Metabolism of the ^{18}O-Methoxy Substituent of 3-Methoxybenzoic Acid and Other Unlabeled Methoxybenzoic Acids by Anaerobic Bacteria

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O-methyl substituents of aromatic compounds can provide C$_1$ growth substrates for facultative and strict anaerobic bacteria isolated from diverse environments. The mechanism of the bioconversion of methoxylated benzoic acids to the hydroxylated derivatives was investigated with a model substrate and cultures of one anaerobic consortium, eight strict anaerobic bacteria, and one facultative anaerobic microorganism. Using high-pressure liquid chromatography and gas chromatography-mass spectral analysis, we found that a haloaromatic dehalogenating consortium, a dehalogenating isolate from that consortium, *Eubacterium limosum*, and a strain of *Acetobacterium woodii* metabolized 3-[methoxy-^{18}O]methoxybenzoic acid (3-anisic acid) to 3-[hydroxy-^{18}O]hydroxybenzoic acid stoichiometrically at rates of 1.5, 3.2, 52.4, and 36.7 nmol/min per mg of protein, respectively. A different strain of *Acetobacterium* and strains of *Syntrophococcus*, *Clostridium*, *Desulfotomaculum*, *Enterobacter*, and an anaerobic bacterium, strain TH-001, were unable to transform this compound. The O-demethylating ability of *E. limosum* was induced only with appropriate methoxylated benzoates but not with D-glucose, lactate, isoleucine, or methanol. Cross-acclimation and growth experiments with *E. limosum* showed a rate of metabolism that was an order of magnitude slower and showed no growth with either 4-methoxysalicylic acid (2-hydroxy-4-methoxybenzoic acid) or 4-anisic acid (4-methoxybenzoic acid) when adapted to 3-anisic acid. However, *A. woodii* NZva-16 showed slower rates and no growth with 3- or 4-methoxysalicylic acid when adapted to 3-anisic acid in similar experiments. The results clearly indicate a methyl rather than methoxy group removal mechanism for such reactions.

Methoxylated aromatic compounds are present as structural units in complex biopolymers like lignin, peat, and coal. The biological or thermochemical destruction of such materials produces methoxylated phenylpropionates, phenols, and benzoates (17, 20). Similar molecules are also found in the effluents of pulp and paper industries (11). These compounds enter the environment and often reside in anoxic habitats. Bacteria metabolizing aryl methoxy substituents have been isolated from a variety of anoxic environments, including sewage sludge (4, 18), sediments (1, 9), soil (10), and the termite gut (J. A. Breznak and J. M. Switzer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I132, p. 194), as well as the ovine (6) and bovine rumen (12, 13). Such substrates provide C$_1$ units to a physiologically diverse group of strict and facultative anaerobic bacteria. In strict anaerobes, such C$_1$ units are fixed into cell carbon, presumably via the autotrophic pathway of acetate biosynthesis (14), the metabolic end products being acetate (1, 4, 6, 10, 13) or butyrate (12). The facultative aerobes do not produce these fatty acid end products under anaerobic conditions (8). Although many such strict and facultative anaerobic bacteria can ferment other carbon substrates, the first step in the utilization of methoxylated aromatic compounds involves the conversion of the parent substrate to the hydroxylated derivative.

This reaction has been referred to as either a demethoxylation (1, 9, 12, 13, 15) or a demethylation (3, 4, 5, 7, 8, 22), implying two distinct mechanisms. The current literature generally supports the demethylation mechanism, but it has not been conclusively proven. The objectives of this study

were (i) to elucidate the mechanism of this reaction with one facultative bacterium, several strict anaerobic microorganisms, and a model aromatic substrate labeled with an ^{18}O-methoxy substituent and (ii) to determine the induction patterns for this reaction by using a model acetogenic bacterium grown on a variety of substrates.

Here we provide evidence that the anaerobic fermentation of aryl methoxy groups by strict anaerobic bacteria involves an O-demethylation mechanism and that this activity is under the control of an inducible enzyme system. This activity is only inducible with a suitable methoxylated aromatic substrate and not with methanol.

MATERIALS AND METHODS

Microorganisms and growth conditions. Table 1 shows the bacteria used in this study, where they were obtained, and references describing the growth media used for culture.

Incubation of substrates and kinetic experiments. All bacteria were tested for 3-anisic acid utilization in their respective growth media amended with 2 mM 3-anisic acid alone or with fructose as a cosubstrate, since some microorganisms only use the methoxybenzoates as electron acceptors (13). Bacteria degrading 3-anisic acid, confirmed by high-pressure liquid chromatography (HPLC), were transferred to the appropriate medium containing 3-[methoxy-^{18}O]anisic acid. The spent culture fluids of these incubations were then subjected to gas chromatography-mass spectral analysis.

Growth experiments were conducted with *Eubacterium limosum* and *Acetobacterium woodii* NZva-16. These microorganisms were transferred twice in a bicarbonate-buffered mineral medium (2) containing 3-anisic acid before growth with other methoxylated benzoates was tested. A 1% inoculum from the same population of cells in late exponential

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TABLE 1. Cultures tested for anaerobic metabolism
of 3-anisic acid

Culture	Source	Reference[a]
Acetobacterium woodii ATCC 29683	ATCC[b]	2
Acetobacterium woodii NZva-16	N. Pfennig	2
Clostridium pfennigii	M. P. Bryant	12
Desulfotomaculum orientis ATCC 19365	ATCC	10
Enterobacter cloacae DG-6	D. Grbić-Galić	2
Eubacterium limosum ATCC 8486	ATCC	2
Syntrophococcus sucromutans	M. P. Bryant	13
Dehalogenating consortium	J. M. Tiedje	18
Strain DCB-1	J. M. Tiedje	18
Strain TM-001	A. C. Frazer and L. Y. Young	4

[a] Includes description of growth medium and conditions.
[b] ATCC, American Type Culture Collection, Rockville, Md.

phase of growth was used for all substrates. Growth in identical media amended with 3-anisic acid, 4-anisic acid (4-methoxybenzoic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid), isovanillic acid (3-hydroxy-4-methoxybenzoic acid), 3-methoxysalicylic acid (2-hydroxy-3-methoxybenzoic acid), 4-methoxysalicylic acid (2-hydroxy-4-methoxybenzoic acid), or 3,5-dimethoxybenzoic acid at an initial concentration of 6.8 mM was measured directly as A_{600} in butyl rubber crimp-sealed culture tubes by using a Spectronic 21 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Culture tubes were pressurized at 138 kPa with a 20%:80% CO_2:N_2 headspace. E. limosum and A. woodii NZva-16 growth tubes were incubated without agitation in the dark at 37 and 30°C, respectively.

Kinetic experiments with E. limosum and A. woodii NZva-16 were performed in growth medium (2) amended with 10 μg of tetracycline (16) per ml and 1.6 mM of the test methoxylated benzoic acid. Serum bottles (50 ml) were incubated with rotary shaking (200 rpm) at 37 and 30°C for E. limosum and A. woodii NZva-16, respectively. Kinetic experiments with the dehalogenating consortium and strain DCB-1 (an isolated dehalogenating bacterium) were performed as described previously (3). Autoclaved inoculated cultures were controls for kinetic experiments, and uninoculated media were used as controls in growth experiments.

Induction and cross-acclimation experiments. A 1% inoculum of E. limosum in late exponential phase of growth with the substrates methanol plus acetate was used in O-demethylation induction experiments. Growth was measured directly in culture tubes containing medium (2) with one of the following supplements: 8.9 mM 3-anisic acid, 14.5 mM D-glucose, 19 mM lactate plus 9.5 mM acetate, 9.3 mM isoleucine plus 9.3 mM acetate, or 70 mM methanol plus 9.4 mM acetate. No growth was observed in control tubes without substrate addition, indicating little methanol carryover. At selected A_{600} values, replicate cultures were transferred to fresh media containing 1.6 mM 3-anisic acid and 10 μg of tetracycline per ml for specific activity measurements. The protein synthesis inhibitor tetracycline was added at concentrations 10 times greater than that required to completely inhibit growth of E. limosum and A. woodii (K. A. DeWeerd and J. M. Suflita, unpublished results) to prevent further growth of the cells during activity measurements.

Cross-acclimation experiments with E. limosum and A.

woodii NZva-16 were performed with cultures grown to an A_{600} of 0.25 and 0.2, respectively, and transferred to media amended with 1.2 mM of the appropriate methoxylated benzoic acid and 10 μg of tetracycline per ml. Rate measurements for induction and cross-acclimation experiments were performed within a 12-h incubation period, with samples periodically taken by syringe, oxidized by exposure to air to stop the reaction, and stored at 4°C for 48 h prior to analysis by HPLC. The aromatic compounds used in this study were all stable under the experimental conditions used. All degradation rates were linear, and growth was not observed during kinetic studies. Protein was measured directly in specific activity assay vessels at the initial and final samplings. Protein concentrations in assay vessels ranged from 34 to 47 and 12 to 20 μg/ml for E. limosum and A. woodii, respectively, in cross-acclimation experiments.

Analytical procedures. Samples of spent culture fluids to be analyzed by HPLC were centrifuged at 8,000 × g for 5 min with a microcentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) to remove particulates and cells. The HPLC conditions used are described in detail elsewhere (3). The product and substrate separations were obtained with a 5-μm C-18 reverse-phase column using an isocratic mobile phase mixture of 50 mM acetate buffer (pH 4.5) and acetonitrile (3:1). Gas chromatography-mass spectral analysis was performed on similar samples analyzed initially by HPLC to confirm the biodegradation of the parent substrate, i.e., 3-[methoxy-^{18}O]anisic acid. Samples were then acidified with concentrated HCl to pH 1 and extracted twice with an equal volume of diethyl ether. The ether phase was concentrated under a nitrogen stream, and trace H_2O was removed with anhydrous Na_2SO_4. The gas chromatography-mass spectra were obtained on an HP 5890 gas chromatograph fitted with an HP-1 capillary column (12 m by 0.2 mm by 0.33 μm) connected to an HP 5790 mass selective detector (all equipment from Hewlett-Packard Co., Palo Alto, Calif.). The electron impact detector was operated at 70 eV. The carrier gas for the gas chromatograph was helium at a flow rate of 1.5 ml/min. The gas chromatograph oven was temperature programmed from 45 to 250°C at 20°C/min. The injection and transfer line temperatures were 250 and 280°C, respectively. Standard compounds (100 ng) were injected to obtain authentic reference spectra.

Protein was measured by the method of Smith et al. (19), with bovine serum albumin as the standard. Cells were washed once with 10 mM PIPES buffer (piperazine-N, N'-bis(2-ethanesulfonic acid; pH 7) and digested with 0.1 N NaOH at 90°C for 30 min prior to protein determinations.

Chemicals. Labeled 3-[methoxy-^{18}O]anisic acid (98.3 atom% ^{18}O) was obtained from Merck & Co., Inc. Rahway, N.J. All other aromatic compounds used were obtained from Aldrich Chemical Co., Milwaukee, Wis.

RESULTS

The dehalogenating consortium, a dehalogenating bacterium, strain DCB-1, E. limosum, and A. woodii NZva-16 transformed 3-anisic acid to 3-hydroxybenzoic acid stoichiometrically with average degradation rates of 1.5, 3.2, 52.4, and 36.7 nmol/min per mg of protein, respectively. Rates differed by less than 15% in duplicate samples, and each determination included at least five datum points taken under nongrowth conditions. No subsequent degradation of the accumulated 3-hydroxybenzoate was observed. However, 3-anisic acid was not metabolized by strain TM-001, A. woodii ATCC 29683, Syntrophococcus sucromutans, Clos-
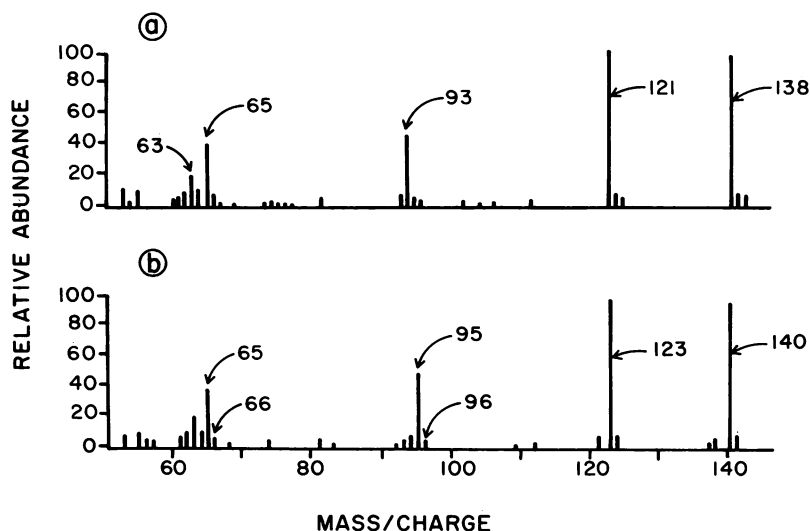
FIG. 1. (a) Mass spectrum of a 3-[hydroxy-^{16}O]hydroxybenzoic acid standard. The metabolite of 3-[methoxy-^{16}O]anisic acid degradation by the dehalogenating consortium, strain DCB-1, E. limosum, and A. woodii NZva-16 possessed the same major mass spectral fragments. (b) Mass spectrum of the 3-[methoxy-^{18}O]anisic acid metabolite produced by A. woodii NZva-16. A metabolite possessing identical major mass spectral fragments was also produced by the dehalogenating consortium, strain DCB-1, and E. limosum when they were grown with the labeled anisic acid.

tridium pfennigii, Desulfotomaculum orientis, or Enterobacter cloacae DG-6. These microorganisms were also tested with fructose as a cosubstrate but were again unable to metabolize 3-anisic acid.

The mass spectrum of the 3-[methoxy-^{18}O]anisic acid metabolite produced by the dehalogenating consortium, strain DCB-1, E. limosum, and A. woodii NZva-16 gave a parent ion at m/z 140 and major mass fragments at m/z 123 and 95 (Fig. 1b). These mass spectral features were 2 mass units greater than 3-[hydroxy-^{16}O]hydroxybenzoic acid standard or the metabolite of 3-[methoxy-^{16}O]anisic acid produced by these bacteria (Fig. 1a). The peaks at 123 and 95 or 121 and 93 undoubtedly reflect the fragmentation of an —OH and —COOH group from the ^{18}O and ^{16}O parent substrates, respectively. This indicates that the heavy oxygen isotope remained intact and that the ether linkage was cleaved between the aryl oxygen and the methyl group. Analysis of mass spectra revealed that the hydroxylated metabolite produced by cultures originally fed labeled anisic acid did not possess detectable quantities of 3-[hydroxy-^{16}O]hydroxybenzoic acid (Fig. 1b). Therefore, the parent material was essentially free of an ^{16}O contaminant, and exchange of the heavy isotope with water was clearly minimal.

Induction experiments. The antibiotic resistance of E. limosum and A. woodii NZva-16 was tested with tetracycline and streptomycin. Both bacteria were susceptible to these antibiotics with 1 µg of tetracycline and 10 µg of streptomycin per ml sufficient to inhibit growth with various substrates (data not shown). E. limosum was cultured on D-glucose, lactate, isoleucine, methanol, or 3-anisic acid, and the specific activity of 3-anisic acid degradation was measured at various growth stages for all these substrates. An example of such an experiment is shown (Fig. 2a). E. limosum showed a proportional increase in specific activity of 3-anisic acid degradation with time when grown with this compound. However, no 3-anisic acid metabolism was observed when E. limosum was grown with methanol (Fig. 2b), D-glucose, lactate, or isoleucine.

Growth and cross-acclimation experiments. E. limosum and A. woodii NZva-16 were transferred twice with 3-anisic acid

as the growth substrate and then tested for the ability to grow with other methoxylated benzoates. E. limosum showed the shortest lag and fastest growth with 3-anisic acid and vanillic acid, followed by 3-methoxysalicylic acid, isovanillic acid, and 3,5-dimethoxybenzoic acid, respectively (Fig. 3a). E. limosum failed to grow on 4-anisic acid or 4-methoxysalicylic acid. A. woodii NZva-16 showed the shortest lag and fastest growth with vanillic or isovanillic acid, followed by 3,5-dimethoxybenzoic acid, 4-anisic acid, and 3-anisic acid, respectively (Fig. 3b). No growth of A. woodii NZva-16 was observed on 3-methoxysalicylic acid or 4-methoxysalicylic acid. Growth yields of both bacteria were doubled with 3,5-dimethoxybenzoic acid compared with the growth yield with monomethoxylated benzoic acids, indicating that both methoxy groups were utilized as C_1 growth substrates.

Cross-acclimation of 3-anisic acid-grown E. limosum cells to other methoxybenzoates showed comparable rates of degradation of 3-anisic acid, vanillic acid, isovanillic acid, 3,5-dimethoxybenzoic acid, and 3-methoxysalicylic acid (Fig. 4 and Table 2). However, the rates of 4-methoxysalicylic acid and 4-anisic acid were less by an order of magnitude (Table 2). When E. limosum was grown with vanillic acid, the rate of 3-anisic acid degradation was comparable to that attained with 3-anisic acid-grown cells. However, similar experiments with isovanillic acid-grown cells showed a decreased 3-anisic acid degradation rate (Table 2).

Cross-acclimation of A. woodii NZva-16 grown with 3-anisic acid to other methoxybenzoates showed comparable rates with 3-anisic acid, 4-anisic acid, vanillic acid, isovanillic acid, and 3,5-dimethoxysalicylic acid. The rate of 3-methoxysalicylic acid utilization was an order of magnitude lower, and 4-methoxysalicylic acid was not metabolized (Table 2).

DISCUSSION

The microorganisms that converted 3-anisic acid to 3-hydroxybenzoic acid stoichiometrically (3) were the dehalo-
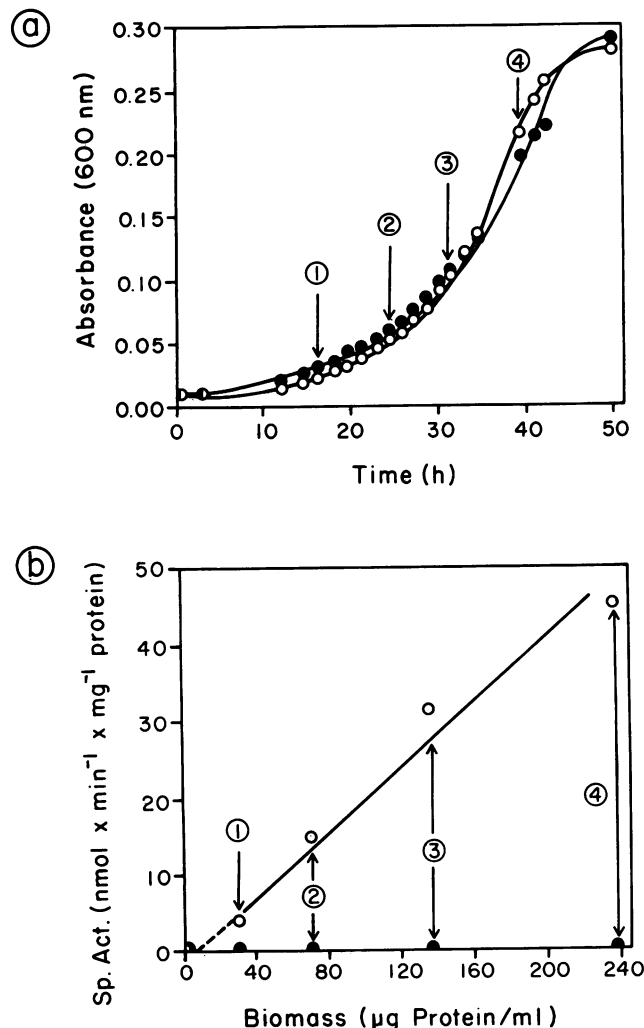
FIG. 2. (a) Growth of *E. limosum* with 8.9 mM 3-anisic acid (O) or 70 mM methanol plus 9.4 mM acetate (●). Numbered arrows indicate times at which specific activity measurements were made. (b) Specific activities of 3-anisic acid degradation at various stages of growth on the two substrates plotted against biomass, determined by protein concentration. Activities were measured in the presence of tetracycline (10 μg/ml). Numbered arrows correspond to those sampling times presented in panel a.

genating consortium, a dehalogenating bacterium, strain DCB-1, *E. limosum*, and *A. woodii* NZva-16. This reaction was previously shown to obey Michaelis-Menten kinetics, indicating the involvement of an enzyme system (3). The rates of 3-anisic acid degradation were an order of magnitude greater for *E. limosum* and *A. woodii* NZva-16 than for either the dehalogenating consortium or strain DCB-1. The faster degradation rates of *A. woodii* NZva-16 and *E. limosum* correlated with the fact that these bacteria grew much faster than the consortium or strain DCB-1. The last two cultures are known to be extremely slow growing (18).

It is interesting to note that other organisms tested were unable to metabolize 3-anisic acid. Most of these cultures were reported to degrade compounds very similar to 3-anisic acid, such as ferulic acid, vanillic acid, syringic acid, or 3,4,5-trimethoxybenzoic acid (4, 7, 10, 12, 13). We successfully cultured many of the bacteria unable to use 3-anisic acid with either vanillic acid, ferulic acid, or 3,5-dimethoxy-
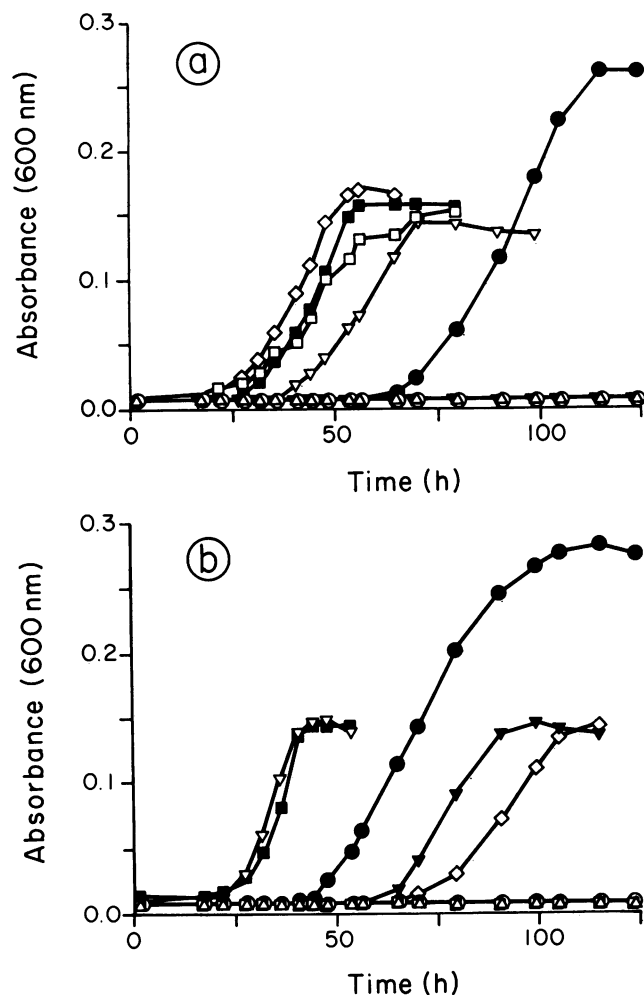

FIG. 3. Growth of *E. limosum* (a) or *A. woodii* NZva-16 (b) at 37 or 30°C, respectively. Both bacteria were cultured with 6.8 mM 3-anisic acid (◇), 4-anisic acid (▼), vanillic acid (▽), isovanillic acid (■), 3-methoxysalicylic acid (□), 4-methoxysalicylic acid (△), 3,5-dimethoxybenzoic acid (●), or no substrate addition (O).

benzoic acid (data not shown). The failure of these organisms to metabolize 3-anisic acid may be due to several reasons, including an inability to transport the substrate, strict O-demethylase specificity, or the failure to induce the requisite enzymes.

The specific activity for 3-anisic acid degradation by *E. limosum* increased with biomass when the bacterium was grown on 3-anisic acid but not when it was grown on methanol or other substrates. This finding indicates that
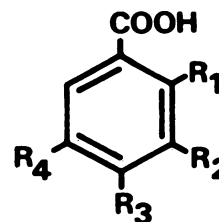

FIG. 4. Generalized structure of methoxybenzoic acids listed in Table 2.

TABLE 2. Cross-acclimation of *E. limosum* and *A. woodii* NZva-16 to various methoxybenzoic acids

Growth substrate[a]	Test substrate	Test substrate substitution pattern[b]	Sp act (nmol/min per mg of protein)[c]	
			E. limosum	*A. woodii* NZva-16
3-Anisic acid	3-Anisic acid	R_1,R_3,R_4 = H; R_2 = OCH_3	52.4 ± 0.2	36.7 ± 5.3
3-Anisic acid	4-Anisic acid	R_1,R_2,R_4 = H; R_3 = OCH_3	1.5 ± 0.7	55.1 ± 4.9
3-Anisic acid	Vanillic acid	R_1,R_4 = H; R_2 = OCH_3; R_3 = OH	45.5 ± 0.4	59.4 ± 4.5
3-Anisic acid	Isovanillic acid	R_1,R_4 = H; R_2 = OH; R_3 = OCH_3	31.4 ± 2.5	35.4 ± 1.3
3-Anisic acid	3,5-Dimethoxybenzoic acid	R_1,R_3 = H; R_2,R_4 = OCH_3	42.3 ± 3.5	47.4 ± 4.2
3-Anisic acid	3-Methoxysalicylic acid	R_1 = OH; R_2 = OCH_3; R_3,R_4 = H	39.7 ± 8.1	3.1[d]
3-Anisic acid	4-Methoxysalicylic acid	R_1 = OH; R_2,R_4 = H; R_3 = OCH_3	2.6 ± 0.6	0
Vanillic acid	3-Anisic acid	R_1,R_3,R_4 = H; R_2 = OCH_3	46.9 ± 6.4	ND[e]
Isovanillic acid	3-Anisic acid	R_1,R_3,R_4 = H; R_2 = OCH_3	11.7 ± 0.1	ND

[a] Cells were grown with 10 mM growth substrate and transferred to media containing 1.6 mM test substrate and 10 μg of tetracycline per ml.
[b] Refer to Fig. 4.
[c] Values are represented as means ± the standard deviations.
[d] Metabolism of test substrate only observed in one replicate.
[e] Specific activity not determined.

another methyl transferase, separate from the methanol methyl transferase, must be produced. Furthermore, O-demethylation activity by this bacterium is inducible. The inability of methanol to act as an inducer and the results of the mass spectral experiments imply that this alcohol may not be the initial C_1 intermediate from the catabolism of methoxylated aromatics by these strict acetogenic bacteria, as was previously hypothesized (1). This conclusion was also reached by Frazer and Young in experiments involving the utilization of a ^{14}C-labeled methoxy substituent of vanillic acid under anaerobic conditions (5).

Comparisons of the growth of *E. limosum* and *A. woodii* NZva-16 show a difference in their preferences for methoxylated benzoic acid substrates. *E. limosum* generally grew better with *meta*-substituted methoxy benzoic acids than with *para*-substituted isomers (Fig. 3a). This microorganism also failed to grow with either 4-anisic acid or 4-methoxysalicylic acid. *A. woodii* NZva-16 grew much better with vanillic or isovanillic acids even though it was initially adapted to grow on 3-anisic acid. This bacterium failed to grow with 3- or 4-methoxysalicylic acids (Fig. 3b). Having both *meta* and *para* positions occupied may facilitate the more efficient removal of arylmethoxy groups by this organism. However, a hydroxyl group *ortho* to the carboxylic acid moiety appears to inhibit utilization of these methoxy groups.

Cross-acclimation studies with *E. limosum* suggest that the enzyme systems responsible for 3-anisic acid O-demethylation activity also are capable of metabolizing other methoxylated benzoic acids. The specific activities were comparable for most of the methoxylated benzoic acids tested except 4-methoxysalicylic acid and 4-anisic acid, which were decreased by an order of magnitude. *E. limosum* grown with vanillic acid showed rates of 3-anisic acid O-demethylation comparable to those of 3-anisic acid-grown cells. However, when this bacterium was cultured with isovanillic acid, 3-anisic acid metabolism was slower than that in cells cultured with 3-anisic acid. Growth of this organism with the *para*-substituted methoxy group of isovanillic acid may induce a different methyl transferase with reduced activity for *meta* isomers.

A. woodii NZva-16 showed less of a preference for *meta*-substituted methoxy groups than *E. limosum*. However, the methoxysalicylic acids were not metabolized by *A. woodii* nearly as well as the other methoxybenzoic acids tested. The *ortho* hydroxy group may sterically hinder the O-demethyl-

ation ability of this organism. The results of cross-acclimation studies with *E. limosum* and *A. woodii* NZva-16 generally correlated with the growth experiments. No growth of *E. limosum* was observed with 4-anisic acid or 4-methoxysalicylic acid, and no growth of *A. woodii* was observed with 3- or 4-methoxysalicylic acid. The same compounds had specific activities an order of magnitude less than those of the other methoxybenzoates. The rate of aryl methoxy group metabolism may be the step limiting microbial growth.

The mass spectral evidence clearly indicates that a methyl transfer is involved in the mechanism of aryl O-demethylation by strict anaerobic microorganisms metabolizing 3-anisic acid. It will be of interest to determine the mechanism of metabolism of aryl methoxy substituents by facultative anaerobes grown aerobically or anaerobically. In *Pseudomonas* sp., the substrate specificity for methoxybenzoate catabolism is different under aerobic and anaerobic conditions (21), perhaps suggesting that different enzymes or mechanisms are used.

The O-demethylation reactions catalyzed by *E. limosum* and *A. woodii* NZva-16 extend what is known about the metabolism of acetogenic eubacteria in two ways. First, when acetogens are grown on methanol, a vitamin B_{12}-dependent methyl transferase activates the one carbon unit for its eventual conversion to the methyl moiety of acetate (14, 23). The data presented here on the induction of an O-demethylating activity for aryl methoxy groups suggest a new mode of entry for one-carbon units into the acetogenic pathway. Second, if the total synthesis of acetate from the methyl moiety of 3-anisic acid in media free of CO_2 and bicarbonate occurs, it would suggest the disproportionation of the one-carbon units to the methyl and carbonyl groups of acetate. Thus, the methyl moiety of 3-anisic acid would serve as the source of both electrons and carbon, analogous to the disproportionation of methanol by these organisms (14, 23). In a preliminary experiment, *E. limosum* was cultured in CO_2-free mineral media with 3-anisic acid, which suggested this possibility (K. A. DeWeerd and J. M. Suflita, unpublished results).

ACKNOWLEDGMENTS

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