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New Metabolites in the Degradation of Fluorene by *Arthrobacter* sp. Strain F101

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Identification of new metabolites and demonstration of key enzyme activities support and extend the pathways previously reported for fluorene metabolism by *Arthrobacter* sp. strain F101. Washed-cell suspensions of strain F101 with fluorene accumulated 9-fluorenone, 4-hydroxy-9-fluorenone, 3-hydroxy-1-indanone, 1-indanone, 2-indanone, 3-(2-hydroxyphenyl) propionate, and a compound tentatively identified as a formyl indanone. Incubations with 2-indanone produced 3-isochromanone. The growth yield with fluorene as a sole source of carbon and energy corresponded to an assimilation of about 34% of fluorene carbon. About 7.4% was transformed into 9-fluorenol, 9-fluorenone, and 4-hydroxy-9-fluorenone. Crude extracts from fluorene-induced cells showed 3,4-dihydrocoumarin hydrolase and catechol 2,3-dioxygenase activities. These results and biodegradation experiments with the identified metabolites indicate that metabolism of fluorene by *Arthrobacter* sp. strain F101 proceeds through three independent pathways. Two productive routes are initiated by dioxygenation at positions 1,2 and 3,4, respectively. *meta* cleavage followed by an aldolase reaction and loss of C-1 yield the detected indanones. Subsequent biological Baeyer-Villiger reactions produce the aromatic lactones 3,4-dihydrocoumarin and 3-isochromanone. Enzymatic hydrolysis of the former gives 3-(2-hydroxyphenyl) propionate, which could be a substrate for a β oxidation cycle, to give salicylate. Further oxidation of the latter via catechol and 2-hydroxymuconic semialdehyde connects with the central metabolism, allowing the utilization of all fluorene carbons. Identification of 4-hydroxy-9-fluorenone is consistent with an alternative pathway initiated by monooxygenation at C-9 to give 9-fluorenol and then 9-fluorenone. Although dioxygenation at 3,4 positions of the ketone apparently occurs, this reaction fails to furnish a subsequent productive oxidation of this compound.

Fluorene is a major component of fossil fuels and their derivatives (41), and together with its ketone 9-fluorenone, it is commonly identified in atmosphere, fresh water, and both riverine and marine sediments (5, 12, 18). Fluorene is highly toxic to fish and aquatic algae (23, 36), and both fluorene and 9-fluorenone have carcinogenic (nongenotoxic) potential (8). This naphthenoaromatic compound bears structural relationships to other chemicals of concern (carbazoles, dibenzothiophenes, dibenzofurans, and dibenzodioxins) and is thus a useful model for biodegradation studies.

The chemical structure of fluorene, including aromatic and alicyclic moieties, offers a variety of possibilities for biochemical attack. *Arthrobacter* sp. strain F101 was shown to grow on fluorene as a sole source of carbon and energy (17). Based on the identification of 9-fluorenol, 9-fluorenone, and 3,4-dihydrocoumarin in culture fluids, two pathways for fluorene metabolism were suggested. A nonproductive route, initiated by monooxygenation at C-9, led to the accumulation of 9-fluorenone. Growth was supported by an alternative pathway involving 3,4 dioxygenation, *meta* cleavage of one of the aromatic rings, and a biological Baeyer-Villiger reaction responsible for 3,4-dihydrocoumarin formation.

Analogous routes have been observed with other bacterial strains. A *Mycobacterium* sp. transforms fluorene, accumulating 1-fluorenol, 1-fluorenone, and 1-indanone (3). *Pseudomonas cepacia* F297, a versatile polycyclic aromatic hydrocarbon degrader, grows on fluorene, accumulating 1-indanone (20). However, there is no evidence of further utilization of 1-indanone by these strains.

An alternative biochemical strategy for the catabolism of fluorene is initiated by monooxygenation at C-9 to give 9-fluorenol, which is then dehydrogenated to 9-fluorenone. These reactions precede a dioxygenase attack adjacent to the carbonyl group, to form the angular diol 1,1a-dihydroxy-1-hydro-9-fluorenone. Cleavage of the five-membered ring generates a substituted biphenyl, whose further degradation by reactions similar to those of biphenyl catabolism leads to the formation of phthalate (19, 29, 37). Phthalate is then metabolized via protocatechuate (19).

Here we identify new metabolites and demonstrate key enzymatic activities that support and extend the pathways previously proposed (17) for the metabolism of fluorene by *Arthrobacter* sp. strain F101. Our results indicate that initial dioxygenation may occur either at positions C-1,C-2 or at C-3,C-4. The corresponding *cis* dihydrodiols undergo dehydrogenation and then *meta* cleavage. After the aldolase reaction and decarboxylation of the fission product, the resulting indanones are substrates for a biological Baeyer-Villiger reaction, yielding the aromatic lactones 3-isochromanone and 3,4-dihydrocoumarin. Enzymatic hydrolysis of the latter and further degradation of the resulting 3-(2-hydroxyphenyl) propionic acid via catechol connect this pathway with the central metabolism, allowing the complete utilization of fluorene. Identifi-

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cation of 4-hydroxy-9-fluorenone is consistent with an alternative route initiated by oxidation at C-9.

MATERIALS AND METHODS

Chemicals. Aromatic compounds used in this study were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Media were obtained from Difco Laboratories, Detroit, Mich. Solvents were obtained from J. T. Baker, Inc. All chemicals and solvents were of the highest purity available.

Microorganism, media, and supply of aromatic compounds. Isolation and identification of *Arthrobacter* sp. strain F101 has been reported previously (17). Bushnell-Haas Broth (BHB) was used as the mineral basal medium throughout the study. When used with fluorene or other aromatic compounds as sole sources of carbon and energy, the mineral medium was supplemented with vitamin B_{12} (40 μg per liter). Aromatic substrates (0.01%) were added to the sterile medium in acetone solution (5%). Prior to inoculation, the acetone was evaporated by shaking the flasks at 30°C for 24 h, which resulted in a fine suspension of the substrate in the liquid medium.

Cells for washed-cell suspensions and enzyme assays were grown in BHB with Bacto Peptone (5 g per liter), yeast extract (1 g per liter), and fluorene. This medium was used either as a liquid or solidified with agar (15 g per liter). Fluorene was supplied to the liquid medium as described above. Fluorene was supplied to the solid medium as a vapor by placing crystals on the lid of the plates.

Utilization of fluorene and accumulation of metabolites. Cells grown for 5 days in solid medium (BHB with Bacto Peptone and yeast extract) and fluorene vapor were used to inoculate (5 μg per ml) replicate batch cultures in 100-ml Erlenmeyer flasks containing 20 ml of BHB, vitamin B_{12}, and fluorene (0.01%). Incubation was performed at 22°C with rotary shaking (200 rpm). Uninoculated flasks and inoculated flasks without fluorene served as controls. Fluorene concentration, accumulation of metabolites, and protein content were determinated at 24-h intervals over the first 4 days and then at 2- or 4-day intervals until the end of incubation (18 days). To determine protein content, cells from one entire flask were harvested by centrifugation, treated with 1 N NaOH at 100°C for 1 min, and analyzed by the method of Lowry et al. (25). To assess fluorene disappearance and metabolite formation, the contents of another flask were adjusted to pH 2.5 by adding 1 N HCl and extracted with ethyl acetate. Then, the extract was dried, redissolved in 1 ml of methanol, and analyzed by high-pressure liquid chromatography (HPLC) as indicated below.

Utilization of presumptive metabolites as sole source of carbon and energy. The utilization of presumptive metabolites of fluorene as growth substrates was tested in a manner similar to that used with fluorene. The differences were that substrate disappearance and protein content were determined in duplicate cultures and datum points were taken only at the time of inoculum and after 10 days of incubation. A given compound was considered to support growth when both replicates showed a disappearance of substrate and an increase in cell protein at least five times greater than that observed with controls.

Washed-cell incubations. For washed-cell incubations with fluorene, cells were pregrown in liquid BHB with Bacto Peptone, yeast extract, and fluorene (0.01%) at 22°C with rotary shaking (200 rpm). Utilization of fluorene in this medium is indicated by the accumulation of a soluble yellow metabolite in the medium (see Results). When liquid cultures reached late exponential phase (48 h, 82 μg of protein/ml), the cells were harvested by centrifugation, washed twice in 50 mM K-Na phosphate buffer (pH 7.2), and resuspended in the same buffer at a concentration of 820 μg of protein/ml. In liquid culture, strain F101 has a clotty growth, which hinders the removal of residual fluorene from the culture. For this reason, cells for incubation with compounds other than fluorene were obtained from solid cultures in the same medium incubated with fluorene crystals on the lid of the plates. In these conditions, utilization of fluorene is also shown by the accumulation of the yellow metabolite in the agar. Bacterial growth (48 h) from the surface of solid medium was recovered and suspended in buffer, and then the resulting suspension was treated as a liquid culture. Cell suspensions were distributed in Erlenmeyer flasks, to which fluorene or other aromatic substrates were added as crystals in excess (0.1%). Incubation was carried out at 22°C and 200 rpm. In all cases, sterile controls were included.

Identification of metabolites. After 48 h of incubation, the cells were removed by centrifugation, and the supernatant was filtered through glass wool to remove the remaining undissolved aromatic substrates. Supernatants were first extracted at pH 7 (three times with an equal volume of ethyl acetate), then acidified to pH 2.5 with HCl (10 N), and extracted again in the same manner. Extracts were dried over anhydrous Na_2SO_4, concentrated by rotary evaporation, and prepared for HPLC and gas chromatography coupled to mass spectrometry (GC-MS) analyses. Samples for HPLC were evaporated to dryness under a nitrogen stream and redissolved in methanol. For GC-MS, the acidic extracts were treated with an excess of BF_3-methyl hydroxide for 12 h at room temperature, then the reaction was stopped with $NaHCO_3$ (1 N), and the methyl esters were extracted with hexane. Metabolites were identified by comparison of their UV-visible and electron impact (EI) MS spectra with those obtained for authentic samples and by coelution in HPLC and GC. Identification of compound IV was completed by high-resolution MS and infrared (IR) spectrometry analysis.

Analytical methods. Reverse-phase HPLC was performed on a Hewlett-Packard model 1050 chromatograph equipped with an HP-1050M diode array UV-visible detector set at 254 nm. Separation was achieved on a 5-μm C_{18} column (25 cm by 4.6 mm [inside diameter]; Chrompack, Middelburg, The Netherlands) with a linear gradient of methanol in water (0 to 100% in 20 min). The injection volume was 10 μl. The amounts of compounds present were estimated from the peak areas of duplicate samples by using standard calibration curves for each chemical. GC-MS analyses were performed on a Hewlett-Packard 5890 gas chromatograph with an HP 5988A mass selective detector. Compounds were separated on an HP-5 capillary column (25 m by 0.2 mm [inside diameter]), with 0.5-μm film thickness and helium as the carrier gas (linear velocity, 25 cm/s). The column temperature was held at 70°C for 1 min and then programmed to 300°C at a rate of 6°C/min. The mass spectrometer was operated at 70 eV of electron ionization energy. The ion source was set at 250°C.

GC-MS in the positive-ion mode was performed with a Finnigan TSQ 700 triple-stage quadrupole (San Jose, Calif.). Methane was used as reagent gas at 0.5 torr in the ion source. High-resolution (10,000) EI MS was performed with a magnetic sector instrument (Fisons, Manchester, United Kingdom). Scans were obtained from *m/z* 60 to 200. IR spectra were recorded on a Bomem model MB120 FT-IR spectrometer (Bomem, Quebec, Canada).

Regulation of fluorene utilization by *Arthrobacter* sp. strain F101. The bacterial strain was grown on BHB with Bacto Peptone and yeast extract until mid-exponential phase. Then, the culture was divided in two portions, one of which received 100 μg of chloramphenicol per ml of culture (growth of this strain was inhibited by <50 μg of chloramphenicol per ml). The subcultures were incubated at 22°C for 2 h with shaking at 200 rpm, and then cells from both subcultures were harvested by centrifugation, washed, and resuspended in sterile 50 mM phosphate buffer. The buffer used for the chloramphenicol-treated subculture contained 100 μg of chloramphenicol per ml. Both aliquots were then supplemented with 0.01% fluorene, incubated, and sampled for spectrometric studies, and their supernatants were analyzed for absorbance at 398 nm, the typical visible absorption maximum of the supernatant from cultures of *Arthrobacter* sp. strain F101 when growing on fluorene (see Results). This method is based on that reported by Foght and Westlake (14).

Preparation of crude extracts and demonstration of enzyme activities. Strain F101 was grown for 3 days on solid BHB medium containing Bacto Peptone and yeast extract and exposed to fluorene vapors. Then, the cells were harvested, washed, and resuspended in 50 mM phosphate buffer, pH 7.0, to give approximately 0.5 g (wet weight) of cells per ml. Cell extracts were prepared by passing the suspension twice through a French pressure cell at 14,000 to 20,000 lb/in^2. Extracts were subjected to centrifugation at 48,000 × *g* for 40 min at 4°C, and the resulting clear supernatant was separated from pelleted cells and debris. These extracts contained 6.4 mg of protein per ml as determined by the method of Lowry et al. (25).

Enzyme activities were assayed by monitoring the disappearance of substrates and appearance of products by UV-visible spectrometry. 3,4-Dihydrocoumarin hydrolase was demonstrated by conversion of 3,4-dihydrocoumarin to a product with the spectral characteristics of authentic 3-(2-hydroxyphenyl) propionate. Catechol 2,3-dioxygenase was demonstrated by conversion of catechol to 2-hydroxymuconic semialdehyde according to the method of Gibson (15). Spectral changes were recorded at room temperature with a Shimadzu UV-265FW spectrophotometer. Reactions were monitored in a cuvette containing 3 ml of K-Na phosphate buffer (50 mM, pH 7.0), 40 μl of cell extract, and 5 μl of a 100 mM solution of 3,4-dihydrocoumarin in ethanol or 40 μl of a 25 mM solution of catechol in phosphate buffer (pH 7.0). Substrate was omitted from reference reaction mixtures. To calculate 3,4-dihydrocoumarin hydrolase activity, product formation was monitored by monitoring the absorbance increase at 270 nm. The molar absorption coefficient of 3-(2-hydroxyphenyl) propionate was 1,870 at pH 7. One unit of enzyme was defined as the amount that produces 1 μmol of 3-(2-hydroxyphenyl) propionate per min under the conditions of the assay. Readings were recorded at 2-min intervals. Catechol 2,3-dioxygenase activity was calculated according to the procedure reported by Gibson (15), modified as indicated above. Readings were recorded at 30-s intervals.

RESULTS

Utilization of fluorene and accumulation of metabolites. *Arthrobacter* sp. strain F101 utilizes fluorene as a sole source of carbon and energy (17). During exponential growth (with an estimated generation time of 37 h), approximately 92.4 μg (556 nmol) of fluorene was converted into 30 μg of protein (amounts per ml) (Fig. 1A). Assuming protein to be about 50% of cell dry weight and cell dry weight to be approximately 50% carbon (26), there appears to be a cellular assimilation of fluorene with an efficiency of about 34%. Growth resulted in the accumulation of a yellow metabolite with an absorption maximum at 398 nm (pH 7). After acidification, the absorption maximum shifted to 376 nm, suggesting the keto-enolic tautomerism characteristic of *meta* cleavage products of aromatic ring systems.

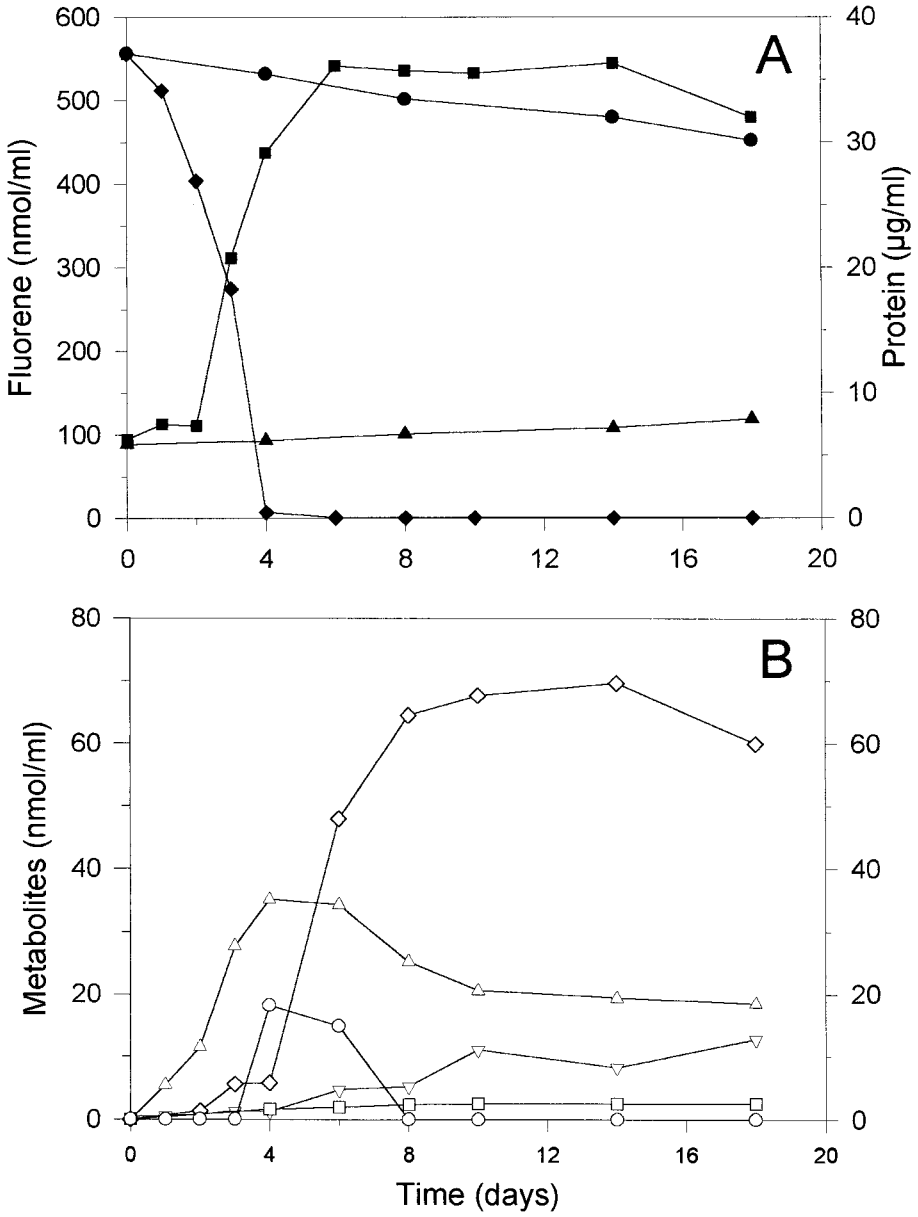

FIG. 1. Utilization of fluorene by *Arthrobacter* sp. strain F101 (A) and accumulation of products formed during growth (B) in liquid mineral medium with fluorene as a sole source of carbon and energy at 22°C and 200 rpm. Growth in cultures (■) and in controls without substrate (▲) is shown as the increase in cell protein. Concentrations of fluorene (◆), cultures; ●, sterile controls), 9-fluorenol (compound I) (△), 9-fluorenone (compound II) (▽), 4-hydroxy-9-fluorenone (compound VIII) (□), 3,4-dihydrocoumarin (compound III) (○), and 3-hydroxy-1-indanone (compound IV) (◇) were determined by HPLC analyses of organic extracts from the cultures.

HPLC analyses of organic extracts from cultures revealed a number of products (Fig. 1B), some of which (9-fluorenol [compound I], 9-fluorenone [compound II], and 3,4-dihydrocoumarin [compound III]) had been previously identified (17). Others (compounds IV, VIII, and X) were later identified as 3-hydroxy-1-indanone, 4-hydroxy-9-fluorenone, and salicylate, respectively (see below). At the end of exponential phase, their concentrations were estimated at 34.3 nm/ml for 9-fluorenol (compound I) (6.2% of starting fluorene), 4.7 nm/ml for 9-fluorenone (compound II) (0.9%), 1.9 nm/ml for 4-hydroxy-9-fluorenone (compound VIII) (0.3%), 14.9 nm/ml for 3,4-dihydrocoumarin (compound III) (2.7%), 48 nm/ml for 3-hydroxy-1-indanone (compound IV) (8.6%), and traces for salicylic acid (compound X). Except for 3,4-dihydrocoumarin and salicylate, which were not further detected, the concentrations of these compounds underwent small changes the next 2 days and became approximately constant for the rest of the incubation (18 days).

No significant changes in biomass were observed in the controls. Fluorene concentration decreased from 556 to 452 nm/ml in sterile controls, which was attributed to loss by volatilization.

Identification of fluorene metabolites. Metabolites IV and X were observed only in growing cultures. Isolation and partial characterization (proton nuclear magnetic resonance [^{1}H NMR] and EI MS) of metabolite IV (1.8 mg) were reported previously (17), suggesting a hydroxy-indanone structure. Further analysis by high-resolution MS and IR spectroscopy in this

TABLE 1. GC R_t and EI mass spectral properties[a] and identification of new products detected in washed-cell incubations of *Arthrobacter* sp. strain F101 with fluorene

Compound no.	R_t (min)	*m/z* of fragment ions (% relative intensity)	Identification
V	16.2	132 (M^+, 97), 104 (M^+ -CO, 100), 103 (57), 85 (8), 78 (47), 77 (48), 76 (26), 63 (22)	1-Indanone
VI	14.7	132 (M^+, 41), 104 (M^+ -CO, 100), 103 (39), 89 (3), 78 (40), 63 (16)	2-Indanone
VII	21.1	160 (M^+, 35), 132 (M^+ -CO, 62), 131 (100), 115 (M^+ -CO-OH, 8), 103 (52), 91 (10), 77 (59), 71 (10), 63 (10)	Formyl-indanone
VIII	30.7	196 (M^+, 100), 168 (M^+ -CO, 51), 152 (M^+ -CO-OH, 1), 139 (61), 113 (10), 98 (8), 87 (9), 74 (10), 63 (16)	4-Hydroxy-9-fluorenone
IX	21	180 (M^+, 17), 149 (M^+ -CH_3O, 11), 148 (M^+ -CH_3OH, 57), 120 (M^+ -$COOCH_3$, 100), 107 (50), 103 (M^+ -$COOCH_3$-OH, 11), 99 (94), 91 (65), 77 (58), 65 (24)	3(2-Hydroxyphenyl)propionic acid (ME[b])

[a] *m/z* (suggested fragmentation and percent relative intensity).
[b] Identified as a methyl derivative.

study confirmed its identification. Positive-ion chemical ionization showed a base peak at $m/z = 149$ $(M + 1)^+$, enabling the assignment of molecular ion (M^+) at $m/z = 148$. High-resolution EI GC-MS gave a mass of 148.0527 for this molecular ion, which is consistent with $C_9H_8O_2$ (mass, 148.0524). IR spectroscopy analysis showed high absorbance between 3,400 and 3,600 cm^{-1}, which is characteristic of the tension of the O-H bond, and a maximum at 1,703 cm/liter, indicative of the tension of the C═O bond (which gives a maximum at 1,700 cm/liter) (34). On the basis of the results and the UV absorption and ^1H NMR results previously published (17), we concluded that the structure of metabolite IV is 3-hydroxy-1-indanone. These data are consistent with those reported by Resnick et al. (27) for this compound. Metabolite X exhibited an HPLC retention time and a UV spectrum indistinguishable from those obtained for authentic salicylic acid (see Table 2).

GC-MS analyses of neutral extracts (42 mg) from washed-cell incubations (400 ml) of strain F101 with fluorene (0.4 g) showed remaining fluorene (11.5%), 9-fluorenone (compound II, 69%), 3,4-dihydrocoumarin (compound III, 2.1%), compound VIII (also observed in growing cultures), and three additional compounds (V, VI, and VII). Compounds V (0.6%), VI (0.1%), and VIII (5.9%) showed retention times (R_ts) and mass spectra indistinguishable from those obtained for authentic samples of 1-indanone, 2-indanone, and 4-hydroxy-9-fluorenone, respectively. The MS spectrum of metabolite VII (1.7%) exhibited molecular ion at $m/z = 160$ (M^+) and a fragmentation pattern consistent with the loss of keto ($M^+ - 28$) and formyl ($M^+ - 29$) groups (32), the latter to give the base peak at $m/z = 131$. This base peak is consistent with a deprotonated indanone. The prominent ion at $m/z = 103$ was also found in the mass spectra of authentic 1- and 2-indanone. Although these data do not allow a definitive identification, they suggest a formyl-indanone structure substituted in the alicyclic ring for metabolite VII. GC-MS analyses of methylated acidic extracts (66 mg) showed a complex mixture of compounds, one of them (compound IX) ($R_t = 21.2$ min, 15%) possessing a retention time and mass spectrum (Table 1) identical to those observed for the methyl ester of authentic 3-(2-hydroxyphenyl) propionic acid. Identification of all of the compounds except compound VII was confirmed by coelution in GC and HPLC with authentic standards (Table 2).

Washed-cell suspensions incubated with 9-fluorenone (compound II) accumulated 4-hydroxy-9-fluorenone (compound VIII) (6.6% of the HPLC chromatogram of the neutral extract) and the yellow metabolite ($\lambda_{max} = 398$ nm) detected in growing cultures with fluorene. Accordingly, we conclude that this yellow compound is a *meta* cleavage product resulting from 9-fluorenone transformation. 9-Fluorenol and 9-fluorenone were not growth substrates for this strain. Incubations with 1-indanone (compound V) accumulated 3,4-dihydrocoumarin (compound III) (3% of the HPLC chromatogram of the neutral extract), 3-hydroxy-1-indanone (compound IV) (7.2% of the HPLC chromatogram of the neutral extract), 3-(2-hydroxyphenyl) propionate (compound IX) (11.2% of the HPLC chromatogram of the acidic extract), and salicylic acid (compound X) (traces by HPLC). When incubated with 2-indanone (compound VI), washed-cell suspensions accumulated a major neutral metabolite (17.3% of the total ion chromatogram) showing a retention time ($R_t = 20.8$ min) and mass spectrum (148 [M^+, 24%], 119 [M^+ -CO-H, 2%], 104 [M^+ -CO-O, 100%], 91 [28%], 78 [25%]) identical to those of authentic 3-isochromanone (compound XI). Identification of this compound was confirmed by HPLC ($R_t = 14.3$ min; $\lambda_{max} = 242$ nm at pH 7.0 and $\lambda_{max} = 260$ nm at pH 7.0). 3,4-Dihydrocoumarin was transformed to 3-(2-hydroxyphenyl) propionate (compound IX) (20.3% of the HPLC chromatogram of the acidic extract) and salicylate (compound X) (traces by HPLC). 1-Indanone, 2-indanone, 3-isochromanone, 3,4-dihydrocoumarin, 3-(2-hydroxyphenyl) propionate, and salicylate were found to be growth substrates for *Arthrobacter* sp. strain F101, as demonstrated by cell protein increase in mineral medium with these compounds as sole sources of carbon and energy. These results strongly support the designation of these compounds as intermediates of the metabolism of fluorene by this strain.

Enzyme activities. Crude extracts from fluorene-induced cells showed the presence of 3,4-dihydrocoumarin hydrolase activity, as demonstrated by UV spectral changes showing the disappearance of 3,4-dihydrocoumarin (III) ($\lambda_{max} = 263$ and 271 nm, at pH 7.0) with accumulation of a product having the spectral characteristics of authentic 3-(2-hydroxyphenyl) propionate ($\lambda_{max} = 271$ nm at pH 7.0) (Fig. 2). The enzyme activity was estimated in 99.5 mU per mg of protein. In the

TABLE 2. HPLC R_t and UV spectral characteristics of new products detected in washed-cell incubations and growing cultures of *Arthrobacter* sp. strain F101

Compound name and no.	R_t (min)	λ_{max} (nm)
1-Indanone (V)	6.2	248, 293
2-Indanone (VI)	7.0	271
4-Hydroxy-9-fluorenone (VIII)	18.9	251
3(2-Hydroxyphenyl)propionate (IX)	4.2	271
Salicylate (X)	4.5	230, 297

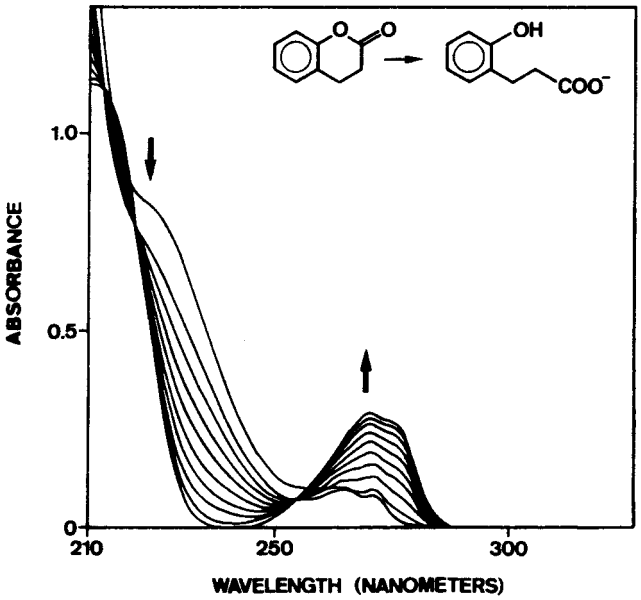

FIG. 2. Metabolism of 3,4-dihydrocoumarin by extracts of fluorene-induced cells of *Arthrobacter* sp. strain F101. The first spectrum was recorded before addition of the cell extract, and subsequent spectra were recorded at 2-min intervals. The enzyme activity was estimated to be 99.45 mU per mg of protein (see the text).

absence of cell extract, no hydrolysis of 3,4-dihydrocoumarin was observed under the conditions of the assay. Crude extracts of cells grown in the presence of fluorene also showed catechol 2,3-dioxygenase, as evidenced by UV spectral changes consistent with conversion of catechol to 2-hydroxymuconic semialdehyde (increase at λ_{max} = 375 nm). The enzyme activity was estimated to be 6.44 mU per mg of protein.

Regulation of fluorene utilization by *Arthrobacter* sp. strain F101. Noninduced cell suspensions incubated with fluorene in the absence of chloramphenicol showed degradation of fluorene after a 4-h lag phase, as demonstrated by UV-visible analyses of the supernatant, showing an increase in absorbance at 398 nm. The supernatant from the same cell suspensions treated with chloramphenicol did not show spectral changes after 8 h of incubation with fluorene. These results indicate that degradation of fluorene by strain F101 of *Arthrobacter* is inducible.

DISCUSSION

The results presented here support and extend the metabolic pathways previously proposed for fluorene degradation by *Arthrobacter* sp. strain F101 (17). Novel key reactions are (i) a 1,2 dioxygenation of the substrate that initiates a set of reactions analogous to those reported for 3,4 dioxygenation, including *meta* cleavage and lactone formation; (ii) an enzymatic hydrolysis of the aromatic lactone (3,4-dihydrocoumarin [compound III]), which allows the complete utilization of the fluorene molecule via 2-(3-hydroxyphenyl) propionate (compound IX) and salicylate (compound X).

Identification of metabolites (GC-MS and HPLC) and demonstration of enzymatic activities, together with previous results, reveal that fluorene catabolism can proceed by three alternative routes (Fig. 3). Two of them support growth and are initiated by dioxygenase attack at either 1,2 or 3,4 positions. The third, involving approximately 7.4% of starting fluorene in growing cultures, is a nonproductive route and is initiated by monooxygenation at C-9. The first steps of the dioxygenation routes are analogous to those described for naphthalene degradation (16, 33). The resulting 1,2 or 3,4 dihydrodiols are dehydrogenated to give the corresponding diols. *meta* cleavage and a subsequent aldolase reaction would give the corresponding formyl indanones (compound VII), presumptively identified here. Identification of 1- and 2-indanone (compounds V and VI, respectively) suggests that loss of the aldehyde substituent occurs after oxidation to carboxyl and subsequent decarboxylation of the resulting β-keto acid. A similar mechanism may account for the compound acenaphthenone observed by Weissenfels et al. (39) in studies of fluoranthene degradation. Heitkamp et al. (22) also propose a mechanism of one-carbon excision in the formation of 4-phenanthroic acid detected in experiments of pyrene degradation by a *Mycobacterium* sp. strain.

As proposed previously (17), the subsequent reaction requires an insertion of one oxygen atom into the carbon ring system by a Baeyer-Villiger reaction (1) after the mechanism of cleavage of alicyclic rings such as cyclohexanol (11) and camphor (7). By this mechanism, 1-indanone (compound V) and 2-indanone (compound VI) would yield 3,4-dihydrocoumarin (compound III) and 3-isochromanone (compound IV), respectively.

1-Indanone (compound V) also appears to be a substrate for aromatic hydroxylation yielding 3-hydroxy-1-indanone (compound IV), probably a dead-end product. Several arene dioxygenases have been described to act as monooxygenases hydroxylating methylenic groups in aromatic compounds (30, 38). Recently, Resnick et al. (27) reported that naphthalene and toluene dioxygenases oxidized 1-indanone to a mixture of 3-hydroxy-1-indanone (91%) and 2-hydroxy-1-indanone (9%). According to this, the hydroxylation of 1-indanone could be explained by a fortuitous action of the arene dioxygenase(s) that initiates the attack of fluorene. At this time there is no evidence of this reaction.

3,4-Dihydrocoumarin (compound III) is enzymatically hydrolyzed by strain F101 to 3-(2-hydroxy phenyl) propionate (compound IX). Although some of the lactones formed by bacterial Baeyer-Villiger oxidations of cyclic ketones are unstable and undergo spontaneous ring opening (9, 21, 40), the majority, including lactones from camphor (6) and cyclohexanol (2, 35) degradation, require subsequent hydrolysis by inducible lactone hydrolases. A 3,4-dihydrocoumarin hydrolase catalyzing the conversion of 3,4-dihydrocoumarin to melilotic acid [3-(2-hydroxy phenyl) propionate] has been purified from the plant *Melilotus alba* and characterized (24). A more recent paper (31) reports that a novel aldonate lactone hydrolase purified from the fungus *Fusarium oxysporum* AKV 3702 also irreversibly hydrolyzes aromatic lactones, including 3,4-dihydrocoumarin and 3-isochromanone. To the best of our knowledge, this is the first time that dihydrocoumarin hydrolase activity has been demonstrated in bacteria.

Weissenfels et al. (39) also proposed a Baeyer-Villiger reaction followed by hydrolysis to explain the degradation of acenaphthenone, an intermediate in the metabolism of fluoranthene by an *Alcaligenes* strain. However, this was based on the identification of the corresponding lactone and a substituted catechol, while hydrolase activity was not reported.

Certain bacterial strains initiate metabolism of 3-phenylpropionate by attack in the aromatic ring. For example, *Escherichia coli* K-12 dioxygenates 3-phenylpropionate and hydroxylates 3-(3-hydroxyphenyl) propionate to give 3-(2,3-dihydroxyphenyl) propionate, which is further degraded via *meta* cleavage (4). Accumulation of salicylate in incubations with fluorene and 3,4-dihydrocoumarin suggests that strain F101 initiates the

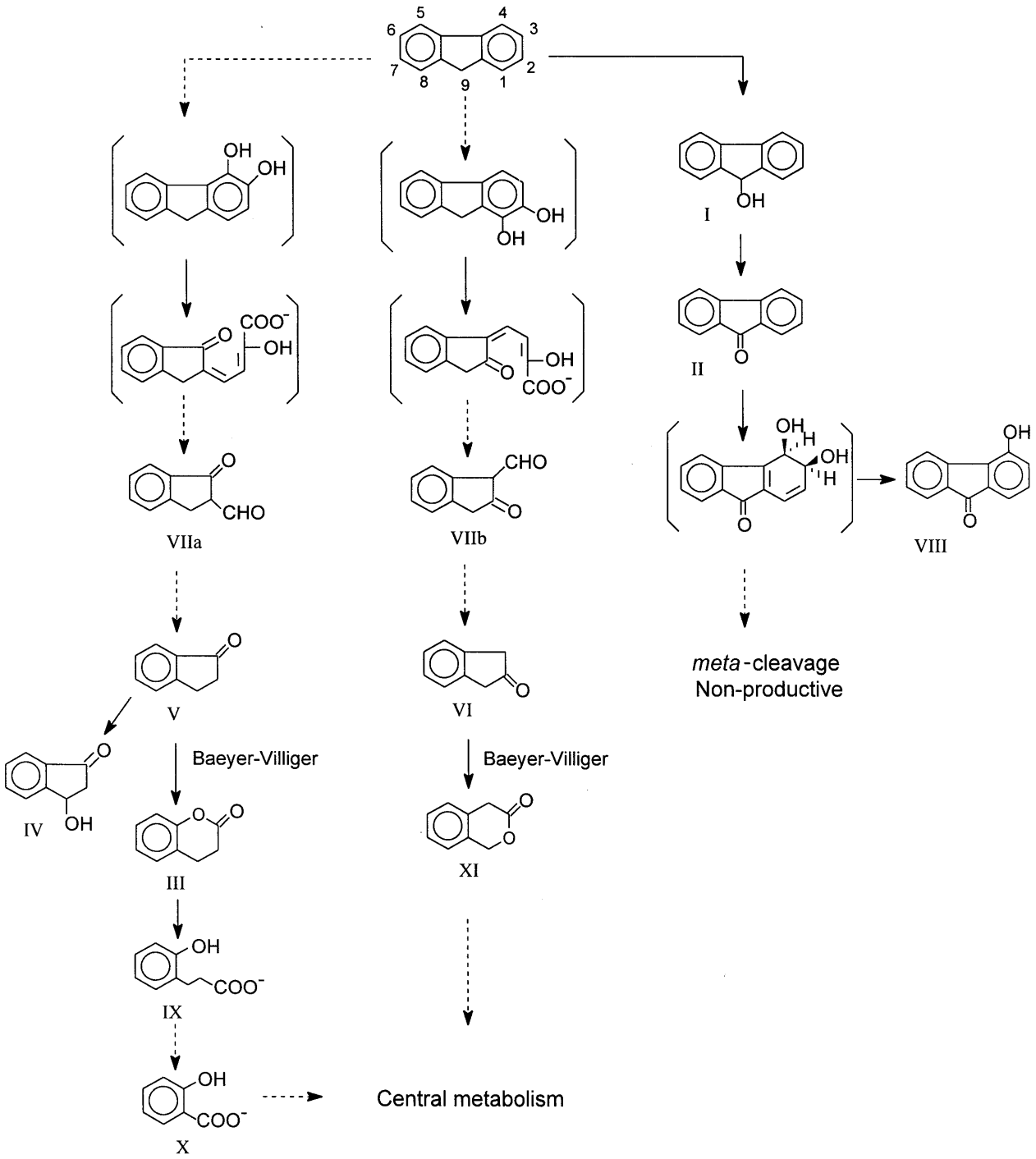

FIG. 3. Schematic pathway proposed for the degradation of fluorene by an *Arthrobacter* sp. Metabolites I, II, and III were identified in a previous publication (17). Structures in brackets are proposed intermediates and have not been identified. Dashed arrows indicate two or more successive reactions. Chemical designations: I, 9-fluorenol; II, 9-fluorenone; III, 3,4-dihydrocoumarin; IV, 3-hydroxy-1-indanone; V, 1-indanone; VI, 2-indanone; VIIa and VIIb, 2-formyl-1-indanone and 1-formyl-2-indanone, respectively (one isomer was tentatively identified, and the position of the substituents was not determined); VIII, 4-hydroxy-9-fluorenone; IX, 3-(2-hydroxyphenyl) propionate; X, salicylate; XI, 3-isochromanone.

attack on 3-(2-hydroxyphenyl) propionate at the side chain. Bacterial metabolism of long-chain alkyl benzenes occurs via β oxidation of the alkylic moiety (16, 33). Rontani et al. (28) reported that 3-phenylpropionate resulting from nonylphenyl degradation by a mixed culture also underwent β oxidation. According to this, the formation of salicylate from 3-(2-hydroxyphenyl) propionate by strain F101 could be explained by release of one molecule of acetate after a single β oxidation cycle.

Salicylate is a key intermediate in the metabolic pathway of naphthalene by pseudomonads (10, 16, 33). In most of the naphthalene degraders investigated, salicylate undergoes oxi-

dative decarboxylation to yield catechol as the substrate for fission of the aromatic nucleus (16). Cells of *Arthrobacter* sp. strain F101 grown on fluorene showed catechol 2,3-dioxygenase, suggesting that salicylic acid could be transformed to catechol and that the latter could undergo *meta* cleavage to connect with the central metabolism. These reactions allow the complete utilization of the fluorene molecules initially attacked by 3,4 dioxygenation.

9-Fluorenone was previously proposed as a dead-end product in a nonproductive route (17). Our results here indicate that dioxygenation of the ketone does occur. Some dihydrodiols resulting from dioxygenase attack of aromatic rings appear to undergo spontaneous dehydration to give the corresponding phenols (22). Consequently, the identification of 4-hydroxy-9-fluorenone could be explained by the nonenzymatic dehydration of the corresponding 3,4-dihydrodiol. In support of this is the detection of the characteristic *meta* cleavage product with absorption maxima at 398 nm, in incubations with fluorene, 9-fluorenol, and 9-fluorenone. A similar absorption maximum has been observed by Foght and Westlake (13) in incubations of a *Pseudomonas* strain with the ketone, while these and other authors reported a maximum of 462 nm for incubations with fluorene (13, 20). Apparently, this compound(s) is not accommodated by subsequent enzymes in the pathway, which prevents release of the pyruvate necessary for growth of the strain.

In accordance with the mass balances of products formed from fluorene in growing cultures, about 7.4% of fluorene would be oxidated throughout the nonproductive route and 92.6% would be degraded by the productive routes. Apparently, some of the molecules following the 3,4 dioxygenation route (8.6%) would be accumulated as 3-hydroxy-1-indanone. The accumulation of dead-end products is consistent with the estimated efficiency in fluorene utilization.

The presence of the five-membered ring with a methylenic group in the fluorene structure prevents its mineralization by reactions previously reported for the degradation of aromatic compounds (16). Some bacterial strains are able to combine monooxygenation at C-9, angular dioxygenation, and reactions similar to those of biphenyl catabolism (19, 37), which resolve this problem. *Arthrobacter* sp. strain F101 has been shown to offer a different and unique metabolic pathway for fluorene utilization. This pathway combines reactions of the metabolism of (i) aromatic compounds, such as dioxygenation; (ii) alicyclics, such as Baeyer-Villiger and enzymatic hydrolysis of the lactone; and (iii) linear fatty acids, such as β oxidation. This and previous reports illustrate the metabolic diversity of fluorene transformation by bacteria.

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