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Aerobic and Anaerobic Catabolism of Vanillic Acid and Some Other Methoxy-Aromatic Compounds by *Pseudomonas* sp. Strain PN-1

BARRIE F. TAYLOR

Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, Florida 33149-1098

Received 13 June 1983/Accepted 6 September 1983

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) supported the anaerobic (nitrate respiration) but not the aerobic growth of *Pseudomonas* sp. strain PN-1. Cells grown anaerobically on vanillate oxidized vanillate, *p*-hydroxybenzoate, and protocatechuic acid (3,4-dihydroxybenzoic acid) with O_2 or nitrate. Veratric acid (3,4-dimethoxybenzoic acid) but not isovanillic acid (3-hydroxy-4-methoxybenzoic acid) induced cells for the oxic and anoxic utilization of vanillate, and protocatechuate was detected as an intermediate of vanillate breakdown under either condition. Aerobic catabolism of protocatechuate proceeded via 4,5-*meta* cleavage, whereas anaerobically it was probably dehydroxylated to benzoic acid. Formaldehyde was identified as a product of aerobic demethylation, indicating a monooxygenase mechanism, but was not detected during anaerobic demethylation. The aerobic and anaerobic systems had similar but not identical substrate specificities. Both utilized *m*-anisic acid (3-methoxybenzoic acid) and veratrate but not *o*- or *p*-anisate and isovanillate. Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), 3-*O*-methylgallic acid (3-methoxy-4,5-dihydroxybenzoic acid), and 3,5-dimethoxybenzoic acid were attacked under either condition, and formaldehyde was liberated from these substrates in the presence of O_2. The anaerobic demethylating system but not the aerobic enzyme was also active upon guaiacol (2-methoxyphenol), ferulic acid (3-[4-hydroxy-3-methoxyphenyl]-2-propenoic acid), 3,4,5-trimethoxycinnamic acid (3-[3,4,5-trimethoxyphenyl]-2-propenoic acid), and 3,4,5-trimethoxybenzoic acid. The broad specificity of the anaerobic demethylation system suggests that it probably is significant in the degradation of lignoaromatic molecules in anaerobic environments.

Lignin is not easily attacked by microorganisms in anaerobic environments (7, 18), although a slow but significant rate of anoxic destruction was detected recently in studies with very long incubation periods (R. Benner, A. E. MacCubbin, and R. E. Hodson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, N80, p. 236). In contrast to lignin, simple methoxy-aromatic compounds, such as vanillic, ferulic, and syringic acids, that are released during its aerobic catabolism (4) are readily attacked by methanogenic microbial consortia from a variety of habitats (2, 8, 9, 12, 18; D. E. Grbic-Galic and L. Y. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, O20, p. 199). Bache and Pfennig (1) recently isolated strains of *Acetobacterium woodii* that anaerobically demethylated a wide range of methoxylated aromatic structures without attacking the benzene nucleus, and pure cultures of sulfate-respiring bacteria which degrade phenylacetate and phenylpropionate but not methoxy-aromatic compounds have been described (F. Widdel, Ph.D. thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1980). This report documents the anoxic breakdown of vanillic acid and some methoxy-aromatic compounds by a facultatively anaerobic bacterium, with destruction of both the methoxy and aromatic moieties.

MATERIALS AND METHODS

Culture methods. The isolation and characteristics of *Pseudomonas* sp. strain PN-1 and the composition of its growth medium have been described previously (17). The organism was grown at 30°C in a mineral salts medium which usually contained 5 mM vanillate as the carbon and energy source and 20 mM KNO_3 to allow anaerobic growth. Veratrate and other aromatic compounds were used at 5 mM; succinate was used at 20 mM. Anaerobic cultures were grown in 18-ml tubes and 180-ml bottles which were filled with medium and closed with rubber-lined screw caps. Cells were grown aerobically in 100-ml batches of medium (lacking KNO_3) contained in 500-ml Erlenmeyer flasks, with rotary shaking at 200 rpm. Growth was followed with a Klett-Summerson colorimeter (red filter; transmission 640 to 700 nm). Cells were harvested by centrifugation

at room temperature (12,000 × g, 10 min), washed, and suspended in 0.05 M phosphate buffer (pH 7.0).

Respirometry. Aerobic respiration was measured at 30°C either manometrically with a Warburg apparatus (Gilson Medical Electronics, Middleton, Wis.) or polarographically with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). In the latter method, the cells were suspended in 5 ml of 0.05 M phosphate buffer (pH 7.0), and after endogenous activity was recorded for about 5 min, 0.2 or 0.5 μmol of substrate was added. Constant volume manometry was used to measure both aerobic and anaerobic respiration. The Warburg flasks usually contained the following: cells in phosphate buffer (pH 7.0), 100 μmol; substrate (in a side arm), 3 μmol; $NaNO_2$ (in a second side arm), 40 μmol (anaerobic respiration); 20% KOH, 0.1 ml (center well); and water to 3.0 ml. Chloramphenicol (33 μg/ml) was added to prevent the synthesis of new enzymes. In anaerobic incubations, the flasks were gassed for 10 min with N_2 before the manometers were closed. After temperature equilibration, the substrates and $NaNO_2$ were added from the side arms at zero time. At the end of the incubations, the flasks were removed from the manometers and cooled in ice. The flask contents were transferred with Pasteur pipettes to tubes that were centrifuged at 5°C to remove intact cells. The cell-free supernatants were either examined immediately by high-pressure liquid chromatography (HPLC) or stored frozen until analyzed.

Chromatography. Aromatic compounds were identified and quantified directly relative to standard compounds in aqueous solution by reverse-phase HPLC. A Waters Associates (Milford, Mass.) chromatograph system was used with a radial compression column (10 cm by 0.8-cm inside diameter) containing octyldecylsilane on silica particles (diameter, 10 μm). The solvent system was either (i) methanol–water–1 M sodium acetate buffer (pH 4.3) (30:69:1 [vol/vol/vol]) or (ii) methanol-water-acetic acid (30:65:5, [vol/vol/vol]) adjusted to pH 3.0 with NaOH. Solvent flow rates were 2 or 3 ml/min with absorbance detection at 254 nm. Examples of retention times with the first solvent system, at a flow rate of 2 ml/min, were as follows: vanillic acid, 3 min 50 s; protocatechuic acid, 3 min 45 s; *p*-hydroxybenzoic acid, 3 min 40 s; *m*-hydroxybenzoic acid, 2 min 45 s; benzoic acid, 7 min; guaiacol, 11 min 10 s; catechol, 5 min 50 s; veratric acid, 5 min 50 s; isovanillic acid, 3 min 40 s; vanillin, 7 min 35 s; *o*-anisic acid, 4 min 25 s; *m*-anisic acid, 7 min 5 s; *p*-anisic acid, 10 min 10 s. Typical retention times with the second solvent system, at a flow rate of 2 ml/min, were as follows: vanillic acid, 3 min 25 s; protocatechuic acid, 2 min 20 s; *p*-hydroxybenzoic acid, 3 min 10 s; *m*-hydroxybenzoic acid, 3 min 35 s; benzoic acid, 8 min 55 s; *o*-anisic acid, 5 min 35 s; veratric acid, 6 min 15 s; isovanillic acid, 3 min 40 s; vanillin, 4 min 35 s; vanillyl alcohol, 2 min 35 s; 3,4-dihydroxybenzaldehyde, 2 min 55 s; catechol, 6 min 30 s; 3,5-dimethoxybenzoic acid, 14 min 40 s (9 min 25 s at 3 ml/min); syringic acid, 3 min 35 s; 3,4,5-trimethoxybenzoic acid, 7 min 20 s; 3-*O*-methylgallic acid, 2 min 25 s; gallic acid, 1 min 50 s; 3,5-dihydroxybenzoic acid (DHB), 2 min; 3,4,5-trimethoxycinnamic acid, 12 min 15 s; ferulic acid, 5 min 10 s; caffeic acid, 3 min 10 s; *p*-hydroxycinnamic acid, 4 min 25 s; gentisic acid, 2 min 10 s.

Methanol and formaldehyde were quantified by gas-liquid chromatography of aqueous samples with an instrument equipped with flame ionization detectors (model 2700; Varian Associates, Walnut Creek, Calif.). The gas flow rates were as follows: N_2 (carrier), 30 ml/min; H_2, 30 ml/min; air, 300 ml/min. Porapak R (80/100 mesh) in a stainless steel column (183 cm by 0.3-cm outer diameter) was used at 150°C. Methanol and formaldehyde had identical retention times in gas-liquid chromatography, and so formaldehyde was also assayed by conversion to its 2,4-dinitrophenylhydrazone (DNP) and quantified by HPLC (13). A 0.5-ml amount of 0.2% (wt/vol) 2,4-dinitrophenylhydrazine in 2 N HCl was added to 1 ml of sample and incubated for 1 h at 25°C. Samples containing nitrite were treated with 0.05 ml of 2 M sulfamic acid before 2,4-dinitrophenylhydrazine was added to prevent its destruction with nitrous acid. After incubation, the formaldehyde-DNP was extracted twice with 1-ml portions of toluene with a Vortex mixer. The toluene layers were retained and assayed for formaldehyde-DNP contents by HPLC on the octyldecylsilane column used above. A solvent system of methanol-water (2:1 [vol/vol]) at 2 ml/min was used with detection at 254 nm. Standard formaldehyde-DNP was prepared by adding excess 37% formaldehyde to 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl, filtering off the precipitate on a Whatman GFF filter, and recrystallizing the product in ethanol. The retention time for formaldehyde-DNP on HPLC was about 4 min.

Other methods. Some substrate transformation experiments were carried out with 50-ml Erlenmeyer flasks or 30-ml serum bottles rather than Warburg flasks. The flasks or bottles were incubated either aerobically or anaerobically after being closed with recessed butyl rubber stoppers and gassed with N_2 at 30°C with rotary shaking (200 rpm). The flasks or bottles usually contained cells suspended in 5 ml of 0.05 M phosphate buffer (pH 7.0) with 1 to 4 mM substrates and, when used, 20 mM KNO_3. Samples were removed from the flasks or bottles with syringes, added to 9 volumes of ice-cold water, and then filtered through Whatman GFF filters to remove whole cells before analysis by HPLC.

The biuret reaction, in which intact cells are used (11) with bovine serum albumin as the standard, was employed to relate Klett units to protein contents. Sulfamic acid was used when it was necessary to remove nitrite from samples before HPLC analysis (3). Chemicals were routinely obtained from either Aldrich Chemical Co. (Milwaukee, Wis.) or Sigma Chemical Co. (St. Louis, Mo.). Organic solvents were supplied by Burdick and Jackson (Muskegon, Mich.). UV-visible spectra were obtained with a Hewlett-Packard (Palo Alto, Calif.) model 8450A spectrophotometer with 1-cm light-path cuvettes.

RESULTS

Growth experiments. Vanillate and *m*-anisate supported anaerobic but not aerobic growth. Vanillin (4-hydroxy-3-methoxybenzaldehyde) and vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) allowed slow aerobic or anaerobic growth, and their use was confirmed by HPLC. The following methoxy-aromatic compounds did not support either oxic or anoxic growth: piper-

TABLE 1. Aerobic oxidation of aromatic compounds

Substrate	Oxygen uptake (nmol/min per mg of protein)[a]			
	p-Hydroxybenzoate[b]	Vanillate[c]	Succinate + veratrate[d]	Succinate + isovanillate[d]
Vanillate	2	89	21	1
Protocatechuate	67	106	10	5
p-Hydroxybenzoate	115	156	10	12
m-Hydroxybenzoate	20	28	0	1
Benzoate	7	23	4	4
Veratrate	NT[e]	8	2	0
Isovanillate	NT	2	0	0

[a] Determined polarographically.
[b] Grown aerobically on 5 mM *p*-hydroxybenzoate.
[c] Grown anaerobically on 5 mM vanillate.
[d] Grown aerobically on 20 mM succinate with either 5 mM veratrate or 5 mM isovanillate.
[e] NT, Not tested.

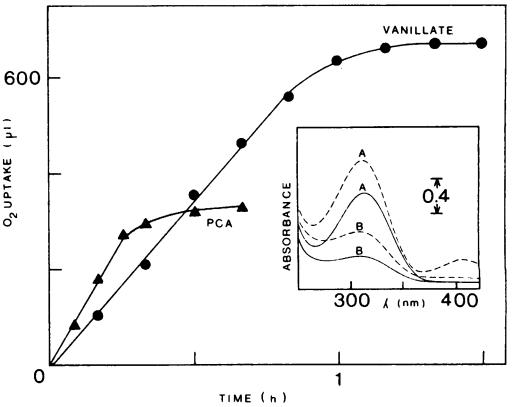

FIG. 1. Aerobic oxidation of vanillate and protocatechuate (PCA) by intact cells grown anaerobically on vanillate. Warburg flasks contained 14.2 mg of cell protein and 10 µmol of substrate; endogenous O_2 uptake rate (no substrate) was subtracted. Symbols: ▲, protocatechuate; ●, vanillate. Inset shows spectra of the products from protocatechuate (A) and vanillate (B) at pH 9.5 (------) and pH 2 (——).

onylate, isovanillate, veratrate, *o*-anisate, *p*-anisate, guaiacol, 3,5-dimethoxybenzoate, 3,4,5-trimethoxybenzoate, syringate, ferulate, and 3,4,5-trimethoxycinnamate.

Vanillate metabolism. Vanillate (5 mM) did not allow aerobic growth and inhibited aerobic growth on *p*-hydroxybenzoate or succinate. However, cells grown anaerobically on vanillate were induced for the aerobic oxidation of vanillate, as were cells grown aerobically on succinate plus veratrate (Table 1). Approximately 5% of the veratrate had been converted into isovanillate when cells grown aerobically on succinate plus veratrate were harvested in the late exponential phase of growth. Aerobic growth on succinate in the presence of isovanillate instead of veratrate produced cells that only weakly oxidized vanillate and other aromatic compounds (Table 1). Cells grown anaerobically on vanillate oxidized vanillate and protocatechuate with an uptake of ca. 3.0 and 1.5 µmol of oxygen, respectively, per µmol of substrate (Fig. 1). The oxidation products were yellow under alkaline conditions but colorless upon acidification. The products from both protocatechuate and vanillate had an absorption maximum at 312 nm (pH 9.5), and another peak at 410 nm was also detected at this pH from the oxidation of protocatechuate (Fig. 1).

The anoxic catabolism of vanillate required nitrate, nitrite, or N_2O. Figure 2 shows the nitrate-dependent use of vanillate by cells grown anaerobically on succinate plus veratrate. Cells grown anaerobically or aerobically on either succinate or succinate plus isovanillate were not induced for either the oxic or anoxic demethylation of vanillate. These results establish veratrate but not isovanillate as an inducer of the aerobic and anaerobic demethylation systems.

Cells grown anaerobically on vanillic acid were induced for the anaerobic catabolism of a range of simple aromatic compounds (Fig. 3). Analysis by HPLC confirmed the complete con-

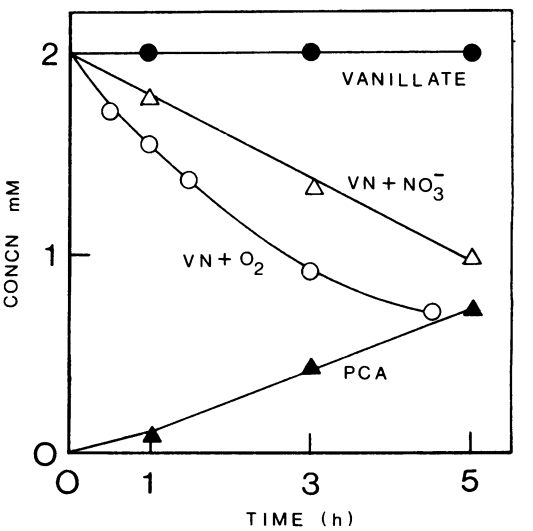

FIG. 2. Catabolism of vanillate (VN) under anerobic and aerobic conditions by cells grown anaerobically on succinate in the presence of veratrate. Flasks contained 10.7 mg of cell protein. Symbols: ●, anaerobic vanillate; ○, vanillate + O_2; △, anaerobic vanillate + KNO_3 (20 mM); ▲, protocatechuate (PCA) produced from vanillate + KNO_3.

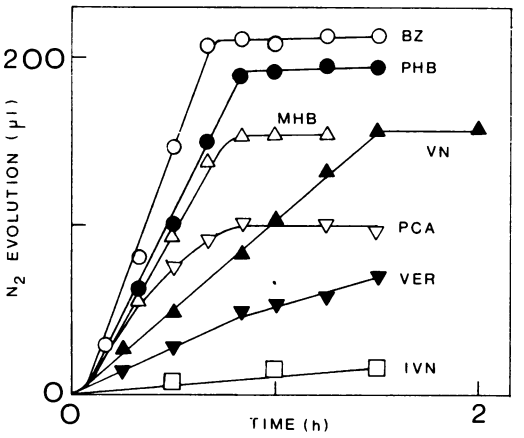

FIG. 3. Anaerobic oxidation of aromatic compounds by cells grown anaerobically on vanillate. Warburg flasks contained 6.1 mg of cell protein, 3 μmol of substrate, and 40 μmol of $NaNO_2$. Endogenous respiration was subtracted. Symbols: ○, benzoate (BZ); ●, *p*-hydroxybenzoate (PHB); △, *m*-hydroxybenzoate (MHB); ▲, vanillate (VN); ▽, protocatechuate (PCA); ▼, veratrate (VER); □, isovanillate (IVN).

sumption of vanillate, protocatechuate, benzoate, and the 3- and 4-hydroxybenzoic acids (Fig. 3). About 75% of the veratrate was used and quantitatively converted to isovanillate, which was not metabolized. When nitrate was present, *m*-anisate was demethylated to 3-hydroxybenzoate under anaerobic conditions by cells grown anaerobically on vanillate. Formaldehyde accumulated during the aerobic but not the anaerobic breakdown of vanillate (Table 2), but methanol was not detected under either condition.

Metabolism of other methoxy-aromatic compounds. Cells grown anaerobically on vanillate were induced for the oxic and anoxic catabolism of vanillyl alcohol, vanillin, and vanillic acid. Under aerobic conditions, vanillin, vanillate, and protocatechuate were detected as intermediates from vanillyl alcohol, vanillin, and vanillate, respectively (Table 3).

Veratrate and *m*-anisate (see above) were anaerobically transformed to isovanillate and 3-hydroxybenzoate, respectively. The same reactions also occurred aerobically (Table 4), and polarographic experiments showed that only *m*-anisate, not *o*- or *p*-anisate, was aerobically attacked.

More methoxy-aromatic compounds were demethylated under anaerobic than aerobic conditions (Table 5). Ferulate and guaiacol were quantitatively converted to caffeic acid (3-[3,4-dihydroxyphenyl]-2-propenoic acid) and catechol, respectively; DHB was detected as an intermediate in the anaerobic breakdown of 3,4,5-trimethoxybenzoate, and 3,4,5-trimethoxycinnamate was catabolized to yield several unidentified intermediates. None of these substrates were attacked aerobically, and *o*-anisate was not transformed under either condition.

Although 3,4,5-trimethoxybenzoic acid was only anaerobically attacked, the related compounds syringic acid, 3-*O*-methylgallate, and 3,5-dimethoxybenzoic acid were subject to both anaerobic and aerobic demethylation (Fig. 4 and 5). Cells grown on vanillate and incubated anaerobically sequentially demethylated syringate to 3-*O*-methylgallate and gallate, followed by dehydroxylation of the gallic acid to DHB (Fig. 4).

TABLE 3. Metabolism of vanillin and vanillyl alcohol by cells grown anaerobically on vanillate[a]

Incubation condition	Substrate	Fall in substrate concn (mM)	Intermediates detected (μM)
Aerobic[b]	Vanillate	1.42	Protocatechuate (28)
	Vanillin	0.53	Vanillate (35)
	Vanillyl alcohol	0.37	Vanillin (108)
Anaerobic[c]	Vanillate	1.47	ND[d]
	Vanillin	2.00	ND
	Vanillyl alcohol	1.21	ND

[a] Cells were incubated for 2 h with 2 mM substrates and chloramphenicol.
[b] Cell concentration of 0.78 mg of protein per ml.
[c] Cell concentration of 1.72 mg of protein per ml.
[d] ND, None detected.

TABLE 2. Formaldehyde production during vanillate oxidation[a]

Incubation condition	Fall in vanillate concn (mM)	HCHO formation (mM)
Aerobic	1.99	0.64
Anaerobic	1.78	0.00

[a] Cells (4.2 mg of protein per ml) were grown anaerobically on vanillate and incubated with 2 mM vanillate for 2 h at 30°C in the presence of chloramphenicol. Anaerobic incubation was under N_2 with 20 mM KNO_3.

TABLE 4. Aerobic transformation of *m*-anisate and veratrate by cells grown anaerobically on vanillate[a]

Substrate	Fall in substrate concn (mM)	Product	Product concn (mM)
Vanillate	1.78	None detected	
m-Anisate	0.95	3-Hydroxybenzoate	0.48
Veratrate	0.83	Isovanillate	0.60

[a] Cells (0.99 mg of protein per ml) were incubated for 3 h with 2 mM substrate and chloramphenicol.

TABLE 5. Anaerobic and aerobic utilization of methoxy-aromatic compounds by intact cells

Incubation condition	Compound	Final concn (mM)	Product and concn (mM)
Anaerobic[a]	Vanillate	0.00	None detected
	Ferulate	0.00	Caffeate (0.94)
	3,4,5-Trimethoxycinnamate	0.05	Several, unidentified
	3,4,5-Trimethoxybenzoate	0.16	DHB (0.66)
	Guaiacol	0.45	Catechol (0.50)
	o-Anisate	1.00	None
Aerobic[b]	Vanillate	0.00	None detected

[a] Cells (1.59 mg of protein per ml) were grown anaerobically on vanillate and then incubated anaerobically with 1 mM substrates, chloramphenicol, and 20 mM KNO_3 for 4 h.

[b] Cells (2.98 mg of protein per ml) were grown aerobically on succinate plus veratrate and then incubated aerobically with 2 mM substrates and chloramphenicol for 26 h. No utilization or products were detected from ferulate, 3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate, guaiacol, or *o*-anisate.

3,5-Dimethoxybenzoate was also degraded in the absence of O_2 (Table 6), with the formation of DHB and an unidentified product that accumulated before DHB, presumably 3-hydroxy-5-methoxybenzoic acid, assuming that demethylation proceeds in a stepwise fashion.

Syringate, 3-*O*-methylgallate, and 3,5-dimethoxybenzoate were aerobically attacked, with the production of formaldehyde (Tables 6 and 7). Furthermore, these substrates were utilized by cells grown aerobically on succinate plus veratrate (Fig. 5; Table 6); this result eliminated a possible contribution from the anaerobic demethylating system since such cells were not induced for the anaerobic degradation of vanillate. DHB was not detected during aerobic metabolism of the three methoxy-aromatic substrates, although a compound that was probably 3-hydroxy-5-methoxybenzoic acid was generated from 3,5-dimethoxybenzoic acid.

DISCUSSION

Vanillate was catabolized either aerobically or anaerobically by *Pseudomonas* sp. strain PN-1, but it only supported anaerobic development. Vanillate even inhibited aerobic growth on *p*-hydroxybenzoate, and possibly formaldehyde generated from the aerobic attack of vanillate is the toxic factor. The spectral characteristics of the products from the aerobic breakdown of vanillate and protocatechuate support earlier data that the aerobic *meta*-cleavage pathway for aromatic compounds operates in *Pseudomonas* sp. strain PN-1 (17); the absorption maxima at 410 and 312 nm correspond to 2-hydroxy-4-carboxy-*cis*,*cis*-muconic semialdehyde (15) and its immediate oxidation product, 2-hydroxy-4-carboxy-*cis*,*cis*-muconic acid (16). *Pseudomo-*

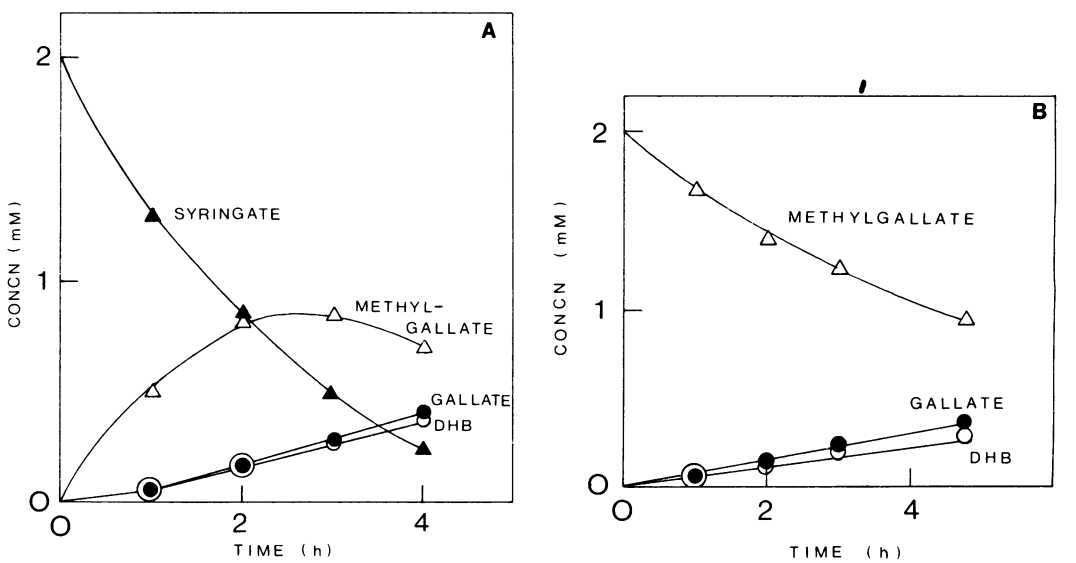

FIG. 4. Anaerobic metabolism of syringate and 3-*O*-methylgallate by cells grown anaerobically on vanillate. (A) Syringate metabolism. Cells (1.72 mg of protein per ml) were incubated with 2 mM syringate, chloramphenicol, and 20 mM KNO_3. (B) 3-*O*-methylgallate metabolism. Cells (0.59 mg of protein per ml) were incubated with 2 mM 3-*O*-methylgallate, chloramphenicol, and 20 mM KNO_3. Symbols: ▲, syringate; △, 3-*O*-methylgallate; ●, gallate; ○, DHB.

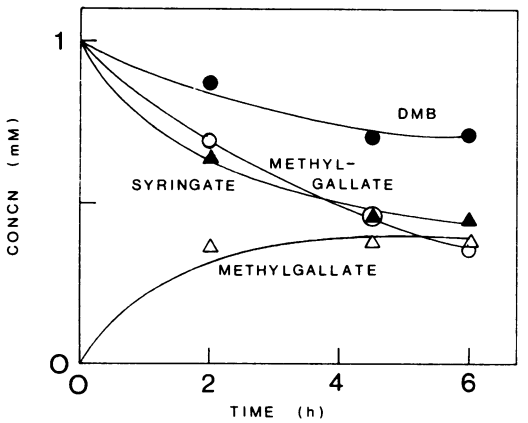

FIG. 5. Aerobic metabolism of syringate, 3-*O*-methylgallate, and DMB by cells grown aerobically in succinate and veratrate cells (1.76 mg of protein per ml) incubated with 1 mM substrates and chloramphenicol. Symbols: ▲, syringate; △, 3-*O*-methylgallate produced from syringate; ○, 3-*O*-methylgallate; ●, 3,5-dimethoxybenzoate (DMB).

nas sp. strain PN-1 grown anaerobically on vanillate was capable of aerobically oxidizing vanillate, protocatechuate, and *p*-hydroxybenzoate. This result is in contrast to the situation for *Rhodopseudomonas palustris*, in which induction of enzymes of the *meta*-cleavage pathway required the presence of O_2 (10), and it also disagrees with earlier data on the levels of protocatechuate-4,5-oxygenase in *Pseudomonas* sp. strain PN-1 grown aerobically and anaerobically on *p*-hydroxybenzoate (17).

Veratrate, which was metabolized both oxically and anoxically to isovanillate, was an inducer for the aerobic and anaerobic enzymes for vanillate breakdown. In contrast, isovanillate, which was not a substrate for either demethylation system, was also a poor inducer of enzymes involved in vanillate and aromatic degradation. Veratrate was probably a less effective inducer than vanillate (or perhaps more correctly, protocatechuate) of the enzymes of aromatic catabolism, since protocatechuate was always detected as an intermediate during the oxic or anoxic attack of vanillate by cells appropriately induced with veratrate. Thus, vanillate was initially converted to protocatechuate by either the aerobic or anaerobic demethylating enzymatic systems, and the production of formaldehyde in the presence of O_2 is consistent with the operation of a monooxygenase (14). Formaldehyde was not detected as a product of the anaerobic breakdown of vanillate, nor was methanol, which might be generated by hydrolytic cleavage of the methoxy-group (1). Identification of the C_1 unit liberated during the anaerobic demethylation of vanillate and other methoxy-aromatic compounds by *Pseudomonas* sp. strain PN-1 requires further study, probably with cell-free systems or mutants or both that are blocked in the oxidation of the C_1 compound.

Pseudomonas sp. strain PN-1 grown anaerobically on vanillate immediately oxidized protocatechuate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, and benzoate with nitrate. This observation supports suggestions of an anaerobic degradative pathway for many substituted aromatic compounds that involves a sequential elimination of substituents to eventually generate benzoic acid, which enters the reductive pathway of aromatic catabolism (6, 9). Neither guaiacol nor catechol, its demethylation product, were ever detected as products of the aerobic or anaerobic dissimilation of vanillate, and so *Pseudomonas* sp. strain PN-1, unlike some bacteria (5), cannot decarboxylate vanillic acid.

Pseudomonas sp. strain PN-1 demethylated methoxy-aromatic compounds either aerobically or anaerobically, but the anaerobic system exhibited the broader substrate specificity. As previously observed (14), the demethylating monooxygenase attacked methoxy groups in the *meta* position relative to the carboxyl group; for example, *m*-anisate and veratrate were converted to *m*-hydroxybenzoate and isovanillate, respectively, but *o*- and *p*-anisate were not attacked. The anaerobic demethylase showed an identical specificity towards these compounds.

TABLE 6. Anaerobic and aerobic metabolism of syringate and 3,5-dimethoxybenzoate[a]

Substrate	Fall in substrate concn (mM) with anaerobic incubation[b]	Aerobic incubation[c]		Aerobic incubation[d]	
		Fall in substrate concn (mM)	HCHO formed (mM)	Fall in substrate concn (mM)	HCHO formed (mM)
Vanillate	0.81	1.99	1.39	1.24	0.53
Syringate	0.72	0.86	0.37	1.05	0.43
3,5-Dimethoxybenzoate	0.51	0.90	0.27	0.39	0.00

[a] Cells were incubated for 1 h with 2 mM substrates and chloramphenicol.
[b] Cells (1.72 mg of protein per ml) were grown anaerobically on vanillate and incubated anaerobically with 20 mM KNO_3.
[c] Cells (3.34 mg of protein per ml) were grown anaerobically on vanillate but incubated aerobically.
[d] Cells (3.56 mg of protein per ml) were grown aerobically on succinate and veratrate.

TABLE 7. Aerobic oxidation of 3-*O*-methylgallate by cells grown anaerobically on vanillate[a]

Substrate	Amt of substrate used[b] (μmol)	O_2 uptake[c] (μmol)	HCHO formed (μmol)
3-*O*-Methylgallate	6.9	10.5	1.3
Vanillate	9.7	26.7	4.5

[a] Warburg flasks were incubated for 145 min and contained cells (6.05 mg of protein) and 10 μmol of substrate.

[b] Confirmed by HPLC. Vanillate (0.3 μmol) and 3-*O*-methylgallate (3.1 μmol) remained, and 0.8 μmol of gallate was produced from 3-*O*-methylgallate.

[c] Endogenous uptake was subtracted (6.5 μmol).

Both enzymatic systems also degraded syringic acid, with a similar pattern of sequential demethylation, to 3-*O*-methylgallate and then gallic acid; under anaerobic conditions, dehydroxylation further transformed gallate into DHB. The inability to detect gallate during the aerobic catabolism of syringate may relate to the oxidation of gallate by protocatechuate-4,5-oxygenase (16). The production of formaldehyde during the aerobic demethylation of syringate, 3-*O*-methylgallate, and 3,5-dimethoxybenzoate is in accord with the action of a monooxygenase on these substrates.

Several lignoaromatic molecules were demethylated anaerobically but not aerobically, i.e., ferulate, guaiacol, 3,4,5-trimethoxycinnamate, and 3,4,5-trimethoxybenzoate, and demethylation of the methoxy group in the *para* position apparently occurred in 3,4,5-trimethoxybenzoate. The broader specificity of the anaerobic demethylating system probably increases its importance to biotechnology and its environmental significance in terms of the anaerobic breakdown of lignoaromatic compounds, especially the conversion of C_1 units derived from their methoxy groups into methane. Further studies of this enzymatic system are clearly warranted.

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