A Description of Some Lignanolytic Soil Bacteria and their Ability to Oxidize Simple Phenolic Compounds

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SUMMARY

Bacteria able to decompose α -conidendrin were isolated from three of five soil samples by enrichment on this compound as sole source of carbon and energy. All the isolates obtained were small Gram-negative motile poorly flagellated rods, which were classified as members of the genus *Agrobacterium*. The ability of these bacteria to oxidize phenolic compounds was examined by manometric studies. From calculations of oxygen uptake, it was concluded that the oxidative rupture of the benzene ring of *m*- and *p*-hydroxybenzoic acid by these bacteria could not be explained entirely by the known metabolic paths which lead from these hydroxybenzoic acids to protocatechuic acid or gentisic acid. It was indicated by simultaneous adaptation technique that neither monohydroxybenzoic acids nor monohydroxycinnamic acids were likely to be formed during the oxidative breakdown of α -conidendrin by these agrobacteria.

INTRODUCTION

In the first paper of this series (Sundman, 1962) a new type of α -conidendrin degradation by bacteria was described. It was shown that in contrast to earlier reported bacterial attacks on α -conidendrin and other methoxylated aromatic compounds (Konetzka, Woodings & Stove, 1957; Tabak, Chambers & Kabler, 1959), which were interpreted to proceed *via* vanillic acid as an intermediate, the present bacteria produce no vanillic acid but considerable amounts of *isovanillic* acid during the breakdown of α -conidendrin. The decomposition was assumed to occur according to Fig. 1, with the 6- α -bound guaiacyl group A being conserved in the *isovanillic* acid, which was believed to be further oxidized, possibly *via* *m*-hydroxybenzoic acid, to protocatechuic acid. In previous work (Konetzka *et al.* 1957; Tabak *et al.* 1959) *p*-hydroxybenzoic acid was reported as intermediate in the bacterial degradation of α -conidendrin *via* vanillic acid, and in the oxidation of trimethoxybenzoic acid by a soil bacterium (Konetzka *et al.* 1957). *p*-Hydroxycinnamaldehyde arises during decomposition of spruce native lignin by various white-rot fungi (Ishikawa, Schubert & Nord, 1963*a, b*). It is not known which metabolic sequences could replace a methoxyl group by a hydrogen atom under aerobic conditions thus forming, e.g. *p*-hydroxybenzoic acid from vanillic acid or *m*-hydroxybenzoic acid from *isovanillic* acid. The position of the mono-substituted benzoic and cinnamic acids in the oxidative breakdown of guaiacyl compounds remains obscure. To acquire more information about the mode of action of the bacteria, this work was enlarged to include the hydroxybenzoic acids and

hydroxycinnamic acids in addition to diphenolic acids and pyrocatechol, which are known as key intermediates in the oxidative breakdown of the aromatic structure (Rogoff, 1961). Further, the oxidation of phenol, benzoic acid and cinnamic acid was investigated. By the simultaneous adaptation technique (Stanier, 1947) organisms

Supposed mode of formation of isovanillic acid from α -conidendrin by the bacteria under investigation (Sundman, 1962).

Metabolic paths of *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid by bacteria.

Fig. 1. Chemical structures of individual compounds and pathways.

of both α -conidendrin-adapted and non-adapted cultures were examined, and an attempt made to determine whether some of these substances were intermediates in the degradation of α -conidendrin.

The α -conidendrin-decomposing bacteria previously described belong to the genera *Pseudomonas* (Tabak *et al.* 1959) and *Flavobacterium* (Konetzka, Pelczar & Gottlieb, 1952). These genera seem to demand special attention in any attempt to

clarify the ligninolytic microflora of the soil. It has been shown that pseudomonads and flavobacteria originating in soil decompose Braun's native lignin, and proliferate on this material as sole source of energy (Sørensen, 1962) and further, that *Pseudomonas fluorescens* strains are capable of oxidizing a number of lignin preparations (Higuchi, Kavamura & Hayashi, 1956). The conidendrin-decomposing bacteria isolated by the author (Sundman, 1962) showed some resemblance to certain pseudomonads but also features of the genus *Agrobacterium*. The present paper examines the results of attempts to determine the taxonomic position of the lignanolytic bacteria which produce isovanillic acid.

METHODS

Soil samples. Five samples, rich in wood residue, were selected. α -Conidendrin-decomposing bacteria were isolated from soil sample Ks, decaying sawdust (Helsinki), pH 5.5; soil sample Ms, forest soil (Helsinki), under *Oxalis* and *Myrtillus*; soil sample O, decaying pile of brush wood (Teisko), pH 5.5. None was obtained from two other samples of forest soil, and a decaying stub, respectively.

Isolation of the bacteria. As previously described (Sundman, 1962), the α -conidendrin-decomposing bacteria were isolated from soil samples by enrichment through several subcultures in a nitrate + mineral salts + vitamin basal medium to which 0.5% (w/v) of α -conidendrin crystals were added as the carbon + energy source. The base solution (K-solution) contained: NaNO_3 , 2.5 g.; KH_2PO_4 , 1.0 g.; CaCl_2 , 0.1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g.; NaCl , 0.1 g.; FeCl_3 , 0.01 g.; vitamin solution (thiamine 10 mg., Ca-pantothenate 10 mg., biotin 20 μg ., B_{12} vitamin 40 μg ., pyridoxin 10 mg., nicotinic acid 10 mg., *p*-aminobenzoic acid 10 mg., folic acid 2 mg., dissolved in 200 ml. of water) 5 ml.; water 1000 ml. final pH 6.5. For isolation, the enrichment cultures were plated on KYE agar (K-solution + Bacto yeast extract 0.1% (w/v) + agar 2% (w/v)). The isolates were replated on the same medium, and the ability to decompose α -conidendrin was noted as a darkening upon growth in the liquid enrichment medium with α -conidendrin as sole source of carbon + energy. About 10 α -conidendrin-decomposing isolates from each soil sample were kept as stock cultures for further work, with monthly transfers on: (a) semi-solid KYE agar (KYE agar with agar content decreased to 0.3% w/v); (b) the liquid α -conidendrin-containing medium.

Manometry. The respirometric experiments were made with washed organisms. These were grown at 28° for 2 days in Roux bottles on KYE agar + glucose 0.2% (w/v) + yeast extract 0.1% (w/v). (Organisms Ms 144412 were difficult to handle when grown on glucose-containing medium because of heavy slime production. Glucose was therefore omitted from the medium for growing this bacterium.) For the production of α -conidendrin-adapted bacteria, α -conidendrin 0.05% (w/v) was added to the medium. The bacteria were harvested by the aid of glass beads and water, and the suspension filtered through glass wool to remove large agar particles. The centrifuged organisms were washed three times with 1/15 M-phosphate buffer pH 5.9, and re-suspended in the same buffer, about 6×10^{10} viable organisms/ml. according to plate counts. The Warburg vessels were filled with: 0.5 ml. suspension of organisms; 1.0 ml. of substrate (1–2.8 μmole in water, pH 7.0); 0.2 ml. 20% (w/v) potassium hydroxide in the centre well. The technique recommended by Umbreit,

Burris & Stauffer (1957) was used. In determining the total oxygen consumption, the substrate was added to the main compartment and the bacteria tipped from the side arm to avoid error caused by droplets adhering to the walls of side arm after tipping. The results given are means of duplicate manometer readings.

Growth experiments. To determine whether a compound served as carbon + energy source for the bacteria, washed organisms were inoculated as stab cultures into semi-solid K agar (K-solution + 0.3%, w/v, Bacto agar), and into similar media with 0.001 and 0.0001 mole/ml., respectively, of compound to be tested. Since these bacteria are not able to utilize agar, the control inoculation into semi-solid K agar served as a check on possible carbon + energy sources transferred with the inoculum or present in the medium. The growth in the tubes provided with compound to be tested is quoted as positive only when the control tube showed no growth.

Phenolic compounds used. α -Conidendrin was kindly supplied by the Crown & Zellerbach Corporation (Camas, Washington state, U.S.A.). The white crystals were dissolved in acetone and re-precipitated from water, m.p. 247–248°. Other compounds were commercially available preparations as follows: *o*-hydroxybenzoic acid (British Drug Houses Ltd, reagent) m.p. 157–158°; *m*-hydroxybenzoic acid (puriss.) m.p. 198–199°; *p*-hydroxybenzoic acid (purum) m.p. 212–214°; *o*-hydroxycinnamic acid (purum) m.p. 207–209°; *m*-hydroxycinnamic acid (purum) m.p. 192–193°; *p*-hydroxycinnamic acid (puriss.) m.p. 214–215°; protocatechuic acid (purum) m.p. 200–201°; gentisic acid (puriss.) m.p. 202–204°; homogentisic acid (purum) m.p. 147–149°, all from Fluka A.G. (Buchs, S.G., Switzerland); pyrocatechol (p.a.) m.p. 104–105°; phenol (p.a. for chromatography) m.p. 39–39.5°, both from Merck Co., Inc. (Darmstadt, Western Germany).

RESULTS

Identification and taxonomy of the α -conidendrin-decomposing bacteria

All the α -conidendrin-decomposing isolates obtained from the three soil samples were motile, non-sporing, Gram-negative single rods, 0.9–1 by 1.3–3.2 μ , with a tendency to uneven staining and Gram variability. No pigment was produced on ordinary media. The isolates from each soil sample showed only minor differences in physiological tests, and were obviously representatives of the same species. One isolate for each soil sample was selected for further study, and replated twice to ensure purity. Of these isolates: Ks 17, O 8, Ms 14, the two first named were used in previous work (Sundman, 1962). The isolates had since been kept as stock cultures for a year. They showed no sign of impurity, but for the identification purposes of the present work their purity was ascertained by repeated platings. Though visually homogeneous, the plates were not considered as proofs of purity until all the colonies from a plate gave visually identical cultures in stabs in semi-solid KYE agar, were positive in the test for α -conidendrin degradation, and showed the same morphology under the phase-contrast microscope. This was achieved after two platings of isolates Ks 17 and O 8, and four platings of isolate Ms 14. The replated cultures are designated Ks 1741, O 811, and Ms 144412.

Each isolate was aerobic, growing as sharply confined surface upon stabbing in semi-solid KYE agar; growth was good at 15° and 28°, the minimum temperature for growth was less than 3°, and the maximum below 37°. Tests for hydrolysis of

gelatin, casein and starch (on KYE agar plates containing 0.4 %, w/v, gelatine, 20 %, w/v, skimmed milk, and 0.2 %, w/v, soluble starch, respectively) were negative. Indole was not produced in the 1 % (w/v) tryptone solution, nor hydrogen sulphide in the mannitol tryptone medium of ZoBell & Feltham (1934). In the Hugh & Leifson (1953) test for the type of carbohydrate metabolism, no acid or gas from glucose was detected during the 10 days of incubation; when incubation was extended to 21 days, the open tube turned yellow. Substances tested as sole carbon + energy sources supporting growth were: glucose, galactose, mannose, xylose,

Table 1. *Characteristics which varied in the α -conidendrins-decomposing isolates Ks 1741, Ms 144412 and O 811*

Determinative test	Isolate		
	Ks 1741	Ms 144412	O 811
Flagella staining	1-2 polar (and/or lateral)	1-5 peritrichous	1-3 polar (and/or subpolar)
Oxidase test (dimethyl- <i>p</i> -phenylene diamine HCl solution poured on KYE agar surface growth)	+	-	+
Nitrite from nitrate (in KYE medium)	+ *	-	+ *
Litmus milk (21 days, 28°), Hofer (1941)	Slight alkalization, reduction at the bottom. No browning	Slimy serum zone at the surface. Milk yellowish brown	Slight alkalization, reduction at the bottom. No browning
Growth on Caglycerophosphate-mannitol agar (Riker <i>et al.</i> 1930)	Brown pigment, surrounded by white precipitate	No colour, abundant white precipitate	Brown pigment, surrounded by white precipitate
Absorption of Congo red	±	+	-
Assimilation of arabinose	-	+	-

* Nitrates completely assimilated, test for nitrite negative upon growth in medium containing glycerol + nitrate (0.025 %, w/v)

mannitol, glycerol, citric acid, gluconic acid, protocatechuic acid, hydroxybenzoic acids, hydroxycinnamic acids (see Table 2). Lactose, saccharose, vanillic acid, vanillin, cinnamic acid, ferulic acid, guaiacol, eugenol, isoeugenol, and phenol did not serve as carbon + energy source. All the isolates were negative in the test for alkaline arginine metabolism (Thornley, 1960), did not produce alkalinity when grown in a medium containing potassium nitrate + glycerol (Sagen, Riker & Baldwin, 1934), and were resistant to 100 i.u. penicillin/ml. KYE agar.

Table 1 illustrates the behaviour of isolates Ks 1741, O 811, and Ms 144412 in some additional tests, in which variable results were obtained. The flagellation of these bacteria, as reported in Table 1, represents the conclusions from numerous tests by Leifson's method, with tannic acid + ethanolic fuchsin (Leifson, 1960), and Fontana's method with silver nitrate as described by Rhodes (1958). Some unknown feature of these organisms made clear-cut flagella-stained preparations difficult to obtain by reason of heavy precipitation of stain around the organisms. In the main, isolates O 811 and Ks 1741 had polar flagellation, but lateral and sub-polar flagella

were noted repeatedly. To decide whether these latter were artefacts caused by overlapping of soma and flagellum (Hodgkiss, 1960) *Agrobacterium tumefaciens* (kindly supplied by Professor O. Pohjakallio, Department of Plant Pathology, University of Helsinki) and *Pseudomonas* spp. (isolated in this laboratory from market milk) were included as peritrichously flagellate and polarly flagellate test organisms, respectively, for the flagella staining techniques used. The preparations obtained with *A. tumefaciens* and α -conidendrin-decomposers Ks 1741 and O 811 were almost indistinguishable, showing few flagella, and in situations which were mainly polar and occasionally lateral or sub-polar. The same precipitation of stain around the organisms was noted in all the preparations. The *Pseudomonas* spp. cultures gave preparations with clear-cut polar flagella only, and without the disturbing precipitate around the organisms. All the isolates were thus to be considered as peritrichously flagellate, and a location in the genus *Pseudomonas* excluded. This is supported by the negative test for alkaline arginine metabolism obtained with the isolates. This test was useful for the differentiation of pseudomonads from other Gram-negative organisms, isolated from spoiled chicken meat, (Thornley, 1960), and according to Brisbane & Rovira (1961) of value also in the separation of soil pseudomonads from other Gram-negative soil bacteria.

In a system for the classification of Gram-negative soil bacteria (Holding, 1960), those aerobic organisms which possess peritrichous flagella and are capable of oxidizing glucose and of growth on inorganic nitrogen are allocated to the genus *Agrobacterium*. According to this classification, the α -conidendrin-decomposing bacteria described here would be considered as agrobacteria. Of the seven *Agrobacterium* species listed in the 7th edition of *Bergey's Manual* (1957), five are plant pathogens, *A. radiobacter* is a common soil organism, and one species, *A. stellulatum*, was isolated from marine mud. On the basis of their growth in litmus milk, and the action on carbohydrates, the present isolates examined here display most resemblance to *A. radiobacter*. Inability to produce hydrogen sulphide, colourless growth on Ca glycerophosphate-mannitol agar (Ms 144412), neutral reaction in nitrate + glycerol medium, growth in litmus milk without production of brown colour (Ks 1741, O 811), and the absorption of congo red (Ms 144412) indicate that none of the isolates listed in Table 1 can be considered as typical *A. radiobacter*. It is believed that they belong to the still rather obscure *A. radiobacter* group of the indigenous soil flora described by Conn (1942, 1948).

Oxidation of aromatic key intermediates

In manometric studies of the oxidative properties of the bacteria, it was found that a common feature of these α -conidendrin-decomposers was a constitutive ability to oxidize protocatechuic acid (PA) and homogentisic acid (HGA) (see Figs. 2, 3). Strains Ms 144412 and O 811 oxidized pyrocatechol (PC) at a comparable rate, and strains O 811 and Ks 1741 also oxidized gentisic acid (GA) at a slower rate (see Fig. 3). No induction was required for the oxidation of these substrates. Organisms grown on yeast extract + glucose started oxygen uptake immediately, with no lag. The *Agrobacterium tumefaciens* strain included lacked the ability to oxidize these phenolic acids. The oxidation of PA, HGA, and PC was viewed in relationship to the ability of the organisms to oxidize various aromatic compounds of lignin-like structure (Sundman, 1964). The simultaneous adaptation technique could

not provide information on whether these aromatic key intermediates were actually intermediates in the α -conidendrin breakdown, since under the experimental conditions the organisms contained the systems for their oxidation independently of whether α -conidendrin was present during growth or not. Protocatechuic acid was indicated by paper chromatography (for technique see Sundman, 1962) in α -conidendrin culture filtrates.

Strain Ms 144412, though constitutively oxidizing homogentisic acid, lacked the ability to oxidize gentisic acid (Table 2). This provided evidence that the homogentisic acid oxidation by this strain did not proceed via gentisic acid formation. This latter compound was assumed to be an intermediate in the oxidation of homogentisic acid by an enzyme preparation of a white-rot fungus (Fukuzumi, 1962).

Fig. 2

Fig. 2. Oxygen uptake by α -conidendrin-adapted organisms of strain Ks 1741 with various phenolic acids and α -conidendrin. Number of viable organisms: 3.88×10^{10} . Amount of substrate: phenolic acids, $2 \mu\text{mole}$; α -conidendrin, $1 \mu\text{mole}$. 1, Endogenous; 2, *m*-hydroxycinnamic acid; 3, protocatechuic acid; 4, homogentisic acid; 5, α -conidendrin; 6, *m*-hydroxybenzoic acid. $\downarrow$ addition of substrate.

Fig. 3

Fig. 3. Oxygen uptake by organisms of strain O 811 (not adapted to α -conidendrin during growth) with phenolic acids. Number of viable organisms: 3.45×10^{10} . Amount of substrate: $2 \mu\text{mole}$. 1, Endogenous; 2, gentisic acid; 3, protocatechuic acid; 4, *p*-hydroxybenzoic acid; 5, *m*-hydroxybenzoic acid. $\downarrow$ = addition of substrate.

Oxidation and metabolic paths of monohydroxybenzoic acids and monohydroxycinnamic acids

From the figures for total oxygen uptake given in Table 2, it can be seen to what extent the hydroxybenzoic acids and hydroxycinnamic acids are oxidized by the present bacteria. It was found that when the acids served as substrate, an induction time of 80–90 min. was required before oxygen uptake started (Fig. 3), *m*-hydroxycinnamic acid being an exception. This substrate was oxidized at increasing rate without a well-defined lag (Fig. 2). Adaptation to α -conidendrin during growth could not suppress the induction time required by the agrobacteria to start oxygen uptake with the hydroxybenzoic acids and hydroxycinnamic acids as substrates. Neither was the slow initial oxygen uptake with *m*-hydroxycinnamic acid as

substrate accelerated by presence of α -conidendrin during growth. There was thus no evidence presented for the position of monohydroxybenzoic acids or monohydroxycinnamic acids as intermediates in the decomposition of α -conidendrin by the present agrobacteria. None of these acids were detected by aid of previously described paper chromatographical technique (Sundman, 1962) in filtrates of α -conidendrin cultures.

Table 2. *Total oxygen uptake (mole oxygen/mole substrate) observed in respirometric experiments with α -conidendrin-decomposing Agrobacterium strains, with washed organisms harvested from glucose + yeast extract + minerals*

Substrate	Organism					
	Ks 1741		Ms 144412		O 811	
	Total oxygen uptake	Growth on substrate as sole carbon + energy source	Total oxygen uptake	Growth on substrate as sole carbon + energy source	Total oxygen uptake	Growth on substrate as sole carbon + energy source
phenol	0	.	0	.	0	.
pyrocatechol	0	—	2.16	+	3.14	+
protocatechuic acid	2.73	+	2.22	+	2.40	+
homogentisic acid	3.15	.	2.39	.	3.21	.
gentisic acid	> 2.36	.	0	.	> 1.43	.
benzoic acid	> 0.68*	.	4.90*	.	> 4.50*	.
<i>o</i> -hydroxybenzoic acid	> 1.52*	.	> 1.97*	.	> 1.60*	.
<i>m</i> -hydroxybenzoic acid	4.39*	+	0	—	4.30*	+
<i>p</i> -hydroxybenzoic acid	0	—	3.29*	+	3.85*	+
cinnamic acid	0	—	.	—	0	—
<i>o</i> -hydroxycinnamic acid	0	.	0	.	0.86	.
<i>m</i> -hydroxycinnamic acid	> 2.44	+	0	—	> 4.10	+
<i>p</i> -hydroxycinnamic acid	0	—	> 1.21*	+	0.88	—

*, Oxidation started after a lag period.

>, Total oxygen uptake could not be determined, since the endogenous respiration rate was not reached during the period of experiment, 5 hr. The total uptake thus exceeded the figure given.

0, Oxygen uptake negligible, proceeding at a linear rate slightly exceeding the rate for endogenous respiration, giving a difference between substrate and endogenous of less than 0.40 mole oxygen/mole substrate in 5 hr.

., not determined.

A comparison of the oxygen uptake with *meta*- and *para*-hydroxybenzoic acids and hydroxycinnamic acids, respectively, as substrates (Table 2), reveals a configurative correlation. The *meta*-hydroxy acids only were oxidized by strain Ks 1741, and utilized as sole carbon + energy sources, whereas strain Ms 144412 was capable of oxidizing and utilizing the *para*-hydroxy acids but not the *meta*-hydroxy acids. Strain O 811 oxidized every one of the four acids, though *p*-hydroxycinnamic acid to a restricted extent, corresponding to 0.88 mole oxygen/mole substrate. This oxygen uptake with *p*-hydroxycinnamic acid was obviously not connected with ring opening and did not provide energy enough to support growth (see Table 2).

The reaction *m*- or *p*-hydroxybenzoic acid $\rightarrow$ protocatechuic acid (Fig. 1) corresponds to an oxygen uptake of 1 atom oxygen/mole hydroxybenzoic acid. Accordingly the difference in oxygen uptake between hydroxybenzoic acid and protocatechuic acid should be 0.5 mole oxygen/mole substrate, provided that the oxidation of the hydroxybenzoic acids proceeds *via* protocatechuic acid, as has been demonstrated to be the case for soil fungi (Henderson, 1960, 1961) and for soil bacteria (Evans, 1947; Sleeper & Stanier, 1950; Yano & Arima, 1958). The oxygen uptake observed in repeated experiments with washed organisms of the present lignanolytic agrobacteria, as indicated in Table 3, presupposes greater differences. Instead of the theoretical difference, 0.5 mole oxygen/mole substrate, between oxygen uptake with protocatechuic acid and the hydroxybenzoic acids, respectively, 1.4 mole oxygen/mole substrate was found to be the actual difference between oxygen taken up with *m*-hydroxybenzoic acid and with protocatechuic acid as

Table 3. *Total oxygen uptake (mole oxygen/mole substrate) observed in repeated experiments with washed organisms grown on glucose + yeast extract + nitrate + mineral salts, using p-hydroxybenzoic acid, m-hydroxybenzoic acid, and protocatechuic acid as substrate, and differences between obtained means for protocatechuic acid oxygen uptake and hydroxybenzoic acid oxygen uptake*

Substrate	Organism		
	Ks 1741	Ms 144412	O 811
<i>p</i> -hydroxybenzoic acid	—	3.28	3.57
		3.29	3.96
		3.90	3.74
		Mean	Mean
		3.69	3.76
<i>m</i> -hydroxybenzoic acid	4.39	—	4.05
	4.02		4.24
	3.93		4.46
	Mean		Mean
	4.11		4.25
protocatechuic acid	2.73	2.22	2.30
	2.43	2.32	3.32
	2.92	2.41	2.92
		2.74	
	Mean	Mean	Mean
	2.69	2.42	2.85
Difference between <i>m</i> -hydroxybenzoic acid and protocatechuic acid oxygen uptakes	1.42	—	1.40
Difference between <i>p</i> -hydroxybenzoic acid and protocatechuic acid oxygen uptakes	—	1.27	0.91

substrates. The corresponding figure for difference between *p*-hydroxybenzoic acid oxidation and protocatechuic acid oxidation was 0.9 or 1.3 mole oxygen/mole substrate. It is believed that the differences actually found give reason to assume that the metabolism of the hydroxybenzoic acids can be explained only partially by the intermediate formation of protocatechuic acid. Furthermore, the oxygen uptake

with *m*-hydroxybenzoic acid was, on repeated occasions, found to be greater than the amount of oxygen consumed in the presence of *p*-hydroxybenzoic acid (see Tables 2, 3), which might indicate that the substrates were metabolized along different paths.

The transformation *m*-hydroxybenzoic acid $\rightarrow$ gentisic acid before ring cleavage (Fig. 1) occurs in pseudomonads (Walker & Evans, 1952; Yano & Arima, 1958). As mentioned above, the agrobacterial strains Ks 1741 and O 811, which are capable of oxidizing *m*-hydroxybenzoic acid, oxidized gentisic acid at a comparatively slow rate (see Fig. 3). The rate of gentisic acid oxidation should be limiting for

Fig. 4. Oxygen uptake by organisms of strain O 811 (not adapted to α -conidendrin during growth) with gentisic acid and *m*-hydroxybenzoic acid. Effect of adaption to *m*-hydroxybenzoic acid. Number of viable organisms: broken curves, 3.5×10^{10} ; whole curves, 1.6×10^{10} . Amount of substrate: $2 \mu\text{mole}$. Endogenous value subtracted. 1, Gentisic acid; 2, *m*-hydroxybenzoic acid. Broken curves = untreated organisms, whole curves = organisms adapted to *m*-hydroxybenzoic acids prior to measurement through shaking with 0.001 M *m*-hydroxybenzoic acid pH 5.5 for 90 min. at 28° .

the *m*-hydroxybenzoic acid oxidation, if this latter proceeds *via* gentisic acid. Organisms adapted to *m*-hydroxybenzoic acid through exposure to this substrate prior to measurement of oxygen uptake showed the same slow oxygen uptake with gentisic acid though the lag of *m*-hydroxybenzoic acid oxidation had been suppressed (Fig. 4). The results obtained provide grounds for the assumption that there was some unknown metabolic system which was working more rapidly than the gentisic acid oxidation, which accounted for the oxidative breakdown of *m*-hydroxybenzoic acid.

DISCUSSION

Classification of the Gram-negative bacteria indigenous to soil is rendered difficult by the lack of knowledge of dividing characteristics. There seems to be a need for a more detailed study of the Gram-negative rods of the soil as a group along the taxometric lines indicated by Gyllenberg (1963) and Hill & Silvestri (1962), in order to establish a useful basis for the separation of this important part

of the soil microflora. In the search for separating tests, the ability to utilize phenolic and lignin-related compounds might be of some value. Such reactions could be well suited to divide organisms which are possibly concerned with the breakdown of lignified plant residue in soil and characterized by inertness in ordinary biochemical tests.

The allocation of the present organisms by genus is a matter for discussion. According to the classification scheme of Holding (1960), followed in this work, the only difference between the criteria for placing an isolate in the genus *Agrobacterium* or *Achromobacter* is the ability to proliferate on inorganic nitrogen; when an organism has this ability it is placed in the genus *Agrobacterium*, and when it has not, in the genus *Achromobacter*. About one half of the *Achromobacter* species listed in the 7th edition of *Bergey's Manual* (1957) are described as having the capacity to use ammonium chloride as nitrogen source; the separation on grounds of nitrogen requirement thus puts together agrobacteria and achromobacteria. On tracing the lignanolytic bacteria examined here through the key to the *Achromobacter* species of the *Bergey's Manual* (1957), they will be considered as *A. cycloclastes* or *A. pestifer*. The former organism has been reported as decomposing phenol and naphthalene. Though none of the present bacteria is able to attack phenol (see Table 2), a relationship to this previously isolated, peritrichously-flagellated, non-pigmented, Gram-negative rod lies near at hand. The type of flagellation (few flagella), Gram reaction (granular staining with a tendency to variability), the small size of the organisms, and reactions typical of *Agrobacterium radiobacter*, listed in Table 1, have contributed to consideration of the present organisms as representatives of the genus *Agrobacterium*.

The oxidation of *p*- and *m*-hydroxybenzoic acid by microbes is known to be initiated by the introduction of a second hydroxyl group, resulting in either gentisic acid or protocatechuic acid, which are further oxidized during the opening of the aromatic ring and the formation of energy-yielding metabolites as shown in Fig. 1. These reactions might be affected by the organisms under discussion, but the stoichiometric differences in oxygen uptake observed with washed organisms for *m*- and *p*-hydroxybenzoic acids and protocatechuic acid, indicates that the *p*-hydroxybenzoic acid oxidation by strain O 811 and Ms 144412, consuming roughly 7 oxygen atoms/mole substrate, cannot proceed only along the path *via* protocatechuic acid, which is oxidized to a degree corresponding to the uptake of 5 atoms oxygen/mole substrate. The probability that the oxidation proceeds *via* protocatechuic acid is still less if *m*-hydroxybenzoic acid oxidation by organisms Ks 1741 and O 811 is in question. This substrate is oxidized to a degree which corresponds to 8 or 9 atoms oxygen/mole *m*-hydroxybenzoic acid. Under the prevailing conditions, the oxidation of gentisic acid by the strains which oxidize *m*-hydroxybenzoic acid is too slow to allow of the evaluation of the total consumption of oxygen. Even if the stoichiometric amount of total oxygen consumed were in agreement with the hypothesis that *m*-hydroxybenzoic acid is oxidized *via* the formation of gentisic acid, the rate of gentisic acid oxidation, in connexion with the inability of *m*-hydroxybenzoic acid-adapted organisms to cause increased oxidation of gentisic acid, cannot satisfactorily support such a view.

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REFERENCES

- Bergey's Manual of Determinative Bacteriology* (1957). 7th ed. Ed. by R. S. Breed, E. D. G. Murray & N. R. Smith. Baltimore: Williams and Wilkins.
- BRISBANE, P. G. & ROVIRA, A. D. (1961). A comparison of methods for classifying Rhizosphere bacteria. *J. gen. Microbiol.* **26**, 379.
- CONN, H. J. (1942). Validity of the genus *Alcaligenes*. *J. Bact.* **44**, 359.
- CONN, H. J. (1948). The most abundant groups of bacteria in soil. *Bact. Rev.* **12**, 257.
- DAGLEY, S., EVANS, W. C. & RIBBONS, D. W. (1960). New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. *Nature, Lond.* **188**, 560.
- EVANS, W. C. (1947). Oxidation of phenol and benzoic acid by some soil bacteria. *Biochem. J.* **41**, 373.
- FUKUZUMI, T. (1962). Enzymatic degradation of lignin. Part II. Oxidation of homogentisic acid and gentisic acid by the enzyme of the wood-rotting fungus *Portia subacida*. *Agr. Biol. Chem., Tokyo*, **26**, 447.
- GYLLENBERG, H. (1963). A general method for deriving determination schemes for random collections of microbial isolates. *Ann. Acad. Sci. fenn. Ser. A*, **4**, No. 69.
- HENDERSON, M. E. K. (1960). The influence of trace elements on the metabolism of aromatic compounds by soil fungi. *J. gen. Microbiol.* **23**, 307.
- HENDERSON, M. E. K. (1961). The metabolism of aromatic compounds related to lignin by some hyphomycetes and yeast-like fungi of soil. *J. gen. Microbiol.* **26**, 155.
- HIGUCHI, T., KAVAMURA, I. & HAYASHI, I. (1956). Biochemical study of wood-rotting fungi. V. The enzymatic oxidation of lignins. *J. Japan. Wood Res. Soc.* **2**, 31.
- HILL, L. R. & SILVESTRI, L. G. (1962). Quantitative methods in the systematics of Actinomycetales. III. The taxonomic significance of physiological-biochemical characters and the construction of a diagnostic key. *Giorn. Microbiol.* **10**, 1.
- HODGKISS, W. (1960). The interpretation of flagella stains. *J. appl. Bact.* **23**, 398.
- HOFFER, A. W. (1941). A characterisation of *Bacterium radiobacter* (Beijerinck and van Delden) Löhnis. *J. Bact.* **41**, 193.
- HOLDING, A. J. (1960). The properties and classification of the predominant Gram-negative bacteria occurring in soil. *J. appl. Bact.* **23**, 515.
- HUGH, R. & LEIFSON, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24.
- ISHIKAWA, H., SCHUBERT, W. J. & NORD, F. F. (1963a). Investigations on lignins and lignification. XXVII. The enzymic degradation of soft wood lignin by white-rot fungi. *Arch. Biochem. Biophys.* **100**, 131.
- ISCHIKAWA, H., SCHUBERT, W. J. & NORD, F. F. (1963b). Investigations on lignins and lignification. XXX. Enzymic degradation of guaiacylglycerol and related compounds by white-rot fungi. *Biochem. Z.* **338**, 153.
- KONETZKA, W. A., PELCZAR, M. J. & GOTTLIEB, S. (1952). The biological degradation of lignin. III. Bacterial degradation of α -conidendrin. *J. Bact.* **63**, 771.
- KONETZKA, W. A., WOODINGS, E. T. & STOVE, J. (1957). Microbial dissimilation of methoxylated aromatic compounds. *Bact. Proc.* p. 135.
- LACK, L. (1959). The enzymic oxidation of gentisic acid. *Biochim. biophys. Acta*, **34**, 117.
- LEIFSON, E. (1960). *Atlas of Bacterial Flagellation*. New York: Academic Press.
- RHODES, M. E. (1958). The cytology of *Pseudomonas* spp. as revealed by a silver-plating staining method. *J. gen. Microbiol.* **18**, 639.
- RIKER, A. J., BANFIELD, W. M., WRIGHT, W. H., KEITT, G. W. & SAGEN, H. E. (1930). Studies on infectious hairy root of nursery apple trees. *J. Agric. Res.* **41**, 507.
- ROGOFF, M. H. (1961). Oxidation of aromatic compounds by bacteria. In *Advances in Applied Microbiology*. Ed. by W. W. Umbreit, vol. 3. New York: Academic Press.

- SAGEN, H. E., RIKER, A. J. & BALDWIN, I. L. (1934). Studies on certain physiological characters of *Phytomonas tumefaciens*, *Phytomonas rhizogenes*, and *Bacillus radiobacter*. Part 1. *J. Bact.* **28**, 571.
- SLEEPER, B. P. & STANIER, R. Y. (1950). The bacterial oxidation of aromatic compounds. I. Adaptive patterns with respect to polyphenolic compounds. *J. Bact.* **59**, 117.
- STANIER, R. Y. (1947). Simultaneous adaption. A new technique for the study of metabolic pathways. *J. Bact.* **54**, 339.
- STANIER, R. Y. & INGRAHAM, J. L. (1954). Protocatechuic acid oxidase. *J. biol. Chem.* **210**, 799.
- SUGIYAMA, S., YANO, K., TANAHARA, H., KOMAGATA, K. & ARIMA, K. (1958). Metabolism of aromatic compounds by bacteria. I. Gentisic acid oxidase and protocatechuic acid oxidase of *Pseudomonas ovalis* S-5. *J. gen. appl. Microbiol., Tokyo*, **4**, 223.
- SUNDMAN, V. (1962). Microbial decomposition of lignins. I. Identification of isovanillic acid as a breakdown product in bacterial degradation of α -conidendrin. *Medd. finska Kemistsamf.* **71**, 26.
- SUNDMAN, V. (1964). The ability of α -conidendrin-decomposing *Agrobacterium* strains to utilize other lignans, and lignin-related compounds (in preparation).
- SÖRENSEN, H. (1962). Decomposition of lignin by soil bacteria and complex formation between autoxidised lignin and organic nitrogen compounds. *J. gen. Microbiol.* **27**, 21.
- TABAK, H. H., CHAMBERS, C. W. & KABLER, P. W. (1959). Bacterial utilization of lignans. *J. Bact.* **78**, 469.
- THORNLEY, M. J. (1960). The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *J. appl. Bact.* **23**, 37.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*. Minneapolis: Burgess Publishing Co.
- WALKER, N. & EVANS, W. C. (1952). Pathways in the metabolism of monohydroxybenzoic acids by soil bacteria. *Biochem. J.* **52**, xxiii.
- YANO, K. & ARIMA, K. (1958). Metabolism of aromatic compounds by bacteria. II. *m*-Hydroxybenzoic acid hydroxylase A and B, 5-dehydroshikimic acid, a precursor of protocatechuic acid, a new pathway from salicylic acid to gentisic acid. *J. gen. appl. Microbiol., Tokyo*, **4**, 241.
- ZOBELL, C. E. & FELTHAM, C. B. (1934). A comparison of lead, bismuth, and iron as detectors of hydrogen sulphide produced by bacteria. *J. Bact.* **28**, 169.