyclohexanone, and 1-methylcyclohexanone. Data were collected from ferulate-metabolizing consortia continuously inhibited with 0.1 mM BESA for 3 months. Results are the average of six replicate consortia.

metabolism can be uncoupled from methanogenesis. However, attempts to isolate the ring-cleaving microorganisms from the consortia remained unsuccessful. The pure cultures which have been isolated (A. C. Frazer, L. Y. Young, and T. L. Genetta, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q49, p. 212; D. Grbić-Galić, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, O45, p. 196) are only transforming or eliminating the substituents from the ring. It can be concluded that the relationships in the consortia are partially syntrophic. In BESA-amended cultures, where the inhibitor might have changed some of the dominant catabolic reactions, other hydrogen-utilizing microorganisms could have taken over the role of methane producers.

Several unusually reduced compounds (toluene, benzene, cyclohexane, and methylcyclohexane) were detected in BESA-amended consortia and nonmethanogenic mixed fermentative subcultures derived from the inhibited consortia. It is suggested that they might be electron and hydrogen sinks under conditions in which the methanogenic bacteria are suppressed. It has been shown for similar complex systems that, in the absence of methanogens, hydrogenogens form more reduced products such as ethanol, propionate, butyrate, lactate, succinate, or adipate (5, 27, 28). Some of these aliphatics have been found in our inhibited consortia also. The only mechanism we can suggest for the formation of toluene and benzene is the reduction and decarboxylation of the intermediates phenylacetate and benzoate; however, there are no data to substantiate this. Toluene and benzene could theoretically give rise to methylcyclohexane and cyclohexane through thermodynamically favorable reduction reactions:

$$\text{toluene} + 3H_2 \rightarrow \text{methylcyclohexane} \qquad \Delta G^{0'} = -96.18 \text{ kJ per reaction}$$

$$\text{benzene} + 3H_2 \rightarrow \text{cyclohexane} \qquad \Delta G^{0'} = -96.27 \text{ kJ per reaction}$$

It is not clear, however, if the proposed reactions are really feasible in anaerobic systems. Both toluene and benzene disappeared with time in the cultures inhibited for 2 months, and so did cyclohexane and methylcyclohexane.

A summary of the detected metabolites and the proposed pathways is illustrated in Fig. 7. The first step is demethoxylation of ferulate to the corresponding hydroxyl compound, caffeate. Bache and Pfennig (1) had first reported the anaerobic demethoxylation of methoxylated aromatics by a strict anaerobe, *Acetobacterium woodii*. After demethoxylation, a reductive dehydroxylation yields *p*-hydroxycinnamate, which is a precursor to cinnamate formed as the result of another dehydroxylation. The dehydroxylation mechanism had been shown to occur in gastrointestinal systems (21). The exact sequence of the intermediates cinnamate-phenylpropionate-phenylacetate has been shown with nonmethanogenic subcultures and with a facultatively anaerobic bacterium isolated from ferulate consortia. They transformed cinnamate to phenylpropionate and phenylpropionate to phenylacetate (Grbić-Galić, submitted for publication). This implies a decarboxylation of phenylpropionate to yield phenylacetate and suggests that benzoate could arise from phenylacetate through another decarboxylation. Anaerobic decarboxylations of aromatic acids had already been reported (10, 22), but the substrates in question had been *p*-hydroxylated; the mechanism we propose has not been described yet.

As already suggested by Healy et al. (13), it appears that

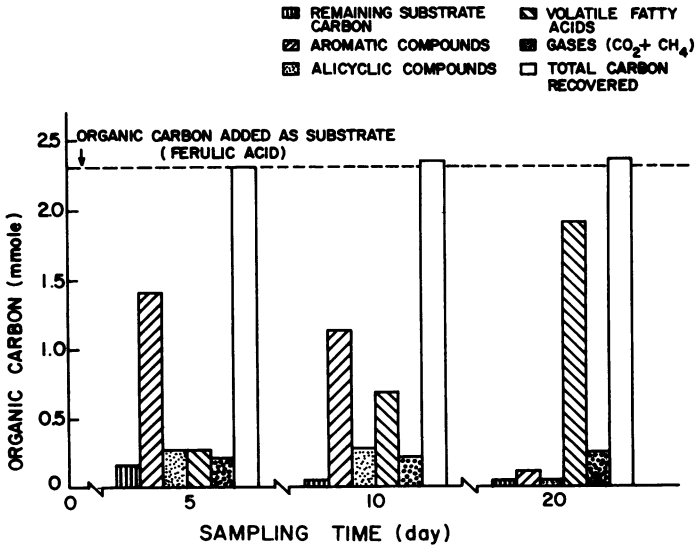

FIG. 5. Carbon recovery for the transformation of ferulate as the sole carbon source by acclimated consortia amended with 0.1 mM BESA for 2 months. Actual values are reported as mean of the results obtained from four replicate consortia.

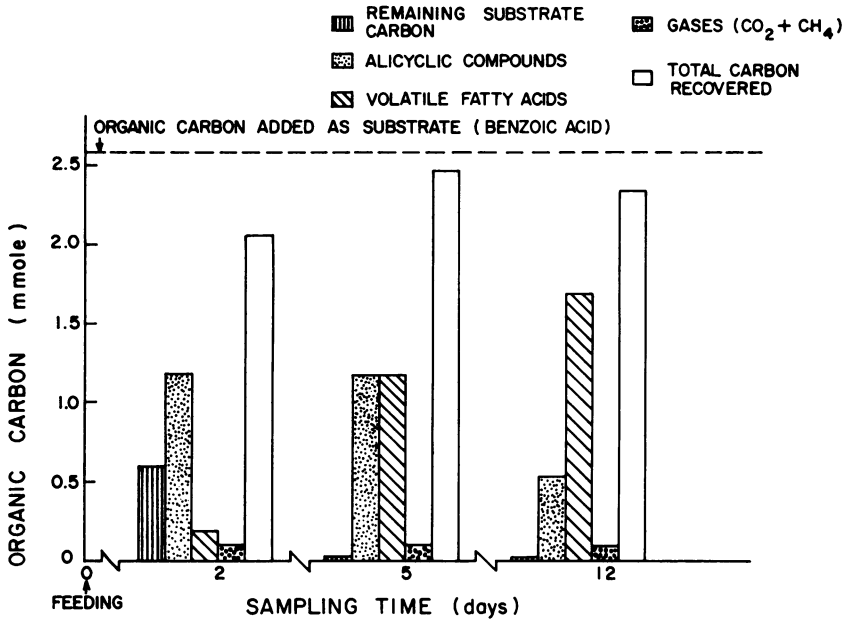

FIG. 6. Carbon recovery for the transformation of benzoate as the sole carbon source by acclimated consortia amended with 1 mM BESA for 2 months. Actual values are reported as mean of the results obtained from four replicate consortia.

the ferulate pathway overlaps that of benzoate. Benzoate is the last aromatic intermediate found in ferulate consortia and most likely the substrate for the ring reduction as described previously (7, 13). This differs from the described pathways for trihydroxylated aromatic compounds (16) where no dehydroxylation occurs before ring reduction and cleavage, and benzoate is not an intermediate. The new alicyclic intermediates of both ferulate and benzoate degradation we

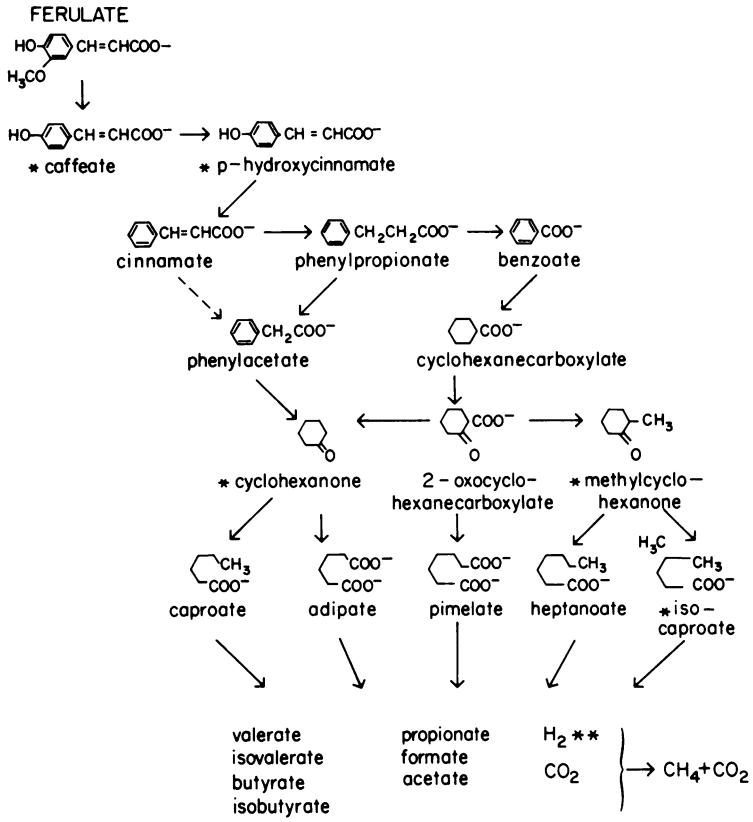

FIG. 7. Proposed pathways illustrating the aromatic and cyclic intermediates of anaerobic ferulate metabolism. All compounds shown were detected in our study except for those denoted by **. Compounds detected for the first time are denoted by *.

report are cyclohexanone and 1-methylcyclohexanone. They could be derived from 2-oxocyclohexanecarboxylate (not found in our consortia but reported by other authors [7, 24]) through a decarboxylation or a hydrogen addition, respectively. These intermediates most likely undergo ring fission (7) to nonvolatile and volatile acids which are subsequently converted to methane precursors. The reasonably good carbon recovery in BESA-amended cultures supports the proposed degradation pathways.

ACKNOWLEDGMENTS

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