The trans-Anethole Degradation Pathway in an Arthrobacter sp.*

Received for publication, October 4, 2001, and in revised form, January 13, 2002
Published, JBC Papers in Press, January 22, 2002, DOI 10.1074/jbc.M109593200

Eyal Shimoni§, Timor Baasov¶, Uzi Ravid¶, and Yuval Shoham‡**

From the §Department of Food Engineering and Biotechnology, ¶Department of Chemistry, and ‡Institute of Catalysis Science and Technology, Technion-Israel Institute of Technology, Haifa 32000, and Agricultural Research Organization, ¶Newe Ya’ar Research Center, P. O. Box 1021, Ramat Yishai 30955, Israel.

A bacterial strain (TA13) capable of utilizing t-anethole as the sole carbon source was isolated from soil. The strain was identified as Arthrobacter aurescens based on its 16 S rRNA gene sequence. Key steps of the degradation pathway of t-anethole were identified by the use of t-anethole-blocked mutants and specific inducible enzymatic activities. In addition to t-anethole, strain TA13 is capable of utilizing anisic acid, anisaldehyde, and anisic alcohol as the sole carbon source. t-Anethole-blocked mutants were obtained following mutagenesis and penicillin enrichment. Some of these blocked mutants, accumulated in the presence of t-anethole quantitative amounts of t-anethole-diol, anisic acid, and 4,6-dicarboxy-2-pyrene and traces of anisic alcohol and anisaldehyde. Enzymatic activities induced by t-anethole included: 4-methoxybenzoate O-demethylase, p-hydroxybenzoate 3-hydroxylase, and protocatechuate-4,5-dioxygenase. These findings indicate that t-anethole is metabolized to protocatechueic acid through t-anethole-diol, anisaldehyde, anisic acid, and p-hydroxybenzoic acid. The protocatechueic acid is then cleaved by protocatechuate-4,5-dioxygenase to yield 2-hydroxy-4-carboxy muconate-semialdehyde. Results from inducible uptake ability and enzymatic assays indicate that at least three regulatory units are involved in the t-anethole degradation pathway. These findings provide new routes for environmental friendly production processes of valuable aromatic chemicals via bioconversion of phenylpropenoids.

Plant essential oils consist of volatile, lipophilic substances that are mainly hydrocarbons or compounds derived from the metabolism of mono- and sesquiterpenes and phenylpropenoids (1). Many of these essential oil components play a role in the plant defense system and, despite their poor solubility, are extremely toxic for microorganisms (2–5). Phenylpropenoids can potentially serve as a good source of starting material for the production of aromatic aldehydes for flavorings and aromas. Several studies have demonstrated that valuable aroma compounds are produced as intermediates in the degradation pathways of such phenylpropenoids (6–11). As a result there is a growing interest in the enzymatic systems leading to their degradation processes of valuable aromatic chemicals via bioconversion of phenylpropenoids.

EXPERIMENTAL PROCEDURES

Enrichment Cultures for t-Anethole-degrading Bacteria—Bacterial strains were isolated from soil samples taken from a greenhouse of spices and herbs at Agricultural Research Organization Newe Ya’ar, and the Technion’s ecological garden. Soil samples (10 g) were suspended in 100 ml of saline, and 2 ml of the dispersion were used as an inoculum. Enrichment medium was composed of inorganic basal salts medium supplemented with 0.1% t-anethole adsorbed on amberlite XAD-2 (Acros Organics, Janssen Pharmaceuticalaan 3a, Geel, Belgium). The basal salts medium contained: 1.2 g of (NH₄)₂SO₄, 0.1 g of CaCl₂ 2H₂O, 0.1 g of MgSO₄ 7H₂O, 0.01 g of FeSO₄ 7H₂O, 0.2 g of KH₂PO₄, 0.1 g of KH₃PO₄, 2.4 l of distilled water. Growth was carried out in shake flasks containing 20% (v/v) medium, at 30 °C (200 rpm). Pure bacterial strains were selected based on their morphological appearance on agar plates. All other growth experiments were carried out using a modified M9 media, containing: Na₃HPO₄ (6.8 g/liter), KH₂PO₄ (3.0 g/liter), NaN(Cl 0.5 g/liter), MgSO₄ 7H₂O (1 g/liter), FeSO₄ (0.01 g/liter), and CaCl₂ (0.02 g/liter).

Identification of Isolated Strains—Bacterial isolates were identified by their fatty acids profile, using the Microbial Identification System (19), at the Plant Diagnostic Laboratory (Plant Protection & Inspection Services, Ministry of Agriculture, Beit Dagan, Israel). Strain TA13 was also identified based on its 16 S rRNA gene sequence (GenBank accession number AF467106), which was obtained via PCR using universal primers for eubacterial 16 S rRNA. Primers were 27F (5’-GAGACTTCTCCGGCTCAG-3’) and 76R (5’-CTGTTTGCCTCCCA-CGGCTTC-3’). DNA sequencing was performed at the Biological Services Unit of the Weizmann Institute, Rehovot, Israel.

Mutagenesis and Selection for t-Anethole-blocked Mutants—Strain TA13 was grown on LB medium for 24 h (30 °C, 200 rpm) and following appropriate dilutions; samples were plated on a LB agar plate, to form

* This work was supported by the Fund for the Promotion of Research at the Technion and grants from the Israeli Ministry of Agriculture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Food Engineering and Biotechnology, Technion-IIT, Haifa 32000, Israel. Tel.: 972-4-8293072; Fax: 972-4-8320742; E-mail: yshoham@tx.technion.ac.il.
a uniform lawn. Crystals of MNNG\(^1\) were placed on agar plates, and the plates were incubated at 30°C for 24 h. Clear zones were formed around the MNNG crystals, and cells from the boundaries of these zones were collected with a sterile bacterial loop, suspended in M9 medium, and washed twice with the same medium (4°C, 10,000 rpm, 10 min). The washed cells were resuspended to a cell density of about 200,000 cells per ml in 0.5 M phosphate buffer (pH 7.0, 24 h, 200 rpm). This overnight culture was diluted to 0.1 \(A_{600}\) in 10 ml of M6 medium + 0.1% t-anethole, incubated at 30°C and 200 rpm up to turbidity of 0.15 \(A_{600}\), and then penicillin G was added to a concentration of 10,000 units/ml. Under these conditions, the final \(R_t\) values were: t-anethole, 0.74; ansailedehyde, 0.63; estragole, 0.73; anisic alcohol, 0.33; anisaldehyde, 0.40; p-hydroxybenzoic acid, 0.11; and protocatechuic acid, 0.05. GC analysis was performed on a Hewlett-Packard 5890 gas chromatograph, using SPB-1 capillary column (30 m, 0.25 mm, 0.50 μm film thickness). The temperature program was as follows: 5.0°C (1 min) + 30°C/min to 100°C, retention times were (min): 1) t-anethole, 7.20; anisaldehyde, 6.55; anisic alcohol, 7.00; anisic acid, 9.10; p-hydroxybenzoic acid, 10.20; and protocatechuic acid, 12.90 (100°C 2 min), 10°C/min, 230°C); 2) t-anethole, 6.88; ansailedehyde, 7.68; anisic alcohol, 8.34; anisic acid, 11.96 (100°C 2 min), 5°C/min, 160°C (1 min). High performance liquid chromatography (HPLC) analyses were performed on a M6000A HPLC system equipped with a Differential Refractometer R401 (Waters Associates Inc., Milford, MA). Aetonitryl/water 80:20 solvent system at 1.5 ml/min was used for analyses of glycerol and glucose by a LiChrospher 100 RP-18 (5 μm, Merck, Darmstadt, Germany). Gas chromatography-mass spectrometry (GC-MS) analysis was performed at the Technion Center for Mass Spectrometry (Technion City, Haifa, Israel) using a Finnigan 4000 and Supelco SPB-5 capillary column (25 m). The mass/charge ratios (m/e) are reported for the molecular ion (M\(^+\)) and for the major fragment ions with m/e ≤ 70 units smaller than that of M\(^+\). Values of m/e with intensities equal or greater than ~5% of the highest were recorded. Mass spectra (chemical ionization (CI)-MS) were obtained by the use of TSG-70B mass spectrophotometer (Finnigan Mat) by CI in isobutyl alcohol or ammonia. \(^1\)H NMR spectra were recorded on a Bruker AM-400 spectrometer and chemical shifts reported (in parts/million) with CDCl\(_3\), C\(_7\)D\(_3\)OD, or D\(_2\)O as the solvents. NMR data are reported as follows: chemical shift as parts/ million relative to the residual solvent signal. For CDCl\(_3\) \((δ = 77.00)\) or to external sodium 2,2-dimethyl 1silapentane tetroxide \((δ = 0.00)\) for D\(_2\)O as the solvent. Chemicals—t-anethole, p-anisaldehyde, m-anisic acid, p-anisic acid, gentisic acid, homogentisic acid, m-hydroxybenzoic acid, protocatechuic acid, NADH, NADP, and NADPH were obtained from Sigma, Catechol, p-hydroxybenzoic acid (99%), and m-cinnamic acid (>99%) were obtained from Fluka Chimica-BioChemica (Fluka Chemie AG, Buchs, Switzerland). Anisyl alcohol (98%), estragole (98%), 4-hydroxy-3-nitrobenzoic acid (98%) was obtained from Acros Organics (Janssen Pharmaceuticaan 3a, Geel, Belgium). Silica gel for column separations was ICN-Silica 62-200, 60A (ICN Biomedicals GmbH, Eschwege, Germany). Amberlite X-2D was obtained either from Sigma or Acros Organics. All other reagents were of analytical grade.

RESULTS

Isolation and Identification of t-Anethole-utilizing Bacteria—In an attempt to isolate bacterial strains capable of utilizing t-anethole, an enrichment procedure using minimal media and t-anethole as the sole carbon source was developed. Initially, t-anethole was added directly to the media. In this procedure we failed to obtain microbial growth presumably due to the toxicity of the carbon source. To overcome this limitation t-anethole was first adsorbed on a hydrophobic carrier (XAD-2), which produced a controlled release system, reducing its toxic effect on the bacterial culture. In this way a stable growth was achieved readily. Following several transfers, two independent strains that grow on t-anethole were isolated. Both strains showed similar morphology on agar plates and appeared as yellow and smooth colonies. One of the strains was designated as TA13 and was chosen for further studies.

Strain TA13 is aerobic, Gram-positive, with a variable rod

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\(^1\)The abbreviations used are: MNNG, N,N-dimethyl-N′-nitro-N′-nitrosoguanidine; GC, gas chromatography; MS, mass spectrometry; CI, chemical ionization; HPLC, high performance liquid chromatography.
shape, which produces yellow pigmentation on agar plate. Isolate TA13 was capable of growing on minimal media in the presence of t-anethole or glucose, resulting in final turbidity of 1.25 OD and 1.95 OD and a doubling time of 4.3 h and 2.1 h, respectively (Fig. 1). Analyses of its fatty acids profile (Table I) suggest that it is an _Arthrobacter_ sp. (similarity index = 0.745). To further identify the strain we utilized universal rRNA primers to amplify the 16 S rRNA gene. The amplification product was sequenced and showed very high homology (identities 1361/1366, 99%) to _Arthrobacter aurescens_ 16 S rRNA gene.

**The Uptake of t-Anethole Is Inducible in Strain TA13**—To test whether a specific metabolic system is responsible for the utilization of t-anethole, the uptake of t-anethole was measured in TA13 cultures that were grown previously on either t-anethole or glucose as the sole carbon source. The t-anethole uptake was followed by monitoring with time the UV absorption at 260 nm of the cell-free supernatant in a resting cells system. As shown in Fig. 2, only cultures grown previously in the presence of t-anethole exhibit uptake capacity, suggesting that t-anethole uptake is inducible. The observations that (a) strain TA13 has an inducible t-anethole degradation system and (b) it is capable of growing on t-anethole as the sole carbon source makes this strain an excellent candidate for characterization of the t-anethole degradation pathway.

**Growth of Strain TA13 on Postulated Intermediates**—The degradation pathway of t-anethole leading to the cleavage of the aromatic ring is likely to include the following steps: oxidation of the propenyl side chain to anisic acid via anisic alcohol and anisaldehyde, followed by demethylation to p-hydroxybenzoic acid and its hydroxylation to protocatechuic acid (Fig. 3). In an attempt to test this pathway, we first tested the ability of TA13 to utilize potential t-anethole degradation products as the sole carbon source. For this purpose, strain TA13 was grown on M9 medium containing the suggested intermediates as the sole carbon source. In addition to t-anethole (I), strain TA13 was capable of growing on anisic alcohol (IV), p-anisaldehyde (V), p-anisic acid (VI), p-hydroxybenzoic acid (VII), and protocatechuic acid (VIII) as the sole carbon source. Interestingly, it failed to utilize catechol or the meta-substituted analogues such as m-anisic acid and m-hydroxybenzoic acid. These results suggest that IV, V, VI, VII, and VIII are possible intermediates in the t-anethole biodegradation pathway. In addition, only t-anethole-induced cells exhibited uptake of the above intermediates in a resting cells system. The observed changes in the UV spectra during the uptake of t-anethole, IV, V, and VI by TA13 cells (previously grown on t-anethole), is shown in Fig. 4.

**Obtaining t-Anethole-blocked Mutants**—Mutants blocked in the degradation pathway are in many cases a valuable tool for identifying intermediates. These mutants can accumulate intermediates obtained before the missing enzymatic step. It should be noted that, since strain TA13 is capable of growing on t-anethole as the sole carbon source on minimal media, it is possible to isolate the required blocked mutants by applying penicillin enrichment. To obtain t-anethole-blocked mutants, mutagenesis was performed by MNNG, followed by penicillin enrichment. After screening over 15,000 colonies, 40 t-anethole-blocked mutants were identified. To obtain t-anethole-blocked mutants, mutagenesis was performed by MNNG, followed by penicillin enrichment. After screening over 15,000 colonies, 40 t-anethole-blocked mutants were identified.

### Table I

| Fatty acid (carbon number) | % | Fatty acid (carbon number) | % |
|---------------------------|---|---------------------------|---|
| 13:0 anteiso              | 0.17| 16:0 iso                  | 8.24|
| 14:0 isoo                 | 2.92| 16:1 w7c/15 iso 2OH       | 0.37|
| 14:0                      | 0.79| 16:0                      | 1.07|
| 15:0 isoo                 | 7.89| Anteiso 17:1 w9c         | 0.23|
| 15:0 anteiso              | 71.41| 17:0 iso                 | 0.46|
| 15:0                      | 0.18| 17:0 anteiso             | 0.02|
| 16:1 iso iso             | 0.24|                           |     |

**Fig. 1.** Growth of strain TA13 on t-anethole (●) or glucose (○) as the sole carbon sources in modified M9 media. Cultures were grown in 50 ml of medium (250-ml flasks) at 30 °C, 200 rpm.

**Fig. 2.** Uptake of t-anethole in resting cells of TA13 initially grown on t-anethole (●) or glucose (○). Cells were grown on modified M9 medium containing 0.1% (w/v) of either t-anethole or glucose. Cells were harvested at mid-logarithmic phase, washed, and resuspended in phosphate buffer (0.1 M, pH 7.0) and ~40 mg t-anethole at 30 °C. The concentration of t-anethole was monitored spectrophotometrically at 260 nm.

**Fig. 3.** Postulated degradation pathway of t-anethole by _Arthrobacter_ strain TA13. Compounds and enzymatic activities identified are in **bold**.
thole-blocked mutants were isolated. The ability of these mutants to accumulate intermediates was tested both in cultures grown on glucose (as the carbon source) in the presence of t-anethole (the inducer), and in a resting cells system, in which t-anethole was added to TA13 mutant cells (grown previously on glucose in the presence of t-anethole) suspended in a buffer. At the end of the incubation period, the cells were extracted with organic solvents, the products were separated on silica gel columns, and their identity and yield were determined. Preliminary TLC analysis of the transformation products showed that out of the 40 blocked mutants, 3 mutants accumulated anisic acid, 4 mutants accumulated traces of anisic alcohol (IV) and anisaldehyde (V), and one mutant accumulated minute amounts of a compound with Rf of 0.08 along with quantitative amounts of a compound that did not migrate on the TLC plate (Rf = 0). The rest of the blocked mutants did not accumulate detectable products, and are presumably blocked after the aromatic ring cleavage, accumulating nonaromatic compounds. No intermediates were detected in control cultures containing only glucose.

In the presence of t-anethole, three metabolic intermediates accumulated by the blocked mutants in high amounts, reaching almost a quantitative conversion. These intermediates were identified by a combination of TLC, GC, MS, and NMR (Table II). Identified compounds were t-anethole-diol (III) anisic acid (VI), and 4,6-dicarboxylate-2-pyrene (XIII). The 1H NMR spectrum of III, presented in Fig. 5, exhibited small peaks that may belong to its stereoisomer. t-Anethole-diol may be formed by the epoxidation of the propene side chain of t-anethole (Fig. 3), followed by the opening of the epoxide by an enzyme or during the acidic extraction (29). Anisic acid is the oxidized product of the propene side chain cleavage and is very likely to be further metabolized to protocatechuic acid (VIII). 4,6-Dicarboxylate-2-pyrene is most probably derived by spontaneous acidic lactonization of 2-hydroxy-4-carboxy muconic acid (X), a metabolite in the meta-cleavage pathway of VIII (30). One possible route for the formation of XIII in strain TA13 is given in Fig. 3. It is likely that mutant 57 lacks the activity of 2-oxopent-4-enoate hydratase (EC 4.2.1.80), which converts 2-carboxy-4-oxo-3-hexenedioate (XI) to 2-carboxy-2-hydroxy-4-oxo-3-hexanediode (XII) (Fig. 3).

Specific Enzymatic Activities Associated with t-Anethole Degradation—As indicated in the previous section, anisic acid and XIII were identified as intermediates in the degradation pathway of t-anethole. The enzymatic reactions leading from VI to 2-hydroxy-4-carboxy muconate semialdehyde (IX) might include the following enzymes (Fig. 3): (i) 4-methoxybenzoate O-demethylase, which removes the methyl of the 4-methoxy group, (ii) 4-hydroxybenzoate 3-hydroxylase, which introduce an hydroxyl group to the benzene ring, and (iii) protocatechuate dioxygenase, which cleaves the benzene ring. To test the presence of these enzymes, their activities were measured in TA13 cultures grown on either t-anethole or glucose. Cells that grow on t-anethole or its p-methoxylated derivatives exhibited 4-methoxybenzoate O-demethylase activity that was over 600-fold higher than glucose grown cultures (Table III). In addition, p-hydroxybenzoic acid-dependent NADH consumption in cell-free extracts was 15-fold higher in cultures grown on t-anethole than in glucose grown cultures. The t-anethole-inducible activity of 4-methoxybenzoate O-demethylase and the p-hydroxybenzoic acid-dependent NADH consumption indicate that VII is an intermediate in the degradation pathway of t-anethole. The cleavage of the protocatechuate acid can be performed by either 1,2-, 3,4-, or 4,5-dioxygenases. The activity of these three enzymes was measured in cell free extract of t-anethole-grown TA13 cells, and only protocatechuate 4,5-dioxygenase activity was detected. This activity was over 4,000-fold higher than that detected for TA13 cells grown on glucose (Table III). These results indicate that t-anethole is metabolized via the meta pathway of protocatechuate acid metabolism.

Regulation of the t-Anethole Degradation Pathway—The experimental approach to explore the regulation of t-anethole degradation pathway in strain TA13 was to grow the culture on M9 media supplemented with the different intermediates and determine the uptakes of various intermediates and enzymatic activities that are induced (Table III). While glucose-grown cells did not exhibit uptake of any intermediate, uptake of t-anethole and V was induced by t-anethole, IV, and V. In addition, cells that were grown on VI or VII did not exhibit uptake of t-anethole or V. The uptake of VI was induced in cells previously grown on t-anethole, IV, V, and VI. p-Hydroxybenzoic acid did not induce the uptake of any of the metabolites that were tested. Surprisingly, the uptake of IV was not detected in cells grown on either of these metabolites.

The regulation of the enzymatic activities leading from VI to IX was tested in cells grown on t-anethole, IV, V, VI, VII, or glucose. All of the activities were induced by t-anethole, anisic alcohol, V, or VI. However, VII and VIII did not induce 4-methoxybenzoate O-demethylase activity. Both VII and VIII induced the activity of their related enzymes: 4-hydroxybenzoate 3-hydroxylase and protocatechuate 4,5-dioxygenase, respectively. Glucose did not induce any of the tested enzymatic activities. These results suggest that at least three regulatory units are involved in the t-anethole degradation pathway.

**DISCUSSION**

Enrichment Culture for t-Anethole-degrading Bacteria—By applying an enrichment media, which is based on minimal media and t-anethole as the sole carbon source, we obtained microbial strains capable of utilizing t-anethole. Since t-anethole is highly toxic, it was necessary to adsorb it first on a hydrophobic carrier such as XAD-2. This procedure produced a "controlled release" system that introduced sub-toxic concentrations of t-anethole to the media. Based on its 16 S rRNA sequence, strain TA13 was identified as *A. aerogenes*. In fact, many *Arthrobacter* spp. are known to utilize aromatic compounds which include VII (31), gentisic acid (32), 4-chlorobenzoate (33–35), mono- and di-chlorinated biphenyls (36), 3-aminoanisaldehyde (37), and many others (38).

The Degradation Pathway of t-Anethole—Several approaches have been used to characterize the t-anethole degradation pathway. Initially it was hypothesized that the degradation pathway (Fig. 3) will be similar to other pathways reported for phenylpropanoides. Growth experiments showed that in addition to t-anethole, *Arthrobacter* TA13 was capable of utilizing related compounds such as IV, V, VI, VII, and VIII, suggesting that these compounds are possible intermediates.
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Cultures of blocked mutants were grown on modified M9 medium supplemented with 0.1% glucose and 0.1% t-anethole. Twenty ml of medium was shaken in 125-ml flasks at 200 rpm and 30 °C for 48–72 h. The transformation products were extracted from the culture by acidification to pH –2.0 and extraction by ethyl acetate.

| Mutant | Intermediates | Identification
|--------|---------------|------------------|
| 57     | t-Anethole-diol<sup>b</sup> | TLC, R<sub>t</sub> = 0.08; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.20 (d, 2H, J = 8.62 Hz, H<sub>j</sub>), 6.83 (d, 2H, J = 8.68 Hz, H<sub>i</sub>), 4.25 (d, 1H, J = 7.84 Hz, H<sub>k</sub>), 3.77 (dt, 1H, J<sub>ij</sub> = 6.27 Hz, J<sub>jk</sub> = 7.84 Hz, H<sub>j</sub>), 3.76 (s, 3H, OCH<sub>3</sub>), 0.97 (d, 3H, J = 6.27 Hz, H<sub>l</sub>); positive CIMS m/z 182.2 (M<sup>+</sup>H<sup>-</sup>), C<sub>9</sub>H<sub>10</sub>O<sub>2</sub> requires 181.2 (9). |
| 8, 17, 27 Anisic acid | TLC, R<sub>t</sub> = 0.40; GC: R<sub>t</sub> = 11.96; GC-MS, 153 (16), 152 (90, M<sup>+</sup>), 135 (100), 77 (7), 63 (18), 53 (9). |
| 57 | 4,6-Dicarboxylate-2-pyrene<sup>c</sup> | TLC: R<sub>t</sub> = 0.00; <sup>1</sup>H NMR (200 MHz, D<sub>2</sub>O): δ 7.29 (s, 1H, H<sub>j</sub>), 6.94 (s, 1H, H<sub>i</sub>), 5.20 (m, 2H, CH<sub>2</sub>), 4.16 (m, 4H, OCH<sub>2</sub>CH<sub>2</sub>); positive CIMS m/z 213.1 (M<sup>+</sup>), C<sub>24</sub>H<sub>14</sub>O<sub>4</sub> requires 214.1 (9). |

<sup>a</sup> TLC analyses were performed on silica gel plates with a solvent system: chloroform/ethyl acetate/formic acid 85:15:1. GC analyses were performed using SPB-1 column (Supelco, 30 m, 0.25 mm inner diameter, 0.25 µm) programmed for 100 °C (2 min.), 5 °C/min. and 160 °C (1 min.).

<sup>b</sup> t-Anethole-diol was purified on silica gel column eluted by a gradient of hexane to hexane/ethyl acetate 25:75.

<sup>c</sup> 4,6-Dicarboxylate-2-pyrene was purified on silica gel column eluted by gradient of chloroform to chloroform/acetic acid 60:20:10.

**Uptake experiments supported this notion (Table III). In addition, t-Anethole-blocked mutants accumulated quantitative amounts of III, VI, and XIII along with traces of IV and V (Table II) in a growing culture and resting cells system containing t-anethole as the sole carbon source. These observations and the detection of inducible enzymatic activities of 4-methoxybenzoate O-demethylase, 4-hydroxybenzoate 3-hydroxylase, and protocatechuate 4,5-dioxygenase (Table III) are in agreement with the suggested pathway for the degradation of t-anethole shown in Fig. 3.

The steps by which anisic acid (VI) is converted to the intermediate IX are supported by the results obtained form specific inducible enzymatic activities. Anisic acid can be converted to IX by demethylation, hydroxylation, and ring cleavage by an extradiol dioxygenase (43, 44). Similar enzymatic activities were reported for other aromatic degrading bacteria, such as *Streptomyces* (22), *Pseudomonas putida*, *P. aeruginosa*, *P. testosteroni*, and *P. fluorescens* (30). The activities of 4-methoxybenzoate O-demethylase, 4-hydroxybenzoate hydroxylase, and protocatechuate 4,5-dioxygenase were detected in TA13 cells that were grown on t-anethole, but not in TA13 cells grown on glucose. These results are in agreement with results obtained for enzymes of the β-ketoisopropyl pathway, where the activity of these enzymes increased 30–1,000-fold in cells grown on a primary substrate of the pathway (45–47).

Surprisingly, in strain TA13 the 4-hydroxybenzoate 3-hydroxylase uses NADH and not NADPH as a cofactor. Monooxygenases of this type usually used NADPH as a cofactor and showed lower affinity for NADH (34, 48). As expected, protocatechuate 4,5-dioxygenase was the only dioxygenase activity detected, indicating that t-anethole is metabolized via the meta-fission pathway.

The t-anethole-inducible activity of these enzymes further indicates that VII, VIII, and IX are intermediates in the degradation pathway of t-anethole.

The conversion of IX to X is supported by the accumulation of 4,6-dicarboxylate-2-pyrene (XIII) in the fermentation broth of t-anethole-blocked mutants grown in the presence of t-anethole (Fig. 3). It should be noted that compound XIII is not a potential intermediate in the t-anethole degradation pathway. Interestingly, this compound was also isolated from cell free extract of *Micrococcus* sp. strain 12B following NADP<sup>+</sup>-dependent oxidation of 2-hydroxy-4-carboxymuconic semialdehyde by alde-
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**TABLE III**

Uptake of intermediates of t-anethole degradation pathway and induction of enzymatic activities in TA13 cells grown on various carbon sources

| Carbon source | Uptake of 4-methoxylated intermediates | Specific activity* |
|---------------|----------------------------------------|--------------------|
|               | t-Anethole | Anisic alcohol | Anisaldehyde | Anisic acid | 4-Methoxybenzoate-O-demethylase⁷ | 4-Hydroxybenzoate-3-hydroxylase⁷ | Protocatechuate-4,5-dioxygenase⁷ |
| t-Anethole    | +          | +             | +            | +          | 6.40                         | 149                            | 4035                         |
| Anisic alcohol| +          | +             | +            | +          | 3.32                         | 581                            | 1953                         |
| Anisaldehyde  | +          | +             | +            | +          | 2.71                         | 480                            | 2989                         |
| Anisic acid   | +          | +             | +            | +          | 4.18                         | 267                            | 2348                         |
| p-Hydroxybenzoic acid | +          | +             | +            | +          | < 0.01                       | 316                            | 820                          |
| Protocatechual acid | ND*       | ND            | ND           | ND         | < 0.01                       | 108                            | 174                          |
| Glucose       | –          | –             | –            | –          | < 0.01                       | 9                              | < 1                          |

* Specific activity = unit/mg protein.  
² Unit = μmol of 3-nitro-4-hydroxybenzoate/min.  
³ Unit = μmol of NADH/min.  
⁴ Unit = 0.001 (OD 410 nm)/min.  
⁵ ND, not determined.

![Diagram](image)

**FIG. 6. Regulation of the postulated t-anethole degradation pathway in Arthrobacter strain TA13 (inducers in arrows).**

The authors suggested that this compound was formed spontaneously from X when the broth was acidified (49). Since in the present study the culture broth was acidified prior to extraction, XIII is likely to be derived from X, which is an intermediate in the degradation pathway of t-anethole. 2-Hydroxy-4-carboxymuconic acid may be produced from VIII in two steps that involve: (a) oxidative cleavage of the vicinal diol to afford 2-hydroxy-4-carboxymuconic acid semialdehyde and (b) further oxidation of the aldehyde to the corresponding carboxylic acid by NAD⁺-dependent dehydrogenase (49–51). These results demonstrate that t-anethole is degraded via VIII, followed by its oxidation using the meta ring-fission mechanism. The detection of XIII indicates that 2-hydroxy-4-carboxymuconic acid semialdehyde is oxidized to X. It is likely that these steps are followed by spontaneous isomerization of X forming a 4-carboxy-2-oxo-3-hexenedioate (XI), which is further degraded to pyruvate and oxaloacetate.

The proposed t-anethole degradation pathway is based on the identification of accumulated intermediates in blocked mutants in presence of t-anethole and inducible enzymatic activities. Three of the accumulated compounds (III, VI, XIII) were produced in a quantitative manner by the blocked mutants. Since the accumulation of these compounds was totally dependent on the presence of t-anethole, it is evident that these intermediates are derived from t-anethole. Two intermediates (IV, V) were detected only in trace amounts presumably because high amounts of them will cause cell death. The proposed epoxide intermediate II was not detected because of its inherent instability under the experimental conditions.

**Regulation of the t-Anethole Degradation Pathway—**Some insights into the regulation of the enzymes involved in the t-anethole degradation pathway were obtained by testing potential inducers of the enzymatic activities. The experimental approach was to grow the culture on the various intermediates and test what enzymatic steps are induced. Fig. 6 presents the suggested regulation map of the pathway. The first conversion steps leading from t-anethole to VI were monitored in a resting cells system. While t-anethole and V induce the uptake of all intermediates, it is evident that VI does not induce the uptake of t-anethole and V. Thus, it is likely that the genes for the degradation of t-anethole to VI are on the same regulatory unit and are induced by t-anethole and V, but not by VI (the end product). The suggestion that the gene for the t-anethole degradation to anisic acid and the genes for 4-methoxybenzoate O-demethylase are on different regulatory units is supported by the work of Priefert et al. (52) on the genes involved in the biocconversion of vanillino to VIII by Pseudomonas sp. strain NR199. This work showed that the genes encoding the two subunits for vanilline demethylase (vanA and vanB), and vanillino dehydrogenase (vdh), are on two different operons.

The enzymatic activities leading from VI to IX were induced by the presence of anisic acid. However, VII did not induce 4-methoxybenzoate O-demethylase activity. Both VII and VIII induced the activity of their related enzymes: 4-hydroxybenzoate 3-hydroxylase and protocatechuate 4,5-dioxygenase. These results suggest that the 4-methoxybenzoate O-demethylase gene is induced by VI and is not on the regulon of the genes coding for 4-hydroxybenzoate 3-hydroxylase and protocatechuate 4,5-dioxygenase. Thus, it is likely that the t-anethole degradation pathway is composed of at least three regulatory units, induced by t-anethole, VI, and VII.

The induction of 4-hydroxybenzoate 3-hydroxylase activity by VIII is unique, since it is usually induced by its substrate, VII. In *Acinetobacter calcoaceticus* the structural genes for the entire VIII pathway are on a single operon, and its expression is elicited by VIII (53, 54). The pob gene, that induces p-hydroxybenzoate hydroxylase, lies beyond them and is not induced by VIII (55). In *Pseudomonas spp.*, VII is the inducer of both p-hydroxybenzoate 3-hydroxylase and protocatechuate-3,4-dioxygenase; however, VIII induces only the activity of protocatechuate-3,4-dioxygenase (47, 56). In *Alcaligenes eutrophus* and *A. calcoaceticus*, p-hydroxybenzoate 3-hydroxylase activity is induced only by VII (45, 46, 57, 58). It is possible that the expression of VII in strain TA13 is regulated by VIII by the induction of specific permeases for VII encoded by genes from the VIII operon. The permease encoded by the pcaK gene in *P. putida* is a membrane protein required for chemotaxis to VII and benzoic acid as well as uptake of VII and VIII (59, 60).

**Summary—**In this study we have isolated a t-anethole degrading bacterium and characterized the key degradation steps. Our data suggest that t-anethole is metabolized to protocatechuc acid through t-anethole-diol, anisaldehyde, anisic acid, and p-
hydroxybenzoic acid. To the best of our knowledge, the degradation pathway for t-anethole in microorganisms was never described previously. One of our main goals in this study was to develop biotransformation processes for valuable aromatic chemicals. Preliminary bioconversion processes with Arthrobacter TA13 and its t-anethole-blocked mutants indicated that they are capable of transforming in high yields (up to 100%) various phenylpropanoides such as eugenol, estragole, and safrole into valuable aromatic compounds (results not shown). These strains are now being evaluated for large scale production of aromatic chemicals in new biotransformation processes.

Acknowledgment—Technical support was provided by the Technion Otto Meyerhof Biotechnology Laboratories established by the Minerva Foundation, Federal Republic of Germany.

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