APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1988, p. 428–433
0099-2240/88/020428-06$02.00/0

Vol. 54, No. 2

Metabolism of 2,6-Dimethylnaphthalene by Flavobacteria

E. A. BARNSLEY

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

Received 18 June 1987/Accepted 3 November 1987

Flavobacteria that were able to grow on 2,6-dimethylnaphthalene (2,6-DMN) were isolated from soil. Most were able to oxidize a broad range of aromatic hydrocarbons after growth on 2,6-DMN at rates comparable to that of the oxidation of 2,6-DMN itself. One small group was neither able to grow on naphthalene nor able to oxidize this compound after growth on 2,6-DMN, but metabolized 2,6-DMN by a pathway which converged with that previously described for naphthalene metabolism in pseudomonads. These organisms could also grow on salicylate or methylsalicylate, and in so doing, early enzymes for 2,6-DMN metabolism were induced.

Alkylated polycyclic aromatic hydrocarbons occur in crude oil in quantities greater than those of the parent hydrocarbons (11, 20). They are acutely toxic to animals (13, 20) but are degraded in microbiological processes when crude oil is shaken with water samples (11). Previously, the conversion of dimethylnaphthalenes (DMNs) to methylnaphthalene carboxylic acids has been reported for *Nocardia* spp. grown on aliphatic hydrocarbons (15, 23), *Pseudomonas* spp. (G. K. Skryabin, I. I. Starovoitov, S. Y. Pshirkov, M. Y. Nefedova, I. I. Chervin, and A. T. Zyakum, Chem. Abstr. **84**:134143s, 1976), *Streptomyces achromogenes* (J. D. Douros, Jr., and R. L. Raymond, Chem. Abstr. **67**:P017381k, 1967). In this study, the method described by Sylvestre (29) to detect hydrocarbon metabolism was used to isolate a large number of species that were able to grow on 2,6-DMN as a sole source of carbon and energy. These belonged to the genera *Pseudomonas*, *Flavobacterium*, *Vibrio*, and *Alcaligenes*. The metabolic pathway in one group of flavobacteria was readily elucidated because it converged with that previously described for naphthalene and was regulated similarly.

MATERIALS AND METHODS

Media. An ethereal solution of 2,6-DMN was sterilely filtered into a sterile conical flask, and ether was allowed to evaporate in a fume hood. Direct autoclaving in a sealed tube caused oxidation to an orange product. Mineral salts (27) were then added. To help initiate the rapid growth of pure strains on the hydrocarbon, it was convenient to add peptone (0.05 g liter^{-1}), yeast extract (0.025 g liter^{-1}), and glucose (0.02 gliter^{-1}); but the strains that were isolated grew without these additives. For strain selection on solidified media, nutrient agar was used, and after growth at 25°C colonies were sprayed with an ethereal solution of 2,6-DMN. Alternatively, mineral salts medium solidified with 2% agar was used, and 2,6-DMN was provided in the lid. Growth at 25°C was always very slow by this method and required at least 7 days to give well-formed colonies of 2 mm in diameter. Some strains which grew well in minimal liquid medium did not grow when 2,6-DMN was provided in the vapor phase.

Strains. Flasks containing 25 ml of sterile mineral salts and 2,6-DMN (0.025 g) were inoculated with about 0.5 g of soil and incubated with shaking at 25°C. Soil samples were from pastureland that was not known to have been treated with herbicide or to have been contaminated with hydrocarbons. Samples were withdrawn at 24-h intervals, diluted serially, and plated onto nutrient agar. After the cells were grown at 25°C, petri dishes with less than confluent growth were sprayed with 2,6-DMN (0.4%) (29) and incubated further. Colonies around which the hydrocarbon cleared were purified on nutrient agar. Many strains that were able to metabolize the hydrocarbon did not use it as a sole source of carbon and energy, and these were not examined further. Determinative tests were made by using the key described by Skerman (27), and strains of the genera *Pseudomonas*, *Vibrio*, *Alcaligenes*, and *Flavobacterium* were identified. Isolates belonging to the genus *Flavobacterium* were gram-negative, very short rods that formed yellow colonies on nutrient agar. Pigment was less marked on minimal media. Some strains were motile, but flagella were not detected (21). All strains were oxidase positive (19), produced acid from glucose aerobically, but could not ferment glucose (14). The flavobacteria were uniformly difficult to maintain and were subcultured on nutrient agar at 14-day intervals.

Cell suspensions and extracts. Cells were grown in 250-ml lots of mineral salts in 1-liter flasks with shaking at 25°C. Either 2,6-DMN or glucose was used as the carbon source. When the A_{600} was greater than 0.5, the cultures were filtered through a thick pad of fine glass wool. Cells were collected by centrifugation, washed with 0.05 M phosphate buffer (pH 7.0), and suspended in buffer (about 1 ml/250 ml of culture). The total protein concentration of cell suspensions (25) was between 10 and 15 mg ml^{-1}. Extracts were prepared from suspensions of cells by sonication (2), followed by centrifugation at 12,800 × *g* for 30 min at 5°C. For studies of metabolite production, cells were grown aerobically in 10-liter batches in a fermentor (Microferm; New Brunswick Scientific Co., Inc., Edison, N.J.).

Respiration measurements. Respiration measurements were made polarographically in phosphate buffer at 25°C. After endogenous respiration was measured, the compound to be examined was added in dimethyl sulfoxide (10 μl of a 10 mM solution into 2 ml of cell suspension). Results were corrected for a small stimulation of respiration caused by the solvent.

Enzymatic activities. 2-Hydroxymethyl-6-methylnaphthalene dehydrogenase was measured spectrophotometrically at 300 nm in 1-cm cuvettes with a reaction mixture containing 50 mM PP_i (pH 8.2), 6-methylnaphthalene-2-aldehyde (33 μM), and NADH (133 μM) at 25°C. The reaction was started by the addition of extract, and the reaction rate was calculated from the rate of change of absorbance by using an extinction coefficient of 13.6 mM^{-1} cm^{-1} (the sum of that of the aldehyde [11.8] and NADH [2.4] minus that of the product 2-hydroxymethyl-6-methylnaphthalene [0.6]). The

substrate concentrations were not saturating and were therefore carefully standardized. 6-Methylnaphthalene-2-aldehyde dehydrogenase was measured spectrophotometrically at 300 nm in 1-cm cuvettes with a reaction mixture containing 50 mM PP_i (pH 8.2), 6-methylnaphthalene-2-aldehyde (33 μM), and NAD (133 μM) at 25°C. The reaction was started by the addition of extract, and the reaction rate was calculated from the rate of change of absorbance by using an extinction coefficient of 6.1 cm^{-1} mM^{-1} (that of the aldehyde minus the sum of those of NADH and 6-methylnaphthalene-2-carboxylic acid [3.3]). The aldehyde was prepared as a 5 mM solution in ethanol. Extracts contained no detectable ethanol dehydrogenase or acetaldehyde dehydrogenase.

1,2-Dihydroxynaphthalene dioxygenase (22), 2-hydroxychromene-2-carboxylic acid isomerase (3), 2′-hydroxybenzalpyruvate aldolase (3), salicyaldehyde dehydrogenase (26), salicylate hydroxylase (2), catechol-2,3-dioxygenase (12), hydroxy muconic acid semialdehyde hydrolase (24), and dehydrogenase (24) were measured by previously published methods. 2,6-DMN oxygenase was measured radiochemically by using whole cells incubated with [4-^{14}C]2,6-DMN in a way similar to that described previously for naphthalene oxygenase (4). Under the conditions of the assay, the unchanged 2,6-DMN extracted into toluene scintillator did not contain detectable amounts of metabolites measured by thin-layer chromatography (TLC) or after evaporation of the 2,6-DMN on filter paper (10).

Compounds that were synthesized. 2-Hydroxymethyl-6-methylnaphthalene (17), 6-methylnaphthalene-2-aldehyde (E. A. Blair, J. J. Melchiore, and I. W. Mills, Chem. Abstr. **58**:491e, 1953), and 6-methylnaphthalene-2-carboxylic acid (1) have been reported previously, but were most conveniently prepared for this study as follows. 2,6-DMN was converted to 2-bromomethyl-6-methylnaphthalene with *N*-bromosuccinimide (6). The bromo derivative (6.02 g, 25.6 mmol) was converted to the aldehyde by heating with dimethyl sulfoxide (40 ml) and $NaHCO_3$ (2.6 g) at 140°C (16). The product was precipitated by pouring the reaction mixture into water (500 ml), filtered, and dried under a vacuum (4.3 g). The dry product was purified by flash chromatography (28) on silica gel (40-μm-diameter particles) by using hexane-dichloromethane (1:1; vol/vol); after evaporation of the solvent, the dry product had a melting point of 120°C and on mass spectrometry gave a molecular ion with *m/e* 170 (Fig. 1). 2-Hydroxymethyl-6-methylnaphthalene was prepared by reduction of the aldehyde (0.60 g) with $NaBH_4$ (0.27 g) in 32 ml of 95% ethanol. After being stirred for 1 h at room temperature, the solution was diluted with 90 ml H_2O and brought to pH 7 to 8 with concentrated HCl to destroy excess $NaBH_4$; the mixture was then extracted with three 50-ml portions of CH_2Cl_2. After drying with Na_2SO_4, the combined extracts were evaporated to dryness. The product that was prepared in this way was substantially pure, whereas attempts to hydrolyze the bromo compound directly to the alcohol by using alkali in a variety of aqueous organic solvents always led to the formation of large proportions of by-products. The crude products were purified by flash chromatography with $CHCl_3$, and after evaporation of solvent the pure compound (0.45 g) had a melting point of 128°C (literature value, 128 to 130°C [19]) and *m/e* 172 (Fig. 1).

6-Methylnaphthalene-2-carboxylic acid was prepared from the aldehyde by oxidation at 25°C with a 0.33 mol proportion of sodium dichromate. The aldehyde (0.549 g) was dissolved in glacial acetic acid (40 ml), and 15 ml of MH_2SO_4 was added. A solution of sodium dichromate (0.319 g) in 5 ml of MH_2SO_4 was added in small portions over a 48-h period. This reaction mixture was diluted with an equal volume of water and extracted with two 50-ml portions of CH_2Cl_2, and the extracts were evaporated to dryness. The mixture was dissolved in 50 ml of CH_2Cl_2 and extracted with sodium bicarbonate solution. Unchanged aldehyde (0.24 g) was recovered from the organic layer. The bicarbonate solution was acidified and extracted with CH_2Cl_2 to obtain crude acids (0.32 g), which were purified by TLC on silica gel plates (2 mm thick) by using ethyl acetate-acetic acid (50:1; vol/vol). The total product recovered (R_f, 0.67) was 0.16 g (melting point, 232°C; literature value [1], 233 to 234°C) with *m/e* (Fig. 1). The acidic by-products had R_f values of 0.60 and 0.53. The acidic by-products accumulated when excess oxidant or elevated temperatures were used.

Metabolite production. Washed cells that were grown on 2,6-DMN were suspended in phosphate buffer at a protein concentration of about 0.5 mg ml^{-1} and shaken with 2,6-DMN at 25°C for 2 h. After filtration through fine glass wool and centrifugation, the clear supernatants from 500 ml of suspension were extracted with CH_2Cl_2 (two times, 100 ml) to give a neutral fraction. The aqueous layer was acidified with HCl and reextracted (acid fraction). Each fraction was dried with Na_2SO_4, evaporated, and separated into subfractions by TLC on silica gel containing a fluorescent indicator. Individual metabolites were extracted with ethyl acetate and rechromatographed before further examination.

High-pressure liquid chromatography of methyl salicylic acids was carried out on a column (4.6 by 250 mm) containing Altex ultrasphere octadecylsilane (5 μm) by using a linear gradient of acetonitrile in water (0 to 100% in 30 min; flow rate, 0.5 ml min^{-1}). The effluent was monitored at 310 nm.

RESULTS AND DISCUSSION

The method described by Sylvestre (29) for the detection of bacteria that are able to metabolize water-insoluble compounds is very valuable because it allows the detection of such bacteria in mixed populations before a significant selective pressure is applied and thus allows the isolation of numerous independent strains, even from a single soil or water inoculum. By this method, bacteria that are able to use 2,6-DMN as a sole source of carbon and energy and that belong to the genera *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, and *Vibrio* were isolated. The flavobacteria were the most numerous (25 of 32 isolates), and they fell into four groups based on hydrocarbon metabolism (Table 1). After growth on 2,6-DMN, all isolates oxidized 2-hydroxymethyl-6-methylnaphthalene, 6-methylnaphthalene-2-aldehyde, and 6-methylnaphthalene-2-carboxylic acid without a lag. Most isolates could also oxidize naphthalene and phenanthrene (although they could not grow on these compounds), but a small group (4 of 25) could not. Of this group two could grow with salicylate (or 3-, 4-, or 5-methyl salicylate) as the sole carbon source and induced all the early enzymes of 2,6-DMN metabolism when grown on either of the salicylates. The properties of this small group of isolates are described below.

Metabolite production. Cultures growing on 2,6-DMN accumulated yellow or orange by-products which streaked on chromatography of culture extracts and obscured possible metabolites. Therefore, the metabolites produced by washed cell suspensions were examined. Because the oxidation of methylnaphthalenes to carboxylic acids has been reported earlier (15, 23) and because the alcohol (compound 2, Fig. 2),

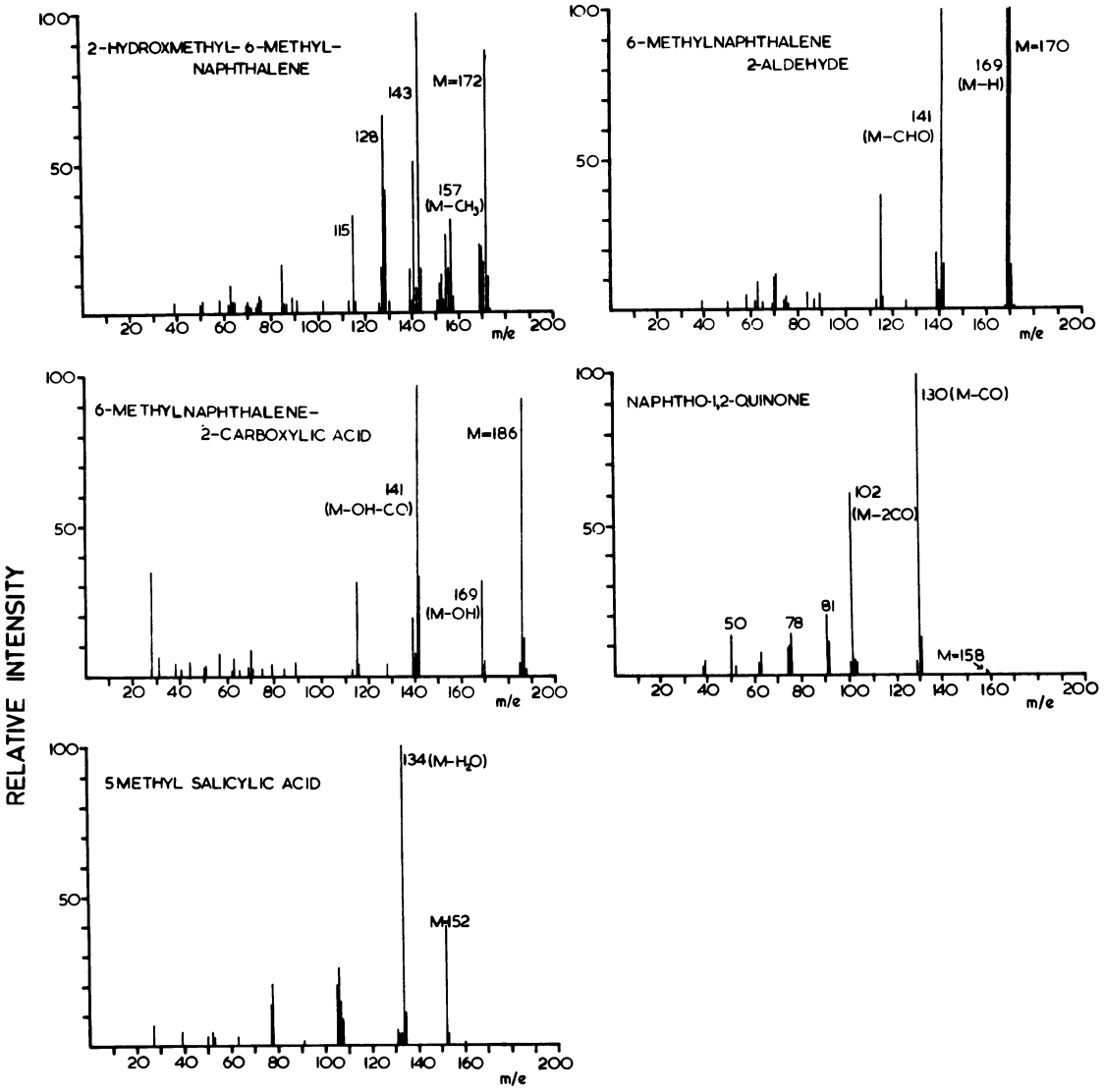

FIG. 1. Mass spectra. The mass spectra were obtained with a mass spectrometer (VG7070HS) by using electron impact at 70 eV. The spectra shown are those of synthetic compounds. When compounds were obtained as metabolites, they were not distinguishable from the synthetic compound. The methyl salicylic acids gave identical fragments, although the relative abundance of fragments was isomer dependent, e.g., the abundance of *m/e* 134 was greater than that of *m/e* 152 for the 4-methyl and 5-methyl isomers but not for the 3-methyl isomer. The molecular ion is indicated in each spectrum by M.

aldehyde (compound 3, Fig. 2), and acid (compound 4, Fig. 2) were oxidized without a lag (Table 1), metabolites were compared chromatographically and by mass spectrometry (Fig. 1) with these compounds. 2-Hydroxymethyl-6-methylnaphthalene (compound 2, Fig. 2) (R_f = 0.62 with ethyl acetate-acetic acid (49:1); R_f = 0.14 with $CHCl_3$) was observed in the neutral fraction, but the aldehyde (compound 3) was not. 6-Methylnaphthalene-2-carboxylic acid (com-

TABLE 1. Properties of flavobacteria that were able to grow on 2,6-DMN

Group	No. of strains	Respiration rate with[a]:						Growth on salicylate	Induction of early enzymes with salicylate
		2,6-DMN (compound 1)	Alcohol (compound 2)	Aldehyde (compound 3)	Acid (compound 4)	Naphthalene	Phenanthrene		
1	2	0.34	0.39	0.29	0.28	<0.01	<0.01	+	+
2	2	0.41	0.39	0.32	0.33	<0.01	<0.01	−	−
3	9	0.38	0.37	0.37	0.34	0.41	0.46	+	+
4	12	0.42	0.38	0.34	0.36	0.34	0.34	+	−

[a] Respiration rate of whole cells at 25°C in the presence of the compound and corrected for endogenous respiration (about 0.05 μmol of O_2/min per mg of protein). After growth on glucose, no significant increase in respiration was caused by these compounds. The measurements reported are for one strain that was representative of each group.

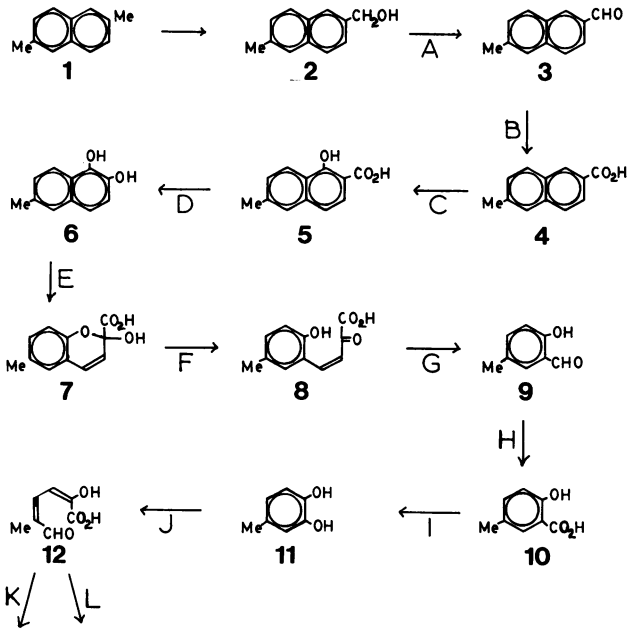

FIG. 2. The proposed metabolic pathway for the dissimilation of 2,6-DMN. Compound 1, 2,6-DMN; compound 2, 2-hydroxymethyl-6-methylnaphthalene; compound 3, 6-methylnaphthalene-2-aldehyde; compound 4, 6-methylnaphthalene-2-carboxylic acid; compound 5, 1-hydroxy-6-methylnaphthalene-2-carboxylic acid; compound 6, 1,2-dihydroxy-6-methylnaphthalene; compound 7, 2-carboxy-2-hydroxy-6-methylchromene; compound 8, 2'-hydroxy-5'-methylbenzalpyruvate; compound 9, 5-methylsalicylaldehyde; compound 10, 5-methyl salicylic acid; compound 11, 4-methylcatechol. The letters A to L identify enzymes whose activities are recorded in Table 2. The reactions catalyzed by D, E, F, G, and H were demonstrated only with the nonmethylated analogs. Me, Methyl.

pound 4) was detected in the acid fraction (R_f = 0.66 with ethyl acetate-acetic acid [49:1]; R_f = 0.27 with CH_2Cl_2-ethyl acetate-acetic acid [40:10:0.4]). The acid fraction also contained a metabolite at the known R_f of the salicylic acids (R_f = 0.45 with ethyl acetate-acetic acid [49:1]). The salicylic acid fraction was therefore methylated briefly with diazomethane in ether, under which conditions methylation was essentially restricted to the carboxylic acid group. The methylated products were rechromatographed with hexane-$CHCl_3$ (35:15; vol/vol), which separated the methyl esters of salicylic and 3-methylsalicylic acid (R_f, 0.32) from those of the 4- and 5-methyl acids (R_f, 0.24). A second processing of the chromatogram in the same solvent improved the separation. The R_f of the methylated metabolite corresponded to that of 4- or 5-methylsalicylic acid. The metabolite was extracted with acetone, and after evaporation of the solvent, it was saponified with 0.05 M NaOH. Acidification, extraction into CH_2Cl_2, and evaporation gave a solid which on mass spectrometry had a molecular ion with *m/e* 152 (Fig. 1) and which on high-pressure liquid chromatography eluted identically with 5-methylsalicylic acid.

The metabolites that were isolated suggested a metabolic pathway (Fig. 2) by which a methyl group was first oxidized to give the acid compound 4, which was then converted to 1,2-dihydroxy-6-methylnaphthalene (compound 6). When compound 6 was metabolized by steps analogous to those described previously for naphthalene (9), it would eventually lead to 4-methyl catechol (compound 11), whose metabolism is recognized (5). Enzymatic activities that catalyzed these reactions were sought.

The metabolism of compound 1 was measured only with whole cells. 2,6-DMN-grown cells metabolized the ^{14}C-labeled compound at a rate of 0.12 mol/min/per mg of protein. The rate of respiration (Table 1) was much higher, indicating the involvement of subsequent oxygen-consuming steps. The initial reaction may involve a monoxygenase system, but it could not be stabilized in disrupted cells. This and subsequent reactions involving the oxidation of compounds 2 and 3 may parallel steps reported previously for the metabolism of toluene (18). The oxidation of compound 4 is analogous to proposals by Davey and Gibson (8). An alcohol dehydrogenase (compound 2 to compound 3) and aldehyde dehydrogenase (compound 3 to compound 4) were present in cell extracts (Table 2). The enzymes had half-lives of about 10 h in cell extracts and were only examined by gel filtration on Sepharose CL-6B, on elution from which they separated poorly from each other at an elution volume corresponding to a molecular weight of 160,000 to 180,000. The alcohol dehydrogenase began to elute first, and the small quantity of enzyme free from the aldehyde dehydrogenase was used for kinetic studies. There was a broad pH optimum at about 8.2, and the K_m values for NADH and 6-methylnaphthalene-2-aldehyde were 0.062 and about 1 mM, respectively. Consequently, concentrations used in the standard method were not saturating with respect to either substrate. That of the aldehyde was, in fact, limited by its low solubility. The activity with NADPH was less than 1% of that with NADH. The reaction could be observed spectrophotometrically in the forward direction (compound 2 to compound 3); but linear initial velocities could not be determined easily, and it was more convenient to measure the reaction of compound 3 to compound 2. The aldehyde dehydrogenase was not obtained free from the alcohol dehydrogenase. Under the conditions of the standard assay, the K_m for the aldehyde was about 0.05 mM. The K_m for NAD estimated from measurements at low concentrations was 0.11 mM, but NAD was inhibitory at high concentrations. The insolubility of the aldehyde and the inhibition by NAD limited the concentrations used in the standard assay. NADP had less than 5% of the activity of NAD. The product of oxidation of the aldehyde was isolated and characterized. To 30 ml of PP_i

TABLE 2. Enzymatic activities of group 1 flavobacteria grown on 2,6-DMN

Enzyme[a]	Sp act after growth on 2,6-DMN[b]
A	1.37
B	0.20
C	0.34
D	0.19
E	3.12
F	0.45
G	0.90
H	0.96
I	0.25
J	0.23
K	0.18
L	0.15

[a] These enzymes catalyzed the reactions identified by letters in Fig. 2.

[b] Specific activities are micromoles per minute per milligram of protein. After growth on glucose, specific activities were ≤0.02. C and D were measured as increased respiration rates with whole cells. D to H inclusive were measured with nonmethylated analogs, but the values given for I, J, and K were similiar to those for the methyl compounds.

were added 0.1 ml of 10 mM aldehyde, 0.2 ml of 10 mM NAD, and 0.08 ml of cell extract. As the reaction ran to completion at intervals of about 5 min, further additions of aldehyde and NAD were made until a total of 6 μmol of aldehyde was reacted. The reaction mixture was extracted with CH_2Cl_2, acidified, and reextracted. After drying, the acid extract was evaporated and chromatographed as a band on silica gel with ethyl acetate-acetic acid (50:1, vol/vol). The product was extracted from the chromatogram with ethyl acetate. It was chromatographically identical to the authentic acid (compound 4) and had an identical mass spectrum (Fig. 1). The yield from the aldehyde was essentially quantitative; in this respect, enzymatic oxidation of the aldehyde is more efficient than chemical oxidation, although it is limited in the quantity that can be prepared conveniently.

6-Methylnaphthalene-2-carboxylic acid (compound 4) was oxidized by 2,6-DMN-grown cells (Table 1), but this activity was not detected in cell extracts either alone or fortified with cofactors. The 1- and 2-naphthoic acids were also oxidized by whole cells. For 6-methyl-2-naphthoic acid, 1-naphthoic acid, and 2-naphthoic acid, the respiration rates after growth on 2,6-DMN were 0.34, 0.13, and 0.30 μmol/min per mg of protein. After growth on glucose, the respiration rates for the three compounds were all less than 0.01 μmol/min per mg of protein. All values were corrected for endogenous O_2 uptake, which was about 0.05 μmol/min per mg of protein. The proposed intermediate compounds 5 to 9 are not available commercially and were not synthesized for this study, but reactions involving the nonmethylated analogs have been reported in studies on naphthalene metabolism. 1-Hydroxy-2-naphthoic acid (analog of compound 5) was oxidized by whole cells and by cell extracts in the presence of NADH. The extent of the reaction measured with extracts of acidified reaction mixture was limited, and the product (naphthoquinone) was obtained in a poor yield (Fig. 3). The primary reaction product was probably compound 6, which, under the conditions of the reaction, undergoes rapid autoxidation to naphtho-1,2-quinone. Naphtho-1,2-quinone was identified in extracts after TLC by its color, absorption spectrum, and mass spectrum with *m/e* 160 (Fig. 1). It is probable that the reactive quinone is responsible for the inhibition of the reaction, and the poor stoichiometry may be due to the binding of quinone to the excess protein that was present when crude cell extracts were used.

The reaction rates for nonmethylated analogs of compounds 6 to 11 are given in Table 2. The muconic acid semialdehyde from catechol was metabolized by both a dehydrogenase and a hydrolase. The activity of the dehydrogenase which produces NADH may have reduced the activity of salicylate hydroxylase that was measured spectrophotometrically by the oxidation of NADH, but it and the catechol oxygenase were not sufficient to mask the salicylate hydroxylase (2).

These observations on the two group 1 organisms strongly support the pathway for 2,6-DMN metabolism given in Fig. 2. Their weakness is the use of nonmethylated compounds for the measurement of reactions D to H inclusive. Although the oxidation of both 6 methylnaphthalene-2-carboxylic acid (compound 4) and 2-naphthoic acid was observed with whole cells, compound 5 has not been identified as a metabolite of the former. On the other hand, induction of the enzymes to catalyze the reactions was dependent on growth on 2,6-DMN or a salicylate. Salicylic acid and its 3-, 4-, and 5-methyl derivatives supported growth of the group 1 organisms and induced the same enzymatic activities. The results

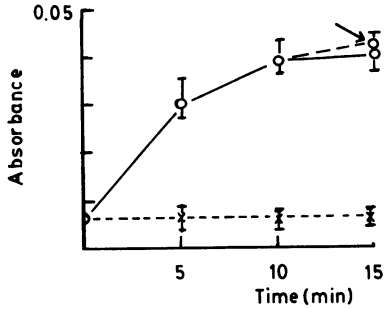

FIG. 3. Formation of naphtho-1,2-quinone from 1-hydroxy-2-naphthoic acid by sonicated cells. Sonicated cells (1 mg of protein), in 1 ml of phosphate (pH 7) containing 10 mM NADH and 100 μl of 10 mM 1-hydroxy-2-naphthoic acid, were incubated aerobically at 25°C in a shaking water bath. The reaction was stopped by shaking with 2 ml of ethyl acetate. After centrifugation ethyl acetate was removed, the extraction was repeated a further two times, the combined extracts were diluted to 10 ml, and the A_{392} was measured. Symbols: ○, test reaction; ×, reaction in the absence of 1-hydroxy-2-naphthoic acid. The bars indicate the range of three replicate assays. The arrow shows the absorbance at 15 min after more NADH was added at 10 min. The results in the absence of NADH were very similar to those in the absence of acid. The absorbance at 15 min (0.04), after correction for the control (0.006), represents a conversion of 14% of the acid to quinone (extinction coefficient, 2.4 mM^{-1} cm^{-1} at 392 nm). Extracts were dried with Na_2SO_4, evaporated in a stream of N_2 at 30°C, and chromatographed by TLC (on silica gel; ethyl acetate-acetic acid [50:1]). The yellow product (R_f, 0.72) was extracted with ethyl acetate and had a mass spectrum identical to that of naphtho-1,2-quinone (Fig. 1).

have not been tabulated but varied between 70 and 115% of those obtained with 2,6-DMN. With respect to induction by salicylate and the reactions by which compound 6 was metabolized, the proposed pathway bears a strong resemblance to that for naphthalene metabolism. Organisms with these properties were, however, a minority among those isolated. Two others could not utilize salicylate and its methyl homologs as sole sources of carbon and energy, and these compounds were not inducers (Table 1).

None of the isolates could grow with naphthalene or phenanthrene as a sole source of carbon and energy, but the majority (groups 3 and 4, Table 1) could oxidize these hydrocarbons after growth on 2,6-DMN. The implication is that either a nonspecific monoxygenase catalyzes the oxidation of all of the hydrocarbons or, alternatively, a typical aromatic dioxygenase is induced coincidentally. The latter might lead to 1,2-dihydroxy-3,7-dimethylnaphthalene. This compound was synthesized (7), but no evidence for its oxidation by extracts of DMN-grown cells was obtained. Such measurements are not simple, however, because 1,2-dihydroxy-3,7-dimethylnaphthalene undergoes autoxidation in aqueous solution more rapidly than does 1,2-dihydroxynaphthalene. The organisms in groups 3 and 4 were all striking because of their ability to oxidize a wide range of aromatic hydrocarbons after growth on 2,6-DMN. This oxidative ability was compared (Table 3) with that of a pseudomonad that was isolated for growth on naphthalene. The flavobacteria maintain high respiration rates compared with those when 2,6-DMN is used at the substrate, whereas the pseudomonads grown on naphthalene are relatively specific. These flavobacteria may be a valuable source of enzymes of broad oxidative ability, although this has not yet been examined either in the laboratory or in the field. Their subdivision in this study into four subgroups is based en-

TABLE 3. Comparison of the respiration rate of a group 3 flavobacterium (strain B233) with that of a pseudomonad (strain PpG7)

Substrate	Respiration rate of strain[a]:		Ratio of B233/PpG7
	B233	PpG7	
Naphthalene	100	100	1
2,6-DMN	100	3	33
Phenanthrene	110	8	14
Fluorene	20	2	10
Fluoranthene	44	<1	>44
2,3-DMN	82	16	5
2-Methylnaphthalene	130	76	2

[a] Each respiration rate is given as a percentage of that obtained with the carbon source used for growth, i.e., 2,6-DMN for strain B233 (absolute rate, 0.4 μmol/min per mg of protein) and naphthalene for strain PpG7 (absolute rate, 0.80 μmol/min per mg of protein).

tirely on examination of a limited number of strains for a limited number of characters (Table 1), and fundamental relationships remain to be determined.

ACKNOWLEDGMENTS

I thank the Natural Sciences and Engineering Research Council, Ottawa, Ontario, Canada, for a grant and B. Gregory, who obtained and interpreted the mass spectra.

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