Xanthobacter tagetidis sp. nov., an Organism Associated with *Tagetes* Species and Able To Grow on Substituted Thiophenes

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Members of the marigold genus of flowering plants (the genus *Tagetes*), which synthesize and accumulate thiophene compounds in their roots, were investigated as potential sources of bacteria able to degrade substituted thiophenes. Batch and continuous enrichment cultures inoculated with compost from root balls of *Tagetes patula* and *Tagetes erecta* reproducibly produced the same predominant type of bacterium when they were supplied with thiophene-2-carboxylate (T2C) or thiophene-2-acetate (T2A) as a carbon and energy substrate. This organism was a yellow-pigmented, neutrophilic, mesophilic, gram-negative, pleomorphic, rod-shaped bacterium, which we classify as a new species of the genus *Xanthobacter*, *Xanthobacter tagetidis*; strain TagT2C (= DSM 11105) is the type strain. Strain TagT2C^T (T = type strain) grew on simple thiophenes, such as T2C, thiophene-3-carboxylate, and T2A, on analogs of these compounds (pyrrole-2-carboxylate and furan-2-carboxylate), and on the condensed thiophene dibenzothiophene. *X. tagetidis* was facultatively autotrophic, fixing carbon dioxide by means of ribulose biphosphate carboxylase, and was able to grow on hydrogen, thiosulfate, or sulfide as an energy substrate. It also grew on a wide range of other heterotrophic, chemolithotrophic, and methylotrophic substrates. Its growth on T2C was optimal at 28 to 31°C and pH 7.6 to 7.8, and the maximum growth rate in batch culture was 0.22 h⁻¹. The DNA base composition of *X. tagetidis* is 68 mol% G+C. A 16S ribosomal DNA sequence analysis of strain TagT2C^T showed that this organism represents a distinct lineage within the *Aquabacter*-*Azorhizobium*-*Xanthobacter* cluster of the alpha-2 subclass of the *Proteobacteria*. Discrimination of *X. tagetidis* from the other genera in this group and from other *Xanthobacter* species is discussed.

In contrast to the extensive studies of bacteria that effect inorganic sulfur turnover in natural environments, soil microorganisms involved in the degradation of organic sulfur compounds other than sulfur amino acids have been almost completely neglected. Plants effect the turnover of enormous amounts of sulfur (up to 10 Gt of S per year), but the microbiological processes associated with this, which involve living plants and decay of plants, have not been comprehensively studied and described.

Tagetes plants are well-documented producers of thiophene compounds (bi- and terthienyls), which can constitute almost 1% of the normal root mass (4, 16, 29, 39). These compounds are variably excreted by the plant cells, in response to altering environmental conditions, and have been shown to have significant fungicidal, insecticidal, and allergenic activities (14, 31, 35, 36). The root balls of growing *Tagetes patula* and *Tagetes erecta* plants have been used to enrich for novel thiophene-degrading microorganisms. In previous studies on the biological degradation of thiophene, workers have used anthropogenically contaminated soil or water as enrichment samples, and many workers have concentrated on dibenzothiophene as a model substrate, particularly for the potential desulfurization of fossil fuels (24, 30, 42). There have been far fewer studies on the microbial breakdown of the simpler substituted thiophenes (1, 2, 6, 7).

In this study we used the simple thiophene derivatives thio-

phene-2-carboxylate (T2C) and thiophene-2-acetate (T2A) as enrichment substrates. Batch and continuous chemostat enrichment cultures were used to isolate pure cultures or consortia capable of T2C or T2A degradation. This work resulted in the isolation of a novel *Xanthobacter* species.

MATERIALS AND METHODS

Growth of *T. patula* and *T. erecta*. *T. patula* (French marigold) and *T. erecta* (African marigold) plants were grown in a commercially available material called Vitax O4 multipurpose compost (Vitax Home and Garden Products, Leicester, United Kingdom) in plastic plant pots (diameter, 9 or 13 cm) in the open air. All references to compost below are references to this support material. Compost samples were obtained from around the roots of plants of each of the species, including both yellow- and orange-flowered varieties of *T. erecta*, by using plants of different ages, and these samples were used as inocula for batch and chemostat enrichment cultures. For batch enrichment cultures we used compost samples from seedlings (height, 4 to 5 cm) and from plants grown to maturity (and flower production). For chemostat enrichment cultures we used compost from mature plants of *T. patula*.

Growth media, methods used for batch culture and continuous culture, and analytical procedures. The medium used for isolation and pure-culture growth and maintenance contained (per liter of distilled water) 7.9 g of Na₂HPO₄ · 2H₂O, 1.5 g of KH₂PO₄, 0.4 g of NH₄Cl, 0.1 g of MgSO₄ · 7H₂O, and 10 ml of a trace metal solution (40); the initial pH of this medium was 7.3. Substrates and phosphates were separately sterilized and mixed with the other components when they were cool. To produce media having different pH values, 1 M NaOH was added as necessary. For solid media, Oxoid bacteriological agar (15 g/liter) was added, and to show pH changes, a saturated phenol red solution (1%, vol/vol) was added. Stock cultures were maintained on agar slants of the basic mineral medium supplemented with T2C (2.5 mM) or sodium acetate (5 mM). For autotrophic growth on hydrogen, the basal medium was supplemented with NiSO₄ (0.1 μM). Erlenmeyer flasks (headspace volume, 300 ml) sealed with Subaseal vaccine stoppers were flushed with H₂ before a bacterial culture (25 ml), NaHCO₃ (10 mM), and O₂ (30 ml) were added. Fixation of dinitrogen was tested by using heterotrophic cultures growing on T2C (2.5 mM) in sealed flasks that either contained normal air or were flushed with a nitrogen-air mixture (95:5, vol/vol) before the flasks were sealed to provide oxygen at a concentration of 1% (vol/vol). To avoid transfer of fixed nitrogen into the nitrogen-free medium, the flasks were inoculated with colonies from agar plates. Growth (with denitrification) on T2C (2.5 mM) or acetate (10 mM) was tested in liquid

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medium (44) supplemented with KNO_3 (25 mM) and NaHCO_3 (24 mM) in argon-flushed flasks or in completely filled bottles (volume, 25 to 100 ml). Aerobic cultures (25 to 100 ml) in 250-ml conical flasks were either shaken in an orbital incubator (New Brunswick Scientific, Hatfield, United Kingdom) at 200 rpm or incubated statically. The routine growth temperature was 30°C. The effects of different temperatures (4 to 69°C) and different pH values (7.3 to 8.7) on growth were tested by using T2C-grown batch cultures (in liquid media or on agar media) and chemostat batch cultures (1.75 liters) growing on thiosulfate (10 mM) that were aerated (100 ml min^{-1}) but otherwise were as described previously (18). Chemostat cultures (1.75 liters) were maintained at $30 \pm 2^\circ\text{C}$ and pH 7.3 ± 0.2 (by automatic titration with 2 M Na_2CO_3) with air flow at a rate of 200 ml/min and were stirred at 200 rpm. The chemostat equipment was supplied by LH Fermentation, Ltd., Reading, United Kingdom, and New Brunswick Scientific, Hatfield, United Kingdom. Growth was monitored by visually observing turbidity, by measuring optical density at 440 nm, and by measuring the disappearance of substrate. Organism dry weights and protein contents were determined as described previously (46). Thiosulfate and tetrathionate were assayed by the cyanolysis method (22, 23), nitrate was assayed by the method of Cawse (5), and nitrite was assayed by using commercial Griess-Ilosvay reagents (Merck, Poole, United Kingdom) and measuring A_{525} . T2C was assayed by monitoring A_{245} (molar extinction coefficient, $8.6 \times 10^3 \text{ liter/mol/cm}$ [7]).

A wide range of growth substrates were tested at the concentrations given below. Substituted thiophenes and other toxic, volatile, or unstable substrates were generally sterilized by filtration, but some compounds (including carbon disulfide, carbonyl sulfide, dimethyl sulfide, methanethiol, thiophene, 3-methylthiophene, and α -terthiophene) were used without prior sterilization. Incubations with volatile substrates were carried out in Quickfit flasks with Subaseal stoppers, into which substrates were injected.

To test T2C as a sulfur, carbon, and energy substrate, the sulfates in the original medium were replaced by chlorides or nitrates, and the initial inoculum was subcultured five times in sulfate-free medium to obtain sulfate-free growth. To test alternative nitrogen sources, NH_4Cl and the trace metal solution were omitted from the medium. An API 20E multitest system (bioMérieux, Marcy l'Etoile, France) was used to determine the physiological characteristics of some isolates.

Antibiotic sensitivity was tested by using Difco Dispence-O-Disc minifilters on agar plates containing T2C and nutrient broth.

Ribulose biphosphate carboxylase was assayed in cell extracts obtained from thiosulfate-grown organisms, as described by Jordan et al. (18).

Enrichment cultures and isolation of organisms. (i) **Batch enrichment cultures.** Samples (10 g) of compost removed from *T. patula* root balls were added to 100-ml portions of liquid medium (pH 7.3) containing T2C or T2A (2.5 mM) as an enrichment substrate and were incubated in 250-ml flasks shaken in air at 30°C. For enrichment cultures for denitrifying organisms we used 2.5-g portions of compost samples in 25-ml bottles completely filled with medium supplemented with T2C and KNO_3 (25 mM). Cultures in which growth occurred were subcultured three times into fresh medium before they were streaked onto agar plates for single-colony isolation. For subsequent enrichment cultures we used standard T2C batch culture conditions and compost samples from root balls of two types (yellow and orange) of *T. erecta* and from root balls of *T. patula*. Samples of unused compost were incubated in medium containing T2C as controls. After incubation, dilution plates of each culture were prepared to check the relative abundance of isolates.

(ii) **Low-dilution-rate continuous enrichment cultures.** Compost samples from *T. patula* root balls were inoculated into three separate chemostats, and a period of initial batch growth was followed by the following conditions: in chemostat i, 2.5 mM T2A and a dilution rate of 0.065 h^{-1} ; in chemostat ii, 2.5 mM T2C and a dilution rate of 0.05 h^{-1} ; and in chemostat iii, 2.5 mM T2C and a dilution rate which increased from 0.013 to 0.039 h^{-1} . Each chemostat culture was grown for 5 to 7 weeks. Dilution plates were prepared during growth in batch culture as the amount of substrate decreased, immediately before substrate pumps were started, at subsequent intervals corresponding to every three culture volume changes, and at the conclusion of the experiment. The substrate used in the agar plates was either acetate (5 mM), nutrient broth (0.1%), thiosulfate (20 mM), T2A (2.5 mM), or T2C (2.5 mM) or there was no substrate, which allowed us to assess the total numbers of heterotrophs, chemolithoautotrophs, primary thiophene users, and oligotrophic opportunists.

Microscopy. Standard light microscopy techniques were used together with scanning (Philips model 501 SEM) and transmission (JEOL model JEM-100 CXII and Philips model 501G) electron microscopes.

Substrate oxidation kinetics. Organisms grown on T2C (2.5 mM) in a chemostat culture (dilution rate, 0.034 h^{-1}) at pH 7.3 and 30°C were used in an oxygen electrode assembly (18) to determine the range of substrates oxidized and the kinetics of substrate oxidation.

DNA G+C ratios. DNA was isolated from cells grown on T2C (2.5 mM) by using a modification of the procedure of Marmur (27), and the G+C content was determined by the spectrophotometric method of Fredericq et al. (13) with a Hewlett-Packard model 8452A diode array spectrophotometer (Anachem, Luton, United Kingdom) or a model CE272 UV spectrophotometer (Cecil Instruments, Cambridge, United Kingdom) and by the melting point method (26) by using 1 or 2°C temperature increments between 75 and 91°C. DNA from *Escherichia coli* B (= ATCC 11303) was used as the standard.

16S rDNA sequence determination and analysis. Genomic DNAs were extracted from strains TagT2C^T (T = type strain) and A2 for PCR-mediated amplification of the 16S ribosomal DNA (rDNA) as described by Rainey et al. (33). The purified PCR products were directly sequenced by using previously described protocols (33), and the sequence reaction mixtures were electrophoresed by using a model 373A automatic DNA sequencer (Applied Biosystems, Foster City, Calif.). The 16S rDNA sequence was manually aligned with the 16S rDNA sequences of members of the alpha-2 subclass of the *Proteobacteria* by using the ae2 editor (25). Evolutionary distances were calculated by the method of Jukes and Cantor (20). Phylogenetic dendrograms were constructed by using tree algorithms contained in the PHYLIP package (12). Tree topologies were evaluated by performing bootstrap analyses (11) of the neighbor-joining data with 1,000 resamplings.

Ubiquinone extraction. The ubiquinone fraction was isolated, purified, and identified by the method described for *Thiobacillus tepidarius* (45) and modified by Jordan et al. (18) by using cells grown in a chemostat on T2C (2.5 mM) at pH 7.3 and a dilution rate of 0.094 h^{-1} . The standards used for ubiquinone chromatography were commercial coenzyme Q10 and coenzyme Q8 prepared from *Thiobacillus ferrooxidans*.

Chemicals. All chemicals were analytical grade unless indicated otherwise. Methanethiol, proteinase K, ubiquinone 30 (coenzyme Q10), RNase, and *E. coli* DNA were obtained from Sigma, Poole, United Kingdom. Methylamines, thiophene compounds, dimethyl sulfide, carbonyl sulfide, and carbon disulfide were obtained from Aldrich Chemical Co., Gillingham, United Kingdom.

Nucleotide sequence accession number. The 16S rDNA sequence determined for strain TagT2C^T has been deposited in the EMBL database under accession number X99469.

RESULTS

Enrichment in batch culture from compost supporting *T. patula* or *T. erecta*.

Aerobic enrichment cultures obtained from the root balls of *T. patula* produced yellow colonies on medium containing T2C; similar colonies also grew on thiosulfate and T2A. A pure culture obtained from the enrichment cultures was designated strain TagT2C^T prior to further characterization. A second series of aerobic enrichment cultures inoculated with compost in which *T. erecta* and *T. patula* had been grown yielded similar yellow isolates in all cases. Recently, an apparently identical organism was isolated from a T2C-containing enrichment culture obtained from the root ball of another species, *Tagetes minuta* (30a). Enrichment cultures prepared with fresh, unsterilized compost in which no *Tagetes* plants had been grown yielded no organisms capable of growth on T2C. No growth on T2C was observed in enrichment cultures under denitrifying conditions.

Enrichment from *T. patula* compost by using continuous culture.

Up to 13 colony types were seen in the initial samples from the chemostats. Most of these colony types accounted for <1% of the total population recovered on agar media containing acetate or nutrient broth or thiosulfate or T2A or T2C as the substrate. In all cases the predominant organisms produced yellow colonies (as did strain TagT2C^T), and this dominance was observed early in the chemostat enrichment cultures, even at the lowest dilution rates. In all three chemostat procedures, the cultures were dominated (92 to 100% of all of the organisms) by strains that produced yellow colonies (TagT2C^T type) on T2C or T2A. One representative isolate, designated strain A2, which was derived from chemostat ii, was used for comparison with strain TagT2C^T from the batch enrichment cultures.

Selection of a pure culture for further study.

Strains TagT2C^T and A2 were found to be indistinguishable from each other on the basis of colony morphology, cell morphology, and growth on T2C. Strain TagT2C^T was studied in detail to determine its characteristics, and a detailed comparison with strain A2 was performed. The results (see below) indicated that these strains were physiologically and biochemically identical, leading to the conclusion that the same type of organism predominated regardless of the conditions employed for enrichment culture.

FIG. 1. Electron micrographs of *X. taetidis* TagT2C^T. (a) Scanning electron microscope image, showing the irregular shape and twisting morphology of the cells. Bar = 1 μ m. (b and c) Transmission electron microscope images of thin sections, showing the irregular cell shape and basal sections of peritrichous flagella. Bars = 0.1 μ m.

Morphology. Colonies of strains TagT2C^T and A2 were egg yolk yellow, low convex, regular, smooth, and mucoid and became slightly raised and irregular in shape with age. As determined by light microscopy, the cells of both strains were short, fat, motile, gram-negative rods (0.5 by 1.0 μ m) with capsules and large, dark inclusion bodies which occurred at one or both ends of the cells, but no endospores were formed. Cells of strain TagT2C^T grown on succinate-containing medium and observed by light microscopy exhibited pleomorphism, including bent cells, as described for the type species of the genus *Xanthobacter*, *Xanthobacter autotrophicus* (43). Scanning electron microscopy images showed that strain TagT2C^T cells were irregular and resembled fusilliform spirals (Fig. 1), while transmission electron microscopy revealed irregular, pleomorphic cells with peritrichous flagella (Fig. 1).

Physiological characteristics. Strains TagT2C^T and A2 were oxidase positive, but were negative for phosphatase activity, indole production, the methyl red test, the Voges-Proskauer test, and glucose fermentation. Strain TagT2C^T was catalase positive (strain A2 was not tested). Strain TagT2C^T grew at temperatures from 4 to 43°C, and the optimum temperature was 28 to 31°C. The pH range for strain TagT2C^T was 6.8 to 8.7, and the optimum pH was 7.6 to 7.8. Growth was not microaerophilic, but under static conditions the batch culture growth rate on T2C was initially as high as the growth rate in a shaken culture (0.13 h⁻¹ at an initial pH of 7.3) and growth was accompanied by less slime formation.

The distinctive yellow pigment was formed during both heterotrophic growth and autotrophic growth. It was not water soluble in exponentially growing cells, but in dying, lysing, or stressed cells some of the pigment diffused into the medium. The identity of the pigment was not investigated further.

As determined with the API 20E test system, strains TagT2C^T

and A2 were identical with respect to the presence of β -galactosidase (weak), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase (weak), gelatinase, and cytochrome oxidase activities; production of acetoin (during fermentation of glucose, glycerol, or pyruvate); use of citrate, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, and arabinose; production of indole (negative); production of hydrogen sulfide from thiosulfate (negative); and production of nitrite from nitrate (negative). These tests showed that the two strains are very similar, and while they did not allow us to determine the genus to which the strains belong, they did eliminate the possibility that the strains are members of a large number of genera, including the genera *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Citrobacter*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Kluyvera*, *Moraxella*, and *Pseudomonas*.

The substrates that supported growth of strain TagT2C^T (as sole sources of energy and carbon), sometimes after lag periods of up to 7 days, were T2C (2.5 mM), T2A (2.5 mM), thiophene-3-carboxylate (2.5 mM), pyrrole-2-carboxylate (10 mM), furan-2-carboxylate (10 mM), dibenzothiophene (2.5 mM), methylamine (20 mM, with NaHCO₃), methanol (20 mM) (weak growth), dimethyl sulfide (2.5 mM), methanethiol (2.5 mM), formate (25 mM), acetate (10 mM), ethanol (20 mM), succinate (10 mM), pyruvate (5 mM), citrate (5 mM), yeast extract (0.01%), nutrient broth (0.1%), galactose (20 mM) (good growth), sucrose (20 mM), fructose (20 mM), and glucose (20 mM) (poor growth). In addition, the following compounds supported growth as energy substrates (with autotrophic fixation of carbon dioxide): thiosulfate (20 mM), tetrathionate (5 mM, with 10 mM NaHCO₃), sulfide (2.5 mM) hydrogen (see Materials and Methods), carbonyl sulfide (4.0 mM), and carbon disulfide (2.5 mM) (best when provided with

FIG. 1—Continued.

0.01% [wt/vol] yeast extract). In sulfate-free medium, T2C supported growth of strain TagT2C^T as a carbon, energy, and sulfur source. Substrates that did not support growth (at a concentration of 2.5 mM) were thiophene, 3-methyl-thiophene, 5-methyl-thiophene-2-carboxylate, 3-methyl-thiophene-2-carboxylate, α -terthiophene, and thiophene-3-acetate. KSCN did not act as a growth substrate or nitrogen source. Molecular dinitrogen served as a sole source of nitrogen for growth on T2C; growth was sustained through three subcultures on me-

dium lacking NH₄Cl and was comparable in flasks with a normal air atmosphere and in sealed flasks containing only 1% (vol/vol) oxygen. The growth yield with dinitrogen as the only source of nitrogen was 50 ± 10 g (dry weight)/mol of T2C (eight determinations), compared with yields of 74 ± 5 g/mol of T2C with NH₄Cl as the nitrogen source. Growth with denitrification did not occur on T2C or acetate, although partial denitrification to nitrite did occur with acetate.

Strains TagT2C^T and A2 appeared to be identical in their

TABLE 1. Kinetics of oxidation of some sulfur compounds by strain TagT2C^T obtained from a chemostat culture growing on 2.5 mM T2C (dilution rate, 0.034 h⁻¹; pH 7.3; 30°C)

Substrate ^a	K _m (μM)	V _{max} (nmol O ₂ /min/mg [dry wt])
T2C	198	304
Thiosulfate	14	81
Tetrathionate	7	75
Sulfide	67	135

^a Substrates were tested at concentrations of 10 to 100 μM.

abilities to grow on T2C, T2A, dibenzothiophene, acetate, nutrient broth, yeast extract, thiosulfate, tetrathionate, sulfide, dimethyl sulfide, methanethiol, and carbon disulfide and in their inability to use 5-methyl-thiophene-2-carboxylate and thiocyanate as growth substrates.

Strain TagT2C^T was sensitive to ampicillin (25 μg) and chloramphenicol (30 μg), but was not sensitive to penicillin (1 U) and tetracycline (5 μg).

Cell extracts of strain TagT2C^T previously grown on thiosulfate had ribulose biphosphate carboxylase activities of 108 ± 6 nmol of CO₂ fixed/min/mg of cell extract protein (eight determinations), which were sufficient to explain autotrophic growth on thiosulfate at the rates observed.

Growth kinetics. Under optimum conditions in shaken batch cultures grown on T2C, the specific growth rate was about 0.22 h⁻¹. Chemostat cultures were not grown at growth rates near this maximum specific growth rate because the organisms leaked a yellow pigment into the medium and produced H₂S at a dilution rate of 0.13 h⁻¹.

Substrate oxidation kinetics. Strain TagT2C^T grown on T2C was able to oxidize the following compounds (each at a concentration of 100 μM): T2C, T2A, thiophene-3-carboxylate, acetate, formate, citrate, thiosulfate, tetrathionate, and sulfide. Organisms grown on T2C did not oxidize glucose (100 μM) or thiophene-3-acetate (10 or 100 μM). Kinetic constants were determined (Table 1), and these constants showed that T2C-grown bacteria had a high affinity for inorganic sulfur species (which may be potentially toxic intermediates in thiophene ring degradation) but a surprisingly low affinity for the growth substrate, T2C.

DNA base composition. The G+C content of strain TagT2C^T was determined by using two separate DNA preparations and two methods, the A₂₆₀/A₂₈₀ ratio method of Fredericq et al. and the melting point method. The G+C contents determined were 67 ± 1 mol% (method of Fredericq et al.; nine determinations) and 71 ± 3 mol% (melting point method; five determinations). The mean ± standard deviation for all determinations was 68 ± 2 mol% G+C.

Ubiquinone content. Strain TagT2C^T contained predominantly coenzyme Q10, as well as some coenzyme Q8, as indicated by a compound that had the same R_f as standard coenzyme Q8 and an absorbance peak at 280 nm.

16S rDNA sequence analysis. An almost complete 16S rDNA sequence comprising 1,417 nucleotides (>95% of the *E. coli* sequence [3]) was determined for strain TagT2C^T. A comparative sequence analysis performed with 16S rDNAs from two other putatively identical strains, including strain A2, showed that all three organisms exhibited 100% homology with each other.

The phylogenetic dendrogram shown in Fig. 2 was constructed from evolutionary distances by the neighbor-joining method (37). A total of 1,371 nucleotides present in all strains between positions 35 and 1488 (*E. coli* positions [3]) were used

for this analysis. The phylogenetic dendrogram (Fig. 2) shows that strain TagT2C^T falls within the radiation of the *Aquabacter-Azorhizobium-Xanthobacter* cluster. It represents a distinct lineage that exhibits levels of 16S rDNA sequence similarity to the other members of the cluster included in the analysis ranging from 96.9 to 98.1% (Table 2). The equidistant position of strain TagT2C^T (Fig. 2) was not supported by the results of the bootstrap analysis, in which values of less than 54% were found for the branch points of the distinct lineages within the *Aquabacter-Azorhizobium-Xanthobacter* cluster. The *Aquabacter-Azorhizobium-Xanthobacter* cluster was recovered in all analyses (100%).

DISCUSSION

The most striking outcome of this study was the finding that enrichment cultures for organisms able to degrade substituted thiophenes, which were prepared by using compost removed from roots of several *Tagetes* varieties, yielded the same morphological type of bacterium regardless of the method of enrichment used or the *Tagetes* species used. The conclusion that the isolates from different enrichment cultures were indeed identical was supported by the results of the physiological tests and 16S rDNA sequence analyses described above. Strain TagT2C^T, which was obtained from a batch enrichment culture, was studied as a typical representative of the T2C-utilizing strains obtained and is described below as a member of a novel *Xanthobacter* species, *Xanthobacter tagetidis*.

Strain TagT2C^T is distinct from the few previously described T2C-using bacteria, which were isolated from sewage or oil-polluted soil and water; these bacteria included *Flavobacterium* (1, 2), *Vibrio* (10), and *Rhodococcus* (21) strains, *E. coli* mutants (19), and an unclassified heterotrophic rod-shaped organism (6, 7). Strain TagT2C^T is striking because of its metabolic versatility; it is able to grow heterotrophically on a wide range of organic nutrients, including substituted thiophenes, chemolithoautotrophically, and methylotrophically. Its ability to grow under static growth conditions may reflect its habitat, root-associated soil particles where aeration may be low. Its production of extracellular slime material is also consistent with its association with root surfaces, where its attachment and survival in the substrate-producing environment may be helped by its slime production. It would be very interesting to

FIG. 2. 16S rDNA sequence-based phylogenetic dendrogram constructed from evolutionary distances by using the neighbor-joining method (37). Scale bar = 2.5 inferred nucleotide substitutions per 100 nucleotides. The numbers at the branch points are bootstrap values.

TABLE 2. Levels of 16S rDNA sequence similarity between strain TagT2C^T and related taxa

Strain	% 16S rDNA sequence similarity							
	Strain TagT2C ^T	<i>Xanthobacter flavus</i> 301 ^T	<i>Xanthobacter flavus</i> H4-14	<i>Azorhizobium caulinodans</i> ORS571 ^T	<i>Xanthobacter agilis</i> SA35 ^T	<i>Aquabacter spiritalensis</i> SPL-1 ^T	<i>Xanthobacter autotrophicus</i> 7C ^T	<i>Xanthobacter autotrophicus</i> JW33
<i>Xanthobacter flavus</i> 301 ^T	98.1							
<i>Xanthobacter flavus</i> H4-14	98.0	99.9						
<i>Azorhizobium caulinodans</i> ORS571 ^T	97.2	98.2	98.2					
<i>Xanthobacter agilis</i> SA35 ^T	97.2	98.1	98.0	97.5				
<i>Aquabacter spiritalensis</i> SPL-1 ^T	97.5	97.1	97.0	96.9	97.0			
<i>Xanthobacter autotrophicus</i> 7C ^T	97.0	97.6	97.5	96.9	96.9	97.3		
<i>Xanthobacter autotrophicus</i> JW33	96.9	97.5	97.4	96.8	96.8	97.2	99.9	
<i>Rhodoplanes roseus</i> DSM 5909 ^T	92.3	92.0	92.0	92.5	92.1	92.0	92.4	92.3

study further the relationship between the plant and the bacterium to determine whether the association is specific, serendipitous, or opportunistic.

The morphological, biochemical, and physiological characteristics of strain TagT2C^T led us to classify this organism as a *Xanthobacter* species (28, 41, 43). The phylogenetic coherence of the genus *Xanthobacter* has, however, recently been shown to be questionable (32). The results of the 16S rDNA sequence analysis clearly show that strain TagT2C^T is a member of the phylogenetic cluster comprising members of the genera *Aquabacter*, *Azorhizobium*, and *Xanthobacter*. In a previous study workers demonstrated that these three genera are intermixed and that there is no clear phylogenetic cluster for the *Xanthobacter* species (32). The addition of the TagT2C^T sequence to the data set has not changed the situation. The

bootstrap analysis showed that although the *Aquabacter*-*Azorhizobium*-*Xanthobacter* cluster was recovered in all cases, the branch points within the cluster were not significant. Consideration of the 16S rDNA sequence data alone does not allow distinction between the following two possibilities: (i) that all members of the *Aquabacter*-*Azorhizobium*-*Xanthobacter* cluster are species of the genus *Xanthobacter*; and (ii) that each distinct lineage represents an individual genus. As comprehensive phenotypic and chemotaxonomic data are not available to provide unequivocal support for either of these options, we sought to establish to which of the three genera strain TagT2C^T is most similar on the basis of physiological and biochemical characteristics.

The three genera have many properties in common, including the same DNA G+C content (Table 3), the inability to

TABLE 3. Distinguishing properties of strain TagT2C^T and the gram-negative genera *Xanthobacter*, *Azorhizobium*, and *Aquabacter*^a

Characteristic	Strain TagT2C ^T	<i>Xanthobacter</i>	<i>Azorhizobium</i>	<i>Aquabacter</i>
Pleomorphism	+ ^b	+	—	—
DNA G+C content (mol%)	68	67–69	66–68	67
Legume nodule symbiont	—	—	+	—
Presence of gas vacuoles	—	—	—	+
Peritrichous flagella	+	±	+	? ^c
Yellow colony pigmentation	+	+	—	—
Cream-white colony pigmentation	—	—	+	+
Slime production	+	±	—	(–) ^d
Fixation of dinitrogen	+	+	+	—
Autotrophic fixation of CO ₂ with ribulose biphosphate carboxylase	+	+	—	—
Growth on:				
Galactose	+	+	—	—
Fructose	+	+	—	—
Sucrose	+	+	—	—
Lactose	+	+	—	—
Formate	+	+	—	—
Methylamine	+	+	—	ND ^e
Methanol	+	+	—	—
Ethanol	+	+	—	—
Hydrogen + CO ₂	+	+	ND	ND
Thiosulfate + CO ₂	+	+	ND	ND

^a Data from references 9, 15, 28, 34, 41, and 43.

^b +, positive; —, negative; ±, characteristic varies in degree among species in response to growth conditions and methods of preparation (43).

^c Organisms are flagellated, but the insertion position has not been reported.

^d A capsule is present, and colonies are shiny, but slime production has not been reported.

^e ND, not determined.

denitrify, and the ability to grow on a variety of common substrates, but they also differ in a number of details (Table 3). It is clear from the characteristics listed in Table 3 that strain TagT2C^T has many more features in common with the genus *Xanthobacter* than with either the genus *Azorhizobium* or the genus *Aquabacter*, including its pleomorphism, pigmentation, chemolithoautotrophy on thiosulfate and hydrogen, and ability to grow on several C₁ compounds and various sugars. These characteristics support inclusion of strain TagT2C^T in the genus *Xanthobacter*, as do the results of the 16S rDNA sequence analysis. In the 16S rDNA sequence analysis strain TagT2C^T fell in the *Aquabacter-Azorhizobium-Xanthobacter* cluster and exhibited 97 to 98% sequence similarity to members of all three genera (Table 2), but was clearly quite distinct at the species level (Fig. 2). Previous work in which DNA-DNA hybridizations among species of these genera were performed showed that members of the genus *Azorhizobium* exhibited less than 20% binding to DNA from *Xanthobacter autotrophicus* or *Xanthobacter flavus* (9). While the relatedness of the genera of this cluster and the relatedness of the *Xanthobacter* spp. were clearly shown by the 16S rDNA sequences and DNA-rRNA hybridization data (9, 32), DNA-DNA hybridization data showed that the *Xanthobacter* spp. are distinct from each other (17), and the levels of DNA-DNA homology among *X. autotrophicus*, *X. flavus*, and *Xanthobacter agilis* were recently shown to range from 17 to 31% (8). These data have little value for making precise phylogenetic placements (38) and did not justify the use of DNA-DNA hybridization with strain TagT2C^T at this time. If a future taxonomic rearrangement were to result in all of the members of this cluster being combined into the genus *Xanthobacter* (32), then strain TagT2C^T would continue to represent a distinct species of this genus.

Description of *Xanthobacter tagetidis* sp. nov. *Xanthobacter tagetidis* (ta.ge.ti'dis. M. L. n., of *Tagetes*, the marigold genus of flowering plants).

(i) **Morphology.** Colonies are low, entire, smooth, convex, mucoid, and egg yolk yellow. Cells are gram-negative, pleomorphic, short, fat rods (0.5 by 1.0 µm) with large, dark inclusion bodies which may occur at one or both ends of the cells. Motile. No spores are produced. A capsule is formed.

(ii) **Cellular characteristics.** The G+C content of the DNA is 68 ± 2 mol% (as determined by the UV spectroscopy and melting point methods). The predominant ubiquinone is coenzyme Q10, and some coenzyme Q8 is present. Ribulose biphosphate carboxylase is present in thiosulfate-grown cells (108 ± 6 nmol of CO₂ fixed/min/mg of cell extract protein). Catalase and oxidase positive.

(iii) **Growth characteristics.** Obligately aerobic but potentially microaerophilic. For growth on T2C the temperature range is 4 to 43°C, the optimum temperature is 28 to 31°C; and the optimum pH is 7.6 to 7.8 (pH range tested, pH 6.8 to 8.7). The initial rates of growth on T2C in static cultures are similar to those in shaken cultures. Static cultures produce less slime than shaken cultures.

(iv) **Metabolic characteristics.** Chemoorganotroph characterized by an ability to grow on substituted thiophenes; facultative chemolithoautotroph on inorganic sulfur compounds and hydrogen; facultative methylotroph. Uses molecular dinitrogen as a nitrogen source. Utilizes T2A, T2C, thiophene-3-carboxylate, dibenzothiophene, furan-2-carboxylate, pyrrole-2-carboxylate, thiosulfate, tetrathionate (with added CO₂), sulfide, hydrogen (with added CO₂), carbonyl sulfide, carbon disulfide, methylamine (with added CO₂), methanol, dimethyl sulfide, methanethiol, formate, acetate, ethanol, succinate, pyruvate, citrate, yeast extract, nutrient broth, galactose, sucrose, fructose, and glucose. In sulfate-free medium, T2C sup-

ports growth as a sole source of carbon, energy, and sulfur. Inhibited by ampicillin and chloramphenicol.

(v) **Habitat.** The organism has been isolated only from the root balls of marigold plants (*T. patula* and *T. erecta*).

(vi) **Type strain.** *X. tagetidis* TagT2C was isolated from a *T. patula* root ball and has been deposited as the type strain of the species in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as strain DSM11105. This strain is one of several apparently identical strains isolated from *T. patula* and *T. erecta*.

We propose that on the basis of its morphological and physiological characteristics, including its facultative autotrophy on hydrogen and thiosulfate, its ability to grow by using dinitrogen as a nitrogen source, the G+C content of its DNA, its levels of 16S rDNA sequence homology with other *Xanthobacter* spp., and its association with thiophene-producing marigold plants, strain TagT2C should be included in the genus *Xanthobacter* as the type strain of a new species, *X. tagetidis*.

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REFERENCES

1. Amphlett, M. J. 1968. The microbiological transformation of sulphur-containing aromatic compounds. Ph.D. thesis. University of Wales, Cardiff, Wales.
2. Amphlett, M. J., and A. G. Calley. 1969. The degradation of 2-thiophenecarboxylic acid by a *Flavobacterium* species. *Biochem. J.* **112**:12-13.
3. Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of the 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **75**:4801-4805.
4. Buitelaar, R. M., M. T. Cesario, and J. Tramper. 1992. Elicitation of thiophene production by hairy roots of *Tagetes patula*. *Enzyme Microb. Technol.* **14**:2-7.
5. Cawse, P. A. 1967. The determination of nitrate in soil solutions by ultraviolet spectrophotometry. *Analyst* **92**:311-315.
6. Cripps, R. E. 1971. Microbial metabolism of aromatic compounds containing sulphur. Ph.D. thesis. University of Warwick, Warwick, United Kingdom.
7. Cripps, R. E. 1973. The microbial metabolism of thiophene-2-carboxylate. *Biochem. J.* **134**:353-366.
8. Doronina, N. V., Y. A. Trotsenko, V. I. Krauzova, and N. E. Suzina. 1996. New methylotrophic isolates of the genus *Xanthobacter*. *Microbiology (Engl. transl. Mikrobiologiya)* **65**:217-224.
9. Dreyfus, B., J. L. Garcia, and M. Gillis. 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-modulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int. J. Syst. Bacteriol.* **38**:89-98.
10. Evans, J. S., and W. A. Venables. 1990. Degradation of thiophene-2-carboxylate, furan-2-carboxylate, pyrrole-2-carboxylate and other thiophene derivatives by the bacterium *Vibrio* YC1. *Appl. Microbiol. Biotechnol.* **32**:715-720.
11. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783-789.
12. Felsenstein, J. 1993. PHYLIP (phylogenetic inference package), version 3.5.1. Department of Genetics, University of Washington, Seattle.
13. Fredericq, E., A. Oth, and F. Fontaine. 1961. The ultraviolet spectrum of deoxyribonucleic acids and their constituents. *J. Mol. Biol.* **3**:11-17.
14. Hausen, B. M., and B. Helmke. 1995. Butenylbithiophene, alpha-terthienyl and hydroxytremetone as contact allergens in cultivars of marigold (*Tagetes* sp.). *Contact Dermatitis* **33**:33-37.
15. Irgens, R. I., K. Kersters, P. Segers, M. Gillis, and J. T. Staley. 1991. *Aquabacter spiritalensis*, gen. nov., sp. nov., an anaerobic, gas-vacuolate aquatic bacterium. *Syst. Appl. Microbiol.* **9**:254-257.
16. Jacobs, J. J. M. R., A. Engelberts, A. F. Croes, and G. J. Wullems. 1994. Thiophene synthesis and distribution in young developing plants of *Tagetes patula* and *Tagetes erecta*. *J. Exp. Bot.* **45**:1459-1466.
17. Jenni, B., M. Aragno, and J. Wiesel. 1987. Numerical analysis and DNA-DNA hybridization studies on *Xanthobacter* and emendation of *Xanthobacter flavus*. *Syst. Appl. Microbiol.* **9**:247-253.

18. Jordan, S. L., A. Kraczkiewicz-Dowjat, D. P. Kelly, and A. P. Wood. 1995. Novel eubacteria able to grow on carbon disulfide. *Arch. Microbiol.* **163**: 131–137.
19. Juhl, M. J., and D. P. Clark. 1990. Thiophene-degrading *Escherichia coli* mutants possess sulfone oxidase activity and show altered resistance to sulfur-containing antibiotics. *Appl. Environ. Microbiol.* **56**:3179–3185.
20. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, New York, N.Y.
21. Kanagawa, T., and D. P. Kelly. 1987. Degradation of substituted thiophenes by bacteria isolated from activated sludge. *Microb. Ecol.* **13**:47–57.
22. Kelly, D. P., and A. P. Wood. 1994. Synthesis and determination of thiosulfate and polythionates, p. 475–501. In H. D. Peck and J. LeGall (ed.), *Methods in enzymology—inorganic sulfur metabolism*. Academic Press, Orlando, Fla.
23. Kelly, D. P., L. A. Chambers, and P. A. Trudinger. 1969. Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. *Anal. Chem.* **41**:898–901.
24. Kilbane, J. J., and K. Jackowski. 1992. Biodesulfurization of water-soluble coal-derived material by *Rhodococcus rhodochrous* IGTS8. *Biotechnol. Bioeng.* **40**:1107–1114.
25. Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese. 1994. The Ribosomal Database Project. *Nucleic Acids Res.* **22**:3485–3487.
26. Mandel, M., and J. Marmur. 1968. Use of ultra-violet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol.* **12B**:195–206.
27. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208–218.
28. Meijer, W. G., L. M. Croes, B. Jenni, L. G. Lehmicke, M. E. Lidstrom, and L. Dijkhuizen. 1990. Characterization of *Xanthobacter* strains H4-14 and 25a and enzyme profiles after growth under autotrophic and heterotrophic conditions. *Arch. Microbiol.* **153**:360–367.
29. Mukundan, U., and M. A. Hjortso. 1990. Thiophene content in normal and transformed root cultures of *Tagetes erecta*: a comparison with thiophene content in roots of intact plants. *J. Exp. Bot.* **41**:1497–1501.
30. Omori, T., L. Mouna, L. Saiki, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1. *Appl. Environ. Microbiol.* **58**:911–915.
- 30a. Padden, A. N., and A. P. Wood. Unpublished data.
31. Perich, M. J., C. Wells, W. Bertsch, and K. E. Tredway. 1995. Isolation of the insecticidal components of *Tagetes minuta* (Compositae) against mosquito larvae and adults. *J. Am. Mosquito Control Assoc.* **11**:307–310.
32. Rainey, F. A., and J. Wiegel. 1996. 16S ribosomal DNA sequence analysis confirms the close relationship between the genera *Xanthobacter*, *Azorhizobium*, and *Aquabacter* and reveals lack of phylogenetic coherence among *Xanthobacter* species. *Int. J. Syst. Bacteriol.* **46**:607–610.
33. Rainey, F. A., N. Ward-Rainey, R. M. Kroppenstadt, and E. Stackebrandt. 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsiaceae* fam. nov. *Int. J. Syst. Bacteriol.* **46**:1088–1092.
34. Reding, H. K., G. L. M. Croes, L. Dijkhuizen, and J. Wiegel. 1992. Emendation of *Xanthobacter flavus* as a motile species. *Int. J. Syst. Bacteriol.* **42**:309–311.
35. Romagnoli, C., D. Mares, M. P. Fasulo, and A. Bruni. 1994. Antifungal effects of alpha-terthienyl from *Tagetes patula* on five dermatophytes. *Phytother. Res.* **8**:332–336.
36. Saenz Carbonell, L. A., I. E. Maldonado Mendoza, O. Moreno Valenzuela, R. Ciauiy, M. Lopez Meyer, C. Oropeza, and V. M. Loyola Vargas. 1993. Effects of medium pH on the secondary metabolites from roots of *Datura stramonium*, *Catharanthus roseus*, and *Tagetes patula* cultured in vitro. *Appl. Biochem. Biotechnol.* **38**:257–267.
37. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
38. Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846–849.
39. Talou, J. R., O. Cascone, and A. M. Giulietta. 1994. Content of thiophenes in transformed root cultures of Argentinian species of *Tagetes*. *Planta Med.* **60**:260–262.
40. Tuovinen, O. H., and D. P. Kelly. 1973. Studies on the growth of *Thiobacillus ferrooxidans*. *Arch. Mikrobiol.* **88**:285–298.
41. Urakami, T., H. Araki, and K. Komagata. 1995. Characteristics of newly isolated *Xanthobacter* strains and fatty acid compositions and quinone systems in yellow-pigmented hydrogen-oxidizing bacteria. *Int. J. Syst. Bacteriol.* **45**:863–867.
42. van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. *Arch. Microbiol.* **153**:324–328.
43. Wiegel, J. 1992. The genus *Xanthobacter*, p. 2365–2383. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York, N.Y.
44. Wood, A. P., and D. P. Kelly. 1983. Autotrophic, mixotrophic and heterotrophic growth with denitrification by *Thiobacillus* A2 under anaerobic conditions. *FEMS Microbiol. Lett.* **16**:363–370.
45. Wood, A. P., and D. P. Kelly. 1985. Physiological characteristics of a new thermophilic obligately chemolithotrophic *Thiobacillus* species, *Thiobacillus tepidarius*. *Int. J. Syst. Bacteriol.* **35**:434–437.
46. Wood, A. P., and D. P. Kelly. 1986. Chemolithotrophic metabolism of the newly-isolated moderately thermophilic, obligately autotrophic *Thiobacillus tepidarius*. *Arch. Microbiol.* **144**:71–77.