Biodegradation **7:** 313–327, 1996.

Microbial metabolism of some 2,5-substituted thiophenes

Phillip M. Fedorak[1], Debora L. Coy[1] & Torren M. Peakman[2]
[1]*Department of Biological Sciences, University of Alberta, Edmonton, Alberta Canada, T6G 2E9;* [2]*School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, Great Britain*

17 June 1996

Key words: bacteria, 2,5-dialkylthiophenes, petroleum, sulfate, 2,5-thiophenedicarboxylic acid, thiophenes

Abstract

2,5-Dialkylthiophenes are found in bitumens and crude oils, and previous studies showed that bacterial metabolism of some with a methyl substituent lead to the formation of 5-methyl-2-thiophenecarboxylic acid, which persisted in the culture medium (Fedorak PM & Peakman TM 1992 *Biodegradation* 2: 223–236). The objectives of this investigation were to study the further metabolism of this acid, and of two dialkylthiophenes, 2,5-diundecylthiophene and 2-(3,7-dimethyloctyl)-5-methylthiophene. Undefined, oil-degrading mixed cultures were used. 5-Methyl-2-thiophenecarboxylic acid was oxidized to 2,5-thiophenedicarboxylic acid which was identified by gas chromatography-mass spectrometry (GC-MS). This dicarboxylic acid was degraded and supported the growth of a mixed microbial population, and approximately 50% of the sulfur in this substrate was detected as sulfate in the medium at the end of the 15-day incubation time. Mixed cultures were incubated with 2,5-diundecylthiophene or 2-(3,7-dimethyloctyl)-5-methylthiophene as their sole carbon source, and at various times some of these were freeze-dried and the residues were treated to form methyl esters of any carboxylic acids produced. GC-MS analyses showed the presence of several dicarboxylic acids, indicating that both alkyl groups were oxidized. A small amount of the dimethyl ester of 2,5-thiophenedicarboxylic acid was detected in the culture grown on 2,5-diundecylthiophene, and 37% of the sulfur from this dialkylthiophene was detected as sulfate in the medium after 35 days of incubation.

Introduction

2,5-Dialkylthiophenes have been identified in bitumens, crude oils and in pyrolysates of kerogens and asphaltenes (Sinninghe Damsté & de Leeuw 1989). Sinninghe Damsté et al. (1987, 1989) identified several classes of these, including 2-alkyl-5-methyl-, 2-alkyl-5-ethyl-, 2-alkyl-5-propyl-, and the so called 'mid-chain' 2,5-dialkyl-thiophenes. The alkyl substituents may be linear or branched (Sinninghe Damsté et al. 1986, 1987, 1989).

The microbial metabolism of six alkylthiophenes was investigated previously (Fedorak & Peakman 1992). The compounds studied had a methyl, ethyl or butyl group in the 2-position of the thiophene ring, and side chains of 10 to 20 carbon atoms in the 5-position. Three of those were *n*-alkyl-substituted, and three were isoprenoidal-substituted. With the exception of 2-(3,7-dimethyloctyl)-5-methylthiophene, the metabolites identified indicated that the long side chains were attacked by *n*-alkane-degrading bacteria. For example, 2-hexadecyl-5-methylthiophene was degraded to 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid that accumulated in cultures that contained no crude oil (Fedorak & Peakman 1992). However, these intermediates underwent further biotransformation to undetected metabolites if crude oil or *n*-hexadecane was added to the culture medium.

There have been several reports on the bacterial metabolism of and/or growth on 2-thiophenecarboxylic acids. Kanagawa & Kelly (1987) showed that *Rhodococcus* spp. isolated from activated sludge utilized 2-thiophenecarboxylic acid, 5-methyl-2-thiophenecarboxylic acid and 2-thiopheneacetic acid as sole carbon and energy sources. In addition, Cripps (1973) showed that 2- thiophenecarboxylic acid was used as the sole carbon, sulfur and energy source for a bacterium designated 'Organism R1'. Evans &

Venables (1990) demonstrated that a *Vibrio* sp. grew on 2-thiophenecarboxylic acid and 2-thiopheneacetic acid as its sole sulfur source. In each case, the sulfur atom was released into the medium as sulfate during growth of these bacteria (Cripps 1973; Kanagawa & Kelly 1987; Evans & Venables 1990). Tanaka et al. (1982) observed that washed cells of a photosynthetic bacterium metabolized 2-thiophenecarboxylic acid to (+)-3-hydroxytetrahydrothiophene-2-carboxylic acid.

In our earlier studies (Fedorak & Peakman 1992), no metabolites of the thiophene carboxylic acids were detected by our routine solvent-extraction and gas chromatography (GC) method. We postulated that some of the dialkylthiophenes may undergo omega-oxidation, as is the case with pristane (Pirnik et al. 1974) leading to dicarboxylic acids, or that the methyl groups of 5-methyl-2-thiophenecarboxylic acid and 5- methyl-2-thiopheneacetic acid may be oxidized to a carboxylic acid, producing a dicarboxylic acid. These dicarboxylic acids would be too polar to be solvent-extracted from the growth medium for GC analysis, and thus would not be detected using the methods outlined in the previous study (Fedorak & Peakman 1992). Evidence of methyl group oxidation was provided when a bacterial strain grown on *n*-tetradecane in the presence of 2-(3,7-dimethyloctyl)-5-methylthiophene yielded 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid (Fedorak & Peakman 1992).

The main goal of this investigation was to provide a better understanding of the metabolism of alkyl thiophenes and thiophenecarboxylic acids by mixed microbial populations. Specific objectives were to determine: (a) whether 5-methyl-2-thiophenecarboxylic acid can be oxidized to 2,5-thiophenedicarboxylic acid by bacterial action; (b) if 2,5-thiophenedicarboxylic acid can be removed from the culture medium by some of the bacterial cultures; (c) whether two alkyl thiophenes, 2,5-diundecylthiophene and 2-(3,7-dimethyloctyl)-5-methylthiophene, are biodegraded via omega-oxidation, yielding dicarboxylic acids as intermediates; and (d) if sulfate is released from some of the 2,5-substituted thiophenes by bacterial action.

Materials and methods

Organosulfur compounds used

5-Methyl-2-thiophenecarboxylic acid was purchased from Aldrich (Milwaukee, WI) and 2,5-thiophenedicarboxylic acid was purchased from Lancaster Synthesis (Windham, NH). 2,5-Diundecylthiophene and 2-(3,7-dimethyloctyl)-5-methylthiophene were synthesized using the methods outlined by Peakman & Kock-van Dalen (1990). Figure 1 shows the structures of the substrates used.

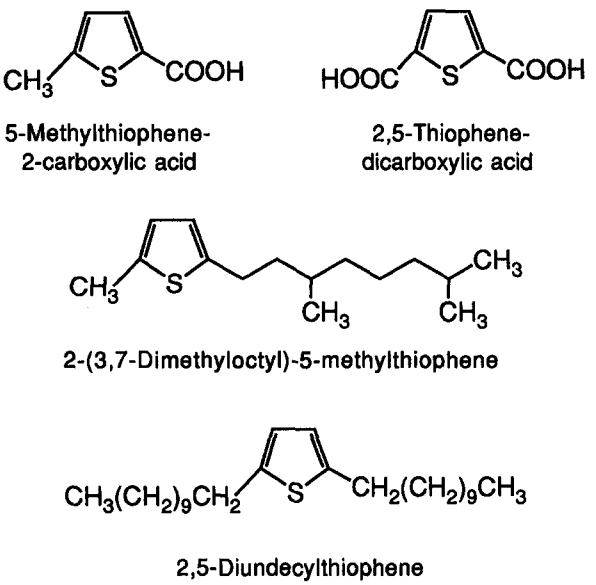

Figure 1. Structures of the 2,5-substituted thiophenes used in this study.

Analytical methods for thiophenecarboxylic acids

Attempts to extract 2,5-thiophenedicarboxylic acid from an aqueous solution (pH<2) into dichloromethane failed, indicating that the dicarboxylic acid was too polar to be partitioned into the organic solvent. Thus, to detect dicarboxylic acids, the pH of a sample was raised to approximately 12 by the addition of 4 M NaOH solution. Then the sample was freeze-dried and the residual material was dissolved in methanol, acidified with a few drops of concentrated H_2SO_4 and refluxed to form the dimethyl ester of the acid (Fedorak & Westlake 1983). The dimethyl esters were readily extracted into hexanes and were amenable to analysis by GC. The utility of the procedure was verified using 2,5-thiophenedicarboxylic acid as a model compound.

The freeze-drying method outline above was too time-consuming for routine use to monitor the formation or metabolism of dicarboxylic acids in the cultures. Therefore, a reversed-phase high performance liquid chromatography (HPLC) method similar to that of

Andersson & Bobinger (1992) with an ion-pair reagent in the mobile phase was used. A Hewlett-Packard (model 1050) instrument was used with the detector set at 270 nm. The mobile phase was methanol:water (50:50) containing 0.005 M PIC®-A Low UV Reagent (Waters) pumped through a LiChrospher 100 RP-18 column (5 μm, 125 mm by 4 mm, Hewlett-Packard) at a flow rate of 1 mL/min. The retention times for 5-methyl-2-thiophenecarboxylic and 2,5-thiophenedicarboxylic acids were 1.8 and 1.3 min, respectively. 2-Naphthoic acid was used as an internal standard for this analysis, and 25 μL of a methanol solution containing 2 mg of the standard/mL were added to a 1-mL sample of the culture prior to HPLC analysis.

GC methods

To screen for the presence of sulfur-containing metabolites, samples were analyzed by capillary GC using a 30 m DB-5 capillary column in a Hewlett-Packard model 5890 gas chromatograph equipped with a flame ionization detector (FID) and a sulfur-selective flame photometric detector (FPD). The temperature program routinely used was: 90°C for 2 min, 4°/min to 250° which was held for 16 min. For the analysis of cultures incubated with 2,5-diundecylthiophene, that would not elute under these conditions, the temperature program was modified to 90°C for 2 min, 8°/min to 250° which was held for 36 min.

GC-mass spectrometry (GC-MS) was done by the personnel in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta. The instrument and conditions were described by Fedorak & Westlake (1986).

Sulfate analysis

The cultures were routinely monitored using the turbidimetric method number 4500-SO_4^{2-}-E given in APHA (1989). However, because the sample volume required was 100 mL, and the total culture volume was only 200 mL, the method was scaled-down by a factor of 100 allowing the use of 1-mL samples of cultures in the analysis. This modified procedure produced a linear calibration curve with standard solutions containing 0.01 to 3.5 mM sulfate.

On some occasions, sulfate analyses were done in the Environmental Engineering Laboratory at the University of Alberta. For each analysis, a 25-μL sample of a culture was injected into a Dionex Ion Chromatography system equipped with temperature- compensated CDM-3 conductivity detector and A1-450 chromatography software. The system was also equipped with an AMMS-II anion microMembrane suppressor for eluent anion suppression. A regeneration solution of 50 mM H_2SO_4 was pumped through the suppressor. The sulfate ions were separated from other ions using an IonPac® AS10 4 mm x 250 mm (Dionex) ion exchange column (with an IonPac® AG10 4 mm x 50 mm guard column) and isocratic elution with 80 mM NaOH at 1 mL/min. The sulfate eluted at approximately 10.4 min. Quantitative results were obtained using an external four-point calibration curve for each set of samples analyzed. A known sample was analyzed at the start and end of each day and errors of <5% were achieved.

Culture media used

All cultures were grown at 28°C in 500-mL shake-flasks containing 200 mL of liquid mineral medium, designated B+NP. Its composition is given by Fedorak & Westlake (1984), and each litre was supplemented with 1 mL of trace metals solution (Fedorak & Grbić-Galić 1991). The medium was inoculated (at 5% v/v) with one of the maintenance cultures described below.

In some experiments designed to detect the release of sulfate from selected thiophenes, sulfate-free medium was used to provide a low background of sulfate. This medium contained (per litre): 0.25 g K_2HPO_4, 1 g NaCl, 0.1 g $MgCl_2 \cdot 6H_2O$, 1 g KNO_3, 0.5 g NH_4Cl, and 0.5 mL of trace metals solution (Fedorak & Grbić-Galić 1991).

Because of their low water-solubility, the thiophene carboxylic acids were routinely added to cultures in a methanol solution. Usually 400 μL of a 12.5 mg/mL solution were added. However, in some experiments, 2,5-thiophenedicarboxylic acid dissolved in 5% (w/v) sodium bicarbonate solution was added to the cultures. 2,5-Diundecylthiophene and 2-(3,7-dimethyloctyl)-5-methylthiophene were added neat using a microsyringe, or as solutions dissolved in dichloromethane.

Bacterial cultures and growth conditions used

Three mixed cultures of oil-degrading bacteria were used for these studies. The cultures were designated SLPB, ERN BIO, and ESSO AG. SLPB was enriched from aviation fuel-contaminated beach material from

Shell Lake, Northwest Territories, Canada, and it has been maintained since 1983 by monthly transfers to fresh medium containing Prudhoe Bay crude oil as the sole carbon source (Fedorak & Peakman 1992). The cultures ERN BIO, and ESSO AG were enriched from samples of activated sludge obtained from wastewater treatment systems at two petroleum refineries in Germany. These have been maintained since 1986 by monthly transfers to fresh medium containing Prudhoe Bay crude oil as the sole carbon source.

In some studies with 2-(3,7-dimethyloctyl)-5-methylthiophene, a pure culture of an *n*-alkane-degrading, Gram-positive bacterium, designated as SE (Foght et al. 1990; Fedorak & Peakman 1992) was used.

Initial experiments to determine whether 5-methyl-2- thiophenecarboxylic acid could be oxidized to 2,5-thiophenedicarboxylic acid used the cultures ERN BIO and ESSO AG as inocula for 200 mL of B+NP medium containing 5 mg (0.035 mmol) of 5-methyl-2-thiophenecarboxylic acid and 200 μL of Prudhoe Bay crude oil. Later, SLPB, ERN BIO, and ESSO AG were used as inocula for 200 mL of B+NP medium containing 5 mg of 5-methyl-2-thiophenecarboxylic acid and 100 μL of *n*-hexadecane. The substrate concentrations in these cultures were monitored by HPLC for 10 days, then one-half of the culture volume was extracted with dichloromethane and the other half was freeze-dried and treated to form methyl esters for GC analyses. Subsequently, cultures of ESSO AG and SLPB were inoculated into 200 mL of medium containing 2.5 mg (0.017 mmol) of 5-methyl-2-thiophenecarboxylic acid and 100 μL of Prudhoe Bay crude oil. These were incubated for 42 days, and then freeze-dried and treated to form methyl esters of the carboxylic acids present.

To demonstrate bacterial utilization of, and growth on 2,5-thiophenedicarboxylic acid, 20-mL portions of the mixed culture SLPB were inoculated into three 500-mL flasks containing 200 mL of sulfate-free medium, and the substrate was added to each flask. Into one culture, 1 mL of a methanol solution (containing 5 mg of 2,5-thiophenedicarboxylic acid, 29 mM) was added, and into the second culture, 1 mL of a 5% sodium bicarbonate solution (containing 5 mg of 2,5-thiophenedicarboxylic acid, 29 mM) was added. The third culture received 4 mL of the latter solution to give 20 mg (0.12 mmol) of 2,5-thiophenedicarboxylic acid in the 200-mL culture. Appropriate sterile controls were prepared containing 5 or 20 mg of this substrate. In addition, the mixed culture SLPB was inoculated into two flasks of sulfate-free medium supplemented with either 1 mL of methanol or 1 mL of 5% sodium bicarbonate solution — these contained no 2,5-thiophenedicarboxylic acid. These cultures and controls were incubated for 6 days, and samples were removed daily for turbidity readings at 600 nm (OD_{600}). At the same times, samples were taken from those flasks that contained 2,5-thiophenedicarboxylic acid for analyses of this compound by HPLC.

Because of the low OD readings observed in the abovementioned experiments, further tests were done to assess growth on 2,5-thiophenedicarboxylic acid. Twenty-millilitre portions of the mixed culture SLPB were inoculated into two flasks of medium containing 200 mL of sulfate-free medium. One received 5 mg (0.029 mmol) of the dicarboxylic acid delivered in 1 mL of bicarbonate solution, and the other received 1 mL of bicarbonate solution, without the dicarboxylic acid. Immediately after inoculation, samples were removed for plate counts. Serial 10-fold dilutions were made, and aliquots of these were plated in triplicate onto Plate Count Agar (Difco, Detroit, MI). The plates were incubated for 3 days at 28°C before the colonies were counted. The concentration of the dicarboxylic acid was determined daily by HPLC, and when it was depleted from the medium, a sample was removed for plate counts, and another 5-mg (0.029-mmol) portion of the dicarboxylic acid, in 1 mL of bicarbonate solution, was added to the shake-flask culture. At the same time, a sample for plate counts was removed from the control shake-flask culture that received no 2,5-thiophenedicarboxylic acid, and the remaining culture received 1 mL of bicarbonate solution devoid of the dicarboxylic acid. These procedures continued until four 5-mg (0.029-mmol) feedings of the dicarboxylic acid were consumed.

To determine whether sulfate was a product of 2,5-thiophenedicarboxylic acid metabolism, triplicate 200-mL cultures of SLPB in sulfate-free medium were fed 5 mg (0.029 mmol) of this substrate four times over a 12-day incubation period. The decrease of 2,5-thiophenedicarboxylic acid was monitored by HPLC, and when it was depleted, another 5 mg of the acid was added to each culture. A control culture which received no 2,5-thiophenedicarboxylic acid was incubated with the test cultures. Sulfate concentrations in the cultures and control were measured at the time of inoculation and after 15 days incubation. This experiment was repeated and the amounts of sulfur associated with the cells used as inocula and with cells that were harvested after growth on 2,5-thiophenedicarboxylic acid were determined. Centrifuged, washed cells were oven dried at 105°C prior to sulfur analysis by the Micro-

analytical Laboratory in the Department of Chemistry, University of Alberta.

To identify metabolites from 2-(3,7-dimethyloctyl)-5-methylthiophene degradation, the following experiments were done. Initial studies used isolate SE that was inoculated into 200 mL of B+NP medium with 2 μL of 2-(3,7-dimethyloctyl)-5-methylthiophene. Each of the duplicate cultures was supplemented with 0.2 mL of Prudhoe Bay crude oil and these cultures and appropriate sterile controls were incubated on a rotary shaker at 28°C. One of the duplicate cultures was extracted after 14 days of incubation, and the other was extracted after 28 days of incubation. The extraction method was designed to separate the acidic metabolites from the residual petroleum. The culture medium was adjusted to pH 12 with a 4 M NaOH solution and extracted five times with 15 mL of dichloromethane. The extracts were pooled, concentrated and analyzed by GC to determine the extent of the petroleum degradation. The aqueous phase was adjusted to pH <2 with H_2SO_4 and this was extracted four times with 15 mL of dichloromethane. The pooled extracts were evaporated to dryness and refluxed in methanol with H_2SO_4 to give methyl esters of the acid metabolites which were analyzed by GC-MS.

To determine whether dicarboxylic acids could be detected as metabolites from 2-(3,7-dimethyloctyl)-5-methylthiophene, the mixed culture SLPB was inoculated into 200 mL of B+NP medium supplemented with 50 μL of pristane and 2 μL of the isoprenoidal thiophene. After 3 days incubation, the culture was adjusted near pH 12, freeze-dried and the residue was refluxed in methanol with H_2SO_4 acid to yield methyl esters of any carboxylic acids that were formed.

The metabolism of 2,5-diundecylthiophene was studied in the following manner. To determine whether this 'mid-chain' 2,5- dialkylthiophene was biodegradable, the mixed culture SLPB was used, and in the initial studies, the following three combinations of carbon sources were added to 200 mL of B+NP medium plus trace metals solution: (a) 3 mg (0.008 mmol) of 2,5-diundecylthiophene; (b) 3 mg (0.008 mmol) of 2,5-diundecylthiophene and 5 mg (0.029 mmol) of 2,5-thiophenedicarboxylic acid; and (c) 5 mg (0.013 mmol) of 2,5-diundecylthiophene and 0.2 mL of Prudhoe Bay crude oil. After 14 days incubation, these cultures and appropriate sterile controls were acidified and extracted for GC-FPD analysis.

2,5-Diundecylthiophene served as the sole carbon source for a later experiment in which the mixed culture SLPB was inoculated into five flasks, each containing 200 mL of sulfate-free medium and 6 mg (0.015 mmol) of the dialkylthiophene. These cultures were incubated for various lengths of time, and at days 0, 5, 7, 12, and 19, a culture was adjusted to near pH 12, freeze-dried and the residue was refluxed in methanol with H_2SO_4 acid to yield methyl esters of any carboxylic acid metabolites that were formed.

To test for the release of sulfate from 2,5-diundecylthiophene, the mixed culture SLPB was inoculated into three flasks of sulfate-free medium and 36 mg (0.092 mmol) of the dialkylthiophene were added as the sole carbon and energy source. This substrate was added as six 6-mg feedings over the course of the experiment. At various times during the 35-day incubation of this culture, samples were removed for HPLC analyses that showed the appearance and subsequent loss of a metabolite that had the same retention time as 2,5-thiophenedicarboxylic acid. At the end of the incubation period, samples were analyzed for sulfate using the Dionex ion chromatography system.

Results

Bacterial oxidation of 5-methyl-2-thiophenecarboxylic acid to 2,5-thiophenedicarboxylic acid

Previous work (Fedorak & Peakman 1992) suggested that the degradation of 5-methyl-2-thiophenecarboxylic acid in mixed cultures required another growth substrate such as crude oil or a fraction of crude oil. Initial experiments used the mixed cultures ERN BIO and ESSO AG with Prudhoe Bay crude oil. Subsequent experiments used SLPB, ERN BIO, and ESSO AG with *n*-hexadecane. Growth, as indicated by increased turbidity, was observed in all five of these cultures during their 14-day incubation period. HPLC analyses of samples from the oil-containing cultures showed slight decreases in the concentration of 5-methyl-2-thiophenecarboxylic acid, but no dicarboxylic acid was detected. No decrease in 5-methyl-2-thiophenecarboxylic acid concentration was observed in the *n*- hexadecane-containing cultures.

Cultures of ESSO AG and SLPB were then incubated for a longer time in medium containing 5-methyl-2-thiophenecarboxylic acid and Prudhoe Bay crude oil. After 42 days of incubation, the concentration of 5-methyl-2-thiophenecarboxylic acid decreased by 40% in the ESSO AG culture and by 60% in the SLPB culture. Then the entire contents of these two cultures

were freeze-dried and the residual material was heated under reflux in methanol containing a few drops of H_2SO_4 acid to form the methyl esters of any carboxylic acids present. GC-FPD analysis of the residue from the SLPB culture showed the presence of the methyl ester of the substrate, 5-methyl-2-thiophenecarboxylic acid, but not that of 2,5-thiophenedicarboxylic acid. In contrast, GC-FPD analysis of the residue from the ESSO AG culture showed the presence of the methyl ester of 5-methyl-2-thiophenecarboxylic acid and that of a sulfur-containing metabolite which had the same retention time as the dimethyl ester of 2,5-thiophenedicarboxylic acid. GC-MS analysis of this sample verified the presence of the dimethyl ester of 2,5-thiophenedicarboxylic acid. The metabolite had the same mass spectrum, with two predominant ions; M^+ at m/z 200, and the base peak at m/z 169 $(M\text{-}OCH_3)^+$ as the dimethyl ester of authentic 2,5- thiophenedicarboxylic acid. These results demonstrated the oxidation of the methyl group of 5-methyl-2-thiophenecarboxylic acid to 2,5-thiophenedicarboxylic acid by an oil-degrading mixed population of bacteria.

Growth of bacterial cultures on 2,5-thiophenedicarboxylic acid

Figure 2 shows the results of an experiment to determine whether 2,5-thiophenedicarboxylic acid would be consumed by cultures of SLPB. The substrate was added as a methanol solution, or as a sodium bicarbonate solution to 200-mL cultures, giving an initial concentration of 25 mg/L (0.15 mM). At the time of inoculation, the methanol-containing culture was at pH 6.7, and the bicarbonate-containing culture was at pH 7.8. Based on the 2,5-thiophenedicarboxylic acid concentrations (Figure 2a), a lag time of 2 days was observed in these cultures. Growth of the cultures, measured by an increase in OD_{600} (Figure 2b), accompanied a decrease in the 2,5-thiophenedicarboxylic acid concentration. The growth in the methanol-containing culture was much greater than that in the bicarbonate-containing culture (Figure 2b). In both cultures, the addition of 5 mg (0.029 mmol) of 2,5-thiophenedicarboxylic acid provided 2.1 mg (0.17 mmol) of organic carbon. However, the addition of 1 mL of methanol provided an extra 250 mg (21 mmol) of organic carbon, which would account for the greater cell density at the end of the incubation period. The corresponding sterile control showed no increase in turbidity (Figure 2b), or any decrease in 2,5-thiophenedicarboxylic acid concentration over the 6-

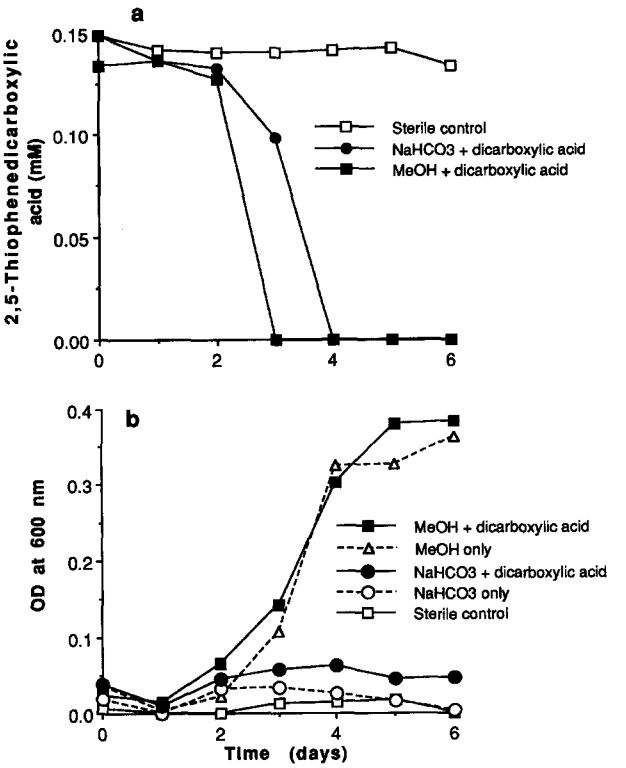

Figure 2. The depletion of 2,5-thiophenedicarboxylic acid (a), and the growth of the mixed culture SLPB (b) in sulfate-free medium initially containing near 0.15 mM dicarboxylic acid which was delivered in 1 mL of a methanol solution, or in 1 mL of a 5% (w/v) sodium bicarbonate solution. The turbidity of the mixed culture in medium without the dicarboxylic acid, but supplemented with 1 mL of methanol or 1 mL of bicarbonate solution, was also monitored.

day incubation time (Figure 2a). Similarly, no increase in OD_{600} was observed in the culture that received sodium bicarbonate solution only (Figure 2b).

A third 200-mL culture in this experiment received 20 mg (0.12 mmol) of 2,5-thiophenedicarboxylic acid from a bicarbonate solution. Because of the volume of bicarbonate solution used (4 mL), the medium was initially at pH 8.4. This was adjusted to pH 7.0 with sterile 1 M HCl prior to inoculation. After a 4-day lag, the substrate concentration began to decrease, and by day 7, the dicarboxylic acid was essentially all consumed. At that time, the OD_{600} was 0.07. These results indicate that 2,5-thiophenedicarboxylic acid at a concentration of 100 mg/L (0.58 mM) inhibited the onset of its degradation by the microbial population in the SLPB culture. Thus, in subsequent experiments, care was taken to keep the concentration of this acid near or below 25 mg/L (0.15 mM).

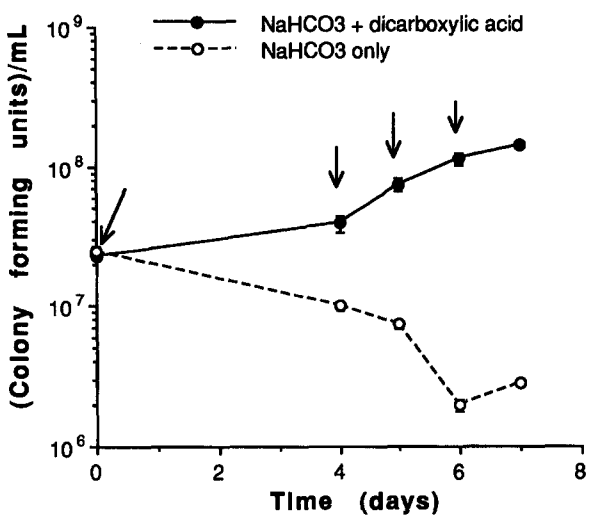

Figure 3. Changes in the viable bacterial counts in shake-flask cultures fed four 5-mg (0.013-mmol) portions of 2,5-thiophenedicarboxylic acid from a sodium bicarbonate solution. The arrows indicate when the substrate was added. The control culture received bicarbonate solution, devoid of 2,5-thiophenedicarboxylic acid. Error bars, which are often smaller than the symbol, show one standard deviation.

Because of the low OD_{600} readings observed for the culture that received the dicarboxylic acid in sodium bicarbonate solution, (Figure 2b) further tests were done to assess the growth of the mixed culture SLPB on 2,5-thiophenedicarboxylic acid. The results in Figure 3 show that there were only small increases in the numbers of viable bacteria with each feeding of the dicarboxylic acid. Over the 7-day period, when the test culture consumed a total of 20 mg (0.12 mmol) of the dicarboxylic acid, the viable count increased only 6-fold. In contrast, the viable count in the control culture decreased over the 7-day period. At the end of the experiment, the viable count in culture that received the dicarboxylic acid was about 60-fold greater than that of the control culture. These results agree with those shown in Figure 2b, verifying low growth yields of this mixed culture on 2,5-thiophenedicarboxylic acid.

Approximately 15 different colonial morphologies were observed on the plates that were inoculated at the time the experiment summarized in Figure 3 started. During the repeated feedings of the dicarboxylic acid, there was a shift in the bacterial population, and four colonial types were predominant when the last plating was done. To date, a bacterium capable of degrading 2,5-thiophenedicarboxylic acid has not been isolated.

Detection of sulfate in bacterial cultures grown on 2,5-thiophenedicarboxylic acid

To improve the detection of sulfate that may result from the growth on 2,5-thiophenedicarboxylic acid, sulfate-free growth medium was used. However, sulfate was introduced into the medium with the SLPB inoculum that was grown on Prudhoe Bay crude oil in sulfate-containing medium. The 10-mL inoculum used in the 200 mL of sulfate-free medium was expected to give a sulfate concentration of about 0.7 mM.

Because of the observed inhibitory effects of elevated concentrations of 2,5-thiophenedicarboxylic acid, multiple feedings of small amounts substrate were used. After each 5-mg (0.029 mmol) feeding, the concentrations of the dicarboxylic acid in the 200-mL cultures were monitored by HPLC analyses, and when the substrate was depleted, more 2,5-thiophenedicarboxylic acid was added.

Table 1 summarizes the sulfate concentrations in the sterile control and the triplicate cultures grown on 2,5-thiophenedicarboxylic acid. Initially, the sulfate concentrations were determined by the turbidimetric method and the low values of the 'percent of theoretical maximum' were suspect. Thus, the samples were also analyzed by the ion chromatography method. For each sample, the ion chromatography method gave higher initial and final sulfate concentrations than the turbidimetric method. However, the size of differences between the final and initial concentrations, given as the 'Increase' in Table 1, was not dependent on the analytical method used. The sterile control showed a slight increase in sulfate concentration over the 15-day incubation period, and these values were used in the calculation of the 'Net increase' of sulfate concentration in the medium.

A total of 20 mg (0.116 mmol) of 2,5-thiophenedicarboxylic acid were added to each culture, and HPLC analyses showed that all of the acid was consumed by the bacteria. Thus the theoretical maximum increase of sulfate concentration was 0.58 mM, assuming all of the sulfur in the added 2,5-thiophenedicarboxylic acid was released as sulfate. The mean value from the turbidimetric method showed that 47% of the theoretical amount of sulfur was recovered as sulfate, and the results from the ion chromatography method gave a mean of 56%. These values were not significantly different based on a Student's t-test ($P<0.05$).

When this experiment was repeated, the mean amount of sulfate detected was 66% of the theoretical amount (range 50 to 80%). Results from the analy-

Table 1. Sulfate analyses of the liquid medium from the 200-mL mixed culture SLPB grown on 20 mg (0.116 mmol) of 2,5-thiophenedicarboxylic acid administered in four 5-mg portions in methanol solution over a 15-day incubation period. Two different analytical methods were used to measure sulfate concentrations.

Analytical method	Culture designation[a]	Sulfate concentration (mM)				% of Th. max.[c]
		Initial	Final	Increase	Net increase[b]	
Turbidimetric	Control[d]	0.76	0.85	0.09	e	e
	Culture 1	0.77	1.17	0.40	0.31	53
	Culture 2	0.78	1.12	0.34	0.25	43
	Culture 3	0.78	1.13	0.35	0.26	45
	Mean					47
Ion Chromatography	Control	1.08	1.14	0.06	e	e
	Culture 1	1.12	1.44	0.32	0.26	45
	Culture 2	1.09	1.44	0.35	0.29	50
	Culture 3	0.90	1.39	0.49	0.43	74
	Mean					56

[a]Triplicate cultures, designated 1, 2 and 3.
[b]Net increase = increased sulfate concentration in culture minus increased sulfate in the control.
[c]Th. max. = theoretical maximum increase in sulfate concentration if all of the sulfur from 2,5-thiophenedicarboxylic acid was converted to sulfate = 0.58 mM.
[d]Control culture was inoculated with SLPB, but received no 2,5- thiophenedicarboxylic acid.
[e]Not applicable.

ses of sulfur in the biomass produced from the growth of the mixed culture on 2,5-thiophenedicarboxylic acid showed that 3.5% of the substrate sulfur was associated with the washed cells.

Microbial metabolism of 2-(3,7-dimethyloctyl)-5-methylthiophene

Preliminary experiments used isolate SE in B+NP medium with 2- (3,7-dimethyloctyl)-5-methylthiophene and Prudhoe Bay crude oil. After 14 days of incubation, GC-FID analysis showed that all of the *n*-alkanes had been degraded, but pristane and phytane remained. These isoprenoidal hydrocarbons also remained after 28 days incubation. The extract from the SE culture was treated to yield methyl esters that were analyzed by GC-MS. Peaks with mass spectra corresponding to the methyl ester of 5-methyl-2-thiopheneacetic acid (mass spectrum shown in Fedorak & Peakman 1992) and the dimethyl ester of 8-(5-carboxy-2-thienyl)-2,6-dimethyloctanoic acid were observed. The mass spectrum of the latter derivatized metabolite (designated compound I) is shown in Figure 4. Chemical ionization GC-MS with ammonia yielded a $(M+1)^+$ ion at m/z 327, confirming the molecular weight of 326, which gave a very weak M^+ ion (Figure 4). Cleavage beta to the thiophene ring, which is characteristic of dialkyl thiophenes (Sinninghe Damsté et al. 1987; Peakman & Kock-van Dalen 1990) would yield the base peak m/z 155 (Figure 4).

Pirnik *et al.* (1974) found that omega-oxidation yielding dicarboxylic acid intermediates plays a key role in the metabolism of the isoprenoid pristane. The detection of compound I was the first evidence of omega-oxidation of the isoprenoidal thiophenes. However, in the pure culture study with isolate SE, this dicarboxylic acid persisted over the 28-day incubation period.

To determine whether other dicarboxylic acids could be detected as metabolites from 2-(3,7-dimethyloctyl)-5-methylthiophene, the mixed culture SLPB was incubated in B+NP medium supplemented with pristane and the isoprenoidal thiophene. After 3 days of incubation, the culture was freeze-dried and the residue reacted to yield methyl esters of any carboxylic acids that were formed. The methyl esters of four metabolites were detected when the culture extract was analyzed by GC-MS. These were designated compounds II to V, and their mass spectra are summarized in Table 2, and their structures are given in Figure 5. The mass spectra of compounds II and III show the characteristic base peak at m/z 111, from the cleavage beta to the thiophene ring. The presence of this base peak indicates that the methyl group is

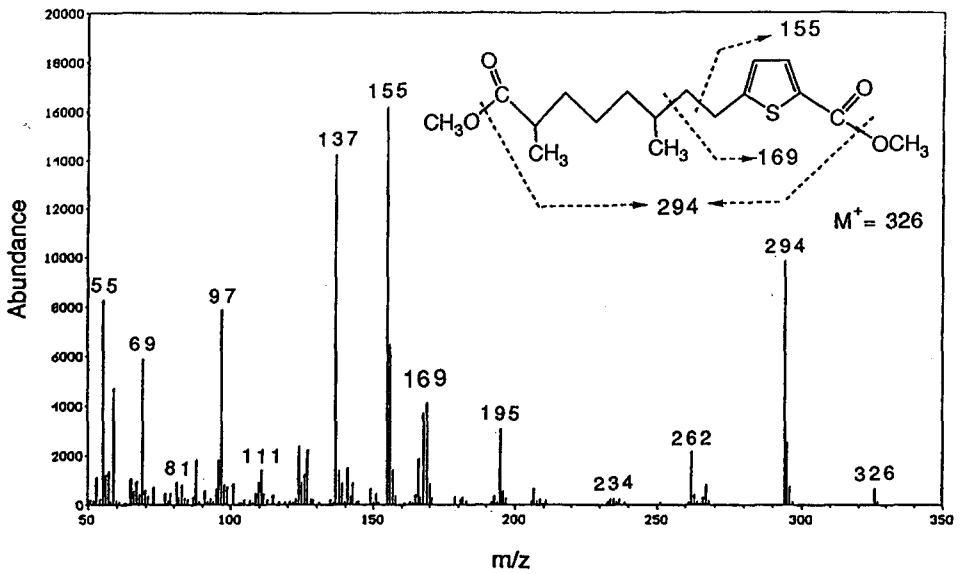

Figure 4. Mass spectrum and proposed structure of a dimethyl ester of a metabolite (compound I) produced by isolate SE grown on Prudhoe Bay crude oil in the presence of 2-(3,7-dimethyloctyl)-5-methylthiophene.

not oxidized. Thus, these two metabolites arose from the oxidation of the isoprenoidal side chain yielding monocarboxylic acids with a methyl group on the thiophene ring (Figure 5). In contrast, the mass spectra of compounds IV and V have abundant ions at m/z 155 (Table 2), which is characteristic of cleavage beta to the ring of a thiophenecarboxylic acid methyl ester. The molecular weights of these metabolites are consistent with the methyl esters of the dicarboxylic acids shown in Figure 5. Therefore, these results demonstrate that both 5-methyl and the isoprenoidal chain of 2-(3,7-dimethyloctyl)-5-methylthiophene were oxidized yielding dicarboxylic acids. However, no 2,5-thiophenedicarboxylic acid was detected in this extract.

Microbial metabolism of 2,5-diundecylthiophene

The metabolism of 2,5-diundecylthiophene, one of the 'mid-chain' 2,5-dialkylthiophenes, was studied in greater detail. It was hypothesized that the C_{11} alkyl side chains would be susceptible to microbial attack, and that these would be removed by a series of beta-oxidations. If this occurred, 2,5-thiophenedicarboxylic acid should be a key intermediate that would undergo further metabolism leading to the release of the sulfur atom that could be detected as sulfate in the medium.

The mixed culture SLPB was used for these investigations. Initially, cultures were incubated for 14 days with a variety of substrate combinations. No trace of 2,5-diundecylthiophene was detected in the extract from the culture that received 5 mg (0.013 mmol) of this compound at the time of inoculation. Thus, the mixed culture bacterial population could attack this compound. Similarly, no residual 2,5-diundecylthiophene was found by the GC analysis of the extract of the culture that initially contained a mixture 2,5-diundecylthiophene and 2,5-thiophenedicarboxylic acid. At several times during the 14-day incubation period, samples were removed from this culture for HPLC analyses to measure the amount of 2,5-thiophenedicarboxylic acid remaining. No loss of this substrate was observed during the first 3 days of incubation, however, after 6 days of incubation, no 2,5-thiophenedicarboxylic acid could be detected in the culture.

Figure 6 shows the chromatograms from the GC-FID analyses of the extract of the culture that originally contained 2,5-diundecylthiophene and Prudhoe Bay crude oil, and that of the corresponding sterile control. *n*-Hexadecane was added as an internal standard. Figure 6b clearly shows the removal of 2,5-diundecylthiophene and the *n*-alkanes from the oil by the microbial action during the 14-day incubation period.

In a subsequent experiment, 2,5-diundecylthiophene served as the sole carbon source for the SLPB

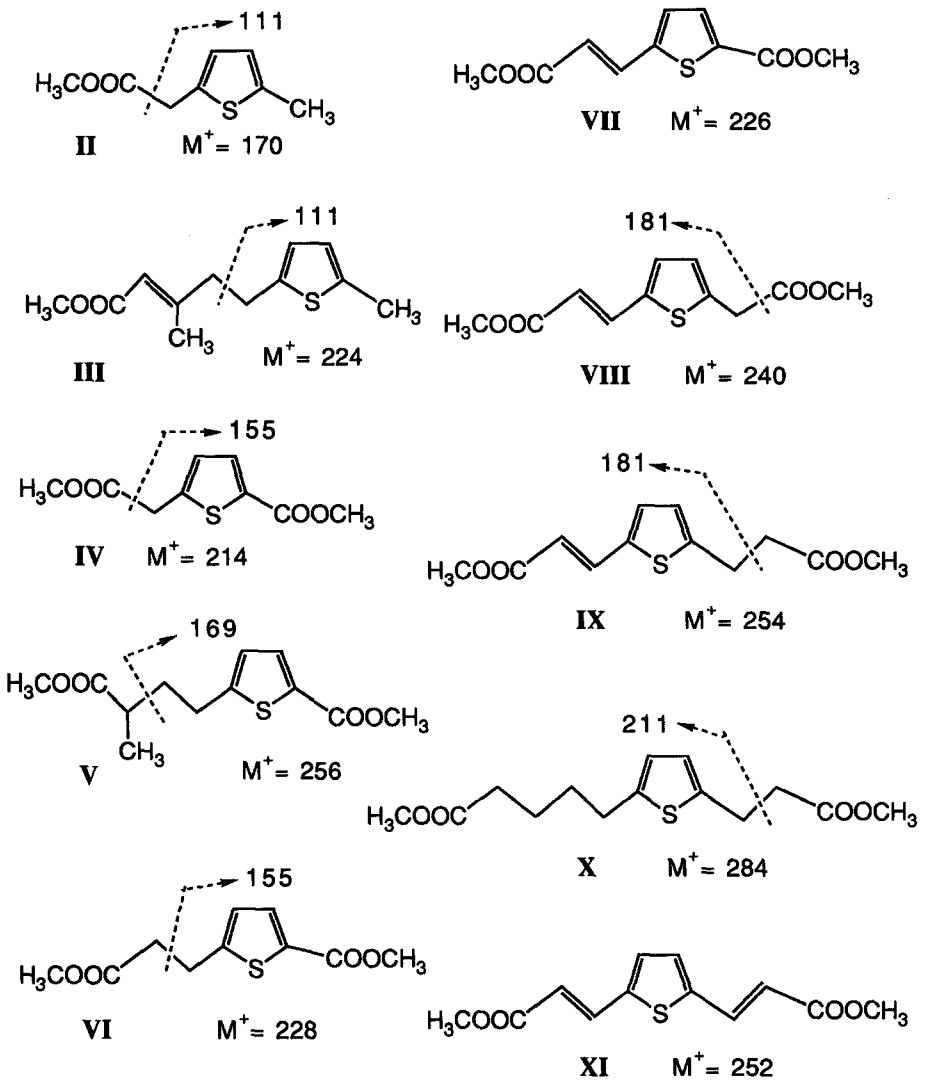

Figure 5. Structures of the methyl esters of metabolites found during this study. Cleavages yielding base peaks and their mass/charge ratios are shown. For those compounds with no fragmentation shown, the $(M- OCH_3)^+$ ions were the major fragment ions in the mass spectra.

mixed population. Replicate cultures were incubated for various lengths of time, and these were freeze-dried and the residue reacted to yield methyl esters of any carboxylic acids. GC-FID analyses showed that the amount of 2,5-diundecylthiophene in these cultures decreased with incubation time, and by day 12, the substrate could not be detected. GC-FPD analysis showed that in the extracts from the day 5, and day 7 cultures, there were about eight major sulfur-containing metabolites. By day 19, only one metabolite was abundant in the GC-FPD chromatogram. This metabolite corresponded to compound VII that was identified by GC-MS (see below).

The extract from the 7-day-old culture was analyzed by GC-MS and the methyl esters of seven thiophene-containing dicarboxylic acids were identified (compounds IV, and VI to XI in Table 2). The structures and some fragmentation patterns are shown in Figure 5. These compounds ranged in molecular weights from 214 (compound IV) to 284 (compound X). Some fragmentations were common. For example, the alpha-cleavage of a methyl ester resulting in loss

Table 2. Summary of the metabolites identifed by GC-MS analyses of extracts of cultures incubated with 2-(3,7-dimethyloctyl)-5-methylthiophene (substrate A) or 2,5-diundecylthiophene (substrate B).

Compound[a]	Substrate	RRT[b]	m/z of major ions from methyl esters (abundance, %)	Identification of methyl ester	Identification of metabolite
II	A	0.43[c]	170(M^+,19), 113(6), 112(8), 111(100), 59(8), 57(7)	Methyl 5-methyl-2-thiopheneacetate	5-Methyl-2-thiopheneacetic acid
III	A	0.83[c]	224(M^+,10), 113(6), 112(17), 111(100), 57(5)	Methyl 5-methyl-2-thiophene-3'-methyl-pent-3'-enoate	3-Methyl-5-(5-methyl-2-thienyl)pent-2-enoic acid
IV	A	0.95[c]	214(M^+,38), 183(16), 156(9), 155(100), 127(14), 96(14), 95(8), 59(9)	Methyl 5-methoxycarbonyl-2-thiophene-ethanoate	5-Carboxy-2-thiopheneacetic acid
	B	2.45[d]			
V	A	1.28[c]	256(M^+,23), 225(16), 169(100), 168(33), 155(39), 137(40), 88(54)	Methyl 5-methoxycarbonyl-2-thiophene-3'-methylbutanoate	4-(5-Carboxy-2-thienyl)-2-methyl-butanoic acid
VI	B	2.83[d]	228(M^+,51), 197(25), 169(26), 168(100), 155(90), 137(52), 127(21), 59(19)	Methyl 5-methoxycarbonyl-2-thiophene-propanoate	5-Carboxy-2-thiophenepropanoic acid
VII	B	3.12[d]	226(M^+,87), 196(13), 195(100), 167(19), 135(14), 108(13), 82(11), 63(11), 58(13)	Methyl 5-methoxycarbonyl-2-thiophene-propenoate	5-Carboxy-2-thiophenepropenoic acid
VIII	B	3.47[d]	240(M^+,51), 209(17), 182(11), 181(100), 122(16), 121(47)	Methyl 5-methoxycarbonylmethyl-2-thiophenepropenoate	5-Carboxymethyl-2-thiophenepropenoic acid
IX	B	3.82[d]	254(M^+,82), 223(30), 222(18), 194(61), 181(100), 163(64), 135(33), 121(46)	Methyl 5-methoxycarbonylethyl-2-thiophene-propenoate	5-Carboxyethyl-2-thiophenepropenoic acid
X	B	4.03[d]	284(M^+,20), 253(13), 212(12), 211(100), 195(8), 165(8), 151(7)	Methyl 5-methoxycarbonylethyl-2-thiophene-pentanoate	5-Carboxyethyl-2-thiophenepentanoic acid
XI	B	4.15[d]	252(M^+,100), 222(12), 221(93), 194(11), 161(13), 134(13), 133(26), 96(13)	Dimethyl 2,5-thiophene-dipropenoate	2,5-Thiophene-dipropenoic acid

[a]See Figure 5 for structures.
[b]RRT=relative retention time.
[c]relative to substrate A.
[d]Relative to methyl 5-methyl-2-thiopheneacetate, a reference compound added to the sample.

of OCH_3 giving the $(M-31)^+$ ion. Cleavage beta to the thiophene ring is commonly observed in substituted thiophenes (Fedorak & Peakman 1992; Sinninghe Damsté et al. 1986). This cleavage yielded the m/z 155 ion from compounds IV and VI; the m/z 181 ion from compounds VIII and IX; and the m/z 211 ion from compound X (see Table 2). Major ions at m/z 168 from compound VI; and at m/z 194 from compound IX are complimentary to the base peak at m/z 104 observed in the mass spectrum of benzenepropanoic acid methyl ester (NIST data base of mass spectra. Spectrum no. 21468).

The microbial degradation of the side chains of 2,5-diundecylthiophene likely occurs by beta-oxidation. During this oxidation process, the action of an acyl dehydrogenase oxidizes a saturated intermediate to give an unsaturated intermediate (Mahler & Cordes 1966). The proposed structures for the compounds VII, VIII, IX and XI (Figure 5) have double bonds. The locations of the double bonds were surmised by the fragmentation patterns and the fact that the biochemical beta-oxidation pathway yields α,β-unsaturated carboxylic acids. The detection of several unsaturated metabolites is consistent with the metabolism of the dialkylthiophene via beta-oxidation.

These results clearly showed that both alkyl chains of 2,5-diundecylthiophene were oxidized yielding a variety of dicarboxylic acids as intermediates. Careful

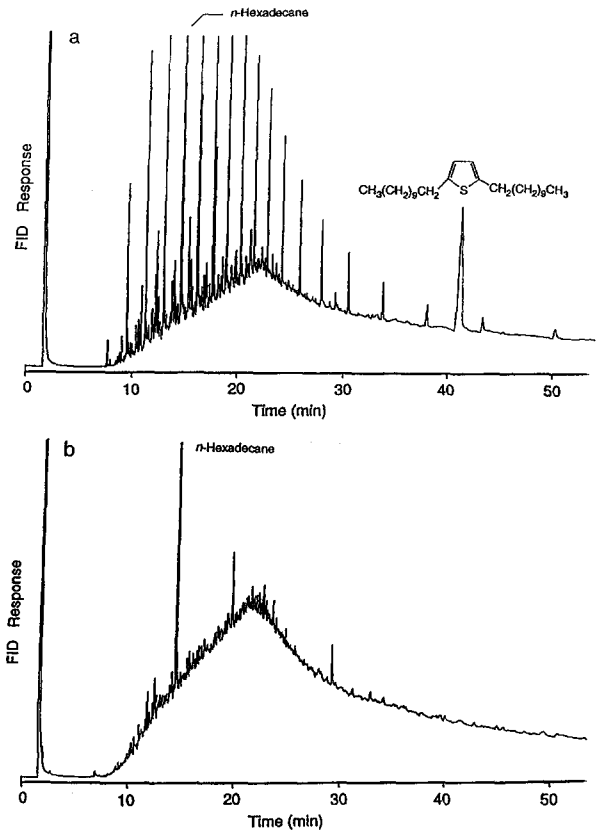

Figure 6. GC-FID analyses of the extract of a sterile control containing 5 mg (0.013 mmol) of 2,5-diundecylthiophene and 0.2 mL of Prudhoe Bay crude oil after 14-days incubation (a), and of the extract from the mixed culture SLPB incubated under the same conditions (b). *n*- Hexadecane was added as an internal standard.

scrutiny of the GC-MS data showed that a trace amount of the dimethyl ester of 2,5-thiophenedicarboxylic acid was present. The mass spectrum showed M^+ at m/z 200 and base peak at m/z 169 $(M\text{-}OCH_3)^+$.

To test for the release of sulfate from 2,5-diundecylthiophene, the mixed culture SLPB was inoculated into three flasks of sulfate-free medium with the dialkylthiophene as the sole carbon and energy source. At various times during the 35-day incubation of this culture, samples were removed for HPLC analyses that showed the appearance and subsequent loss of a metabolite that had the same retention time as 2,5-thiophenedicarboxylic acid. At the end of the incubation period, samples were analyzed for sulfate using the ion chromatography system. The final mean sulfate concentration in the viable cultures was 0.17 mM above that in the sterile controls. If all of the sulfur in the 2,5-diundecylthiophene had been converted to sulfate, the theoretical increase would have been 0.46 mM. Thus, the amount of sulfate detected was 37% of the stoichiometric amount, and these results indicate that cleavage of the thiophene ring occurred, with the subsequent release of the sulfur atom that was found as sulfate.

Discussion

These investigations showed that 2,5-thiophenedicarboxylic acid is a key intermediate in the metabolism of some 2,5-dialkylthiophenes by mixed cultures of oil-degrading bacteria. This acid was formed from the oxidation of the methyl group of 5-methyl-2-thiophenecarboxylic acid, which was identified as a metabolite in pure culture studies (Fedorak & Peakman 1992). However, we have not been able to isolate a pure culture of bacteria that will grow on 5-methyl-2-thiophenecarboxylic acid. 2,5-Thiophenedicarboxylic acid was also found as an intermediate in the degradation of 2,5-diundecylthiophene by the mixed culture SLPB. A thorough search of the literature failed to find any previous studies on the microbial metabolism of 2,5-thiophenedicarboxylic acid.

Kanagawa & Kelly (1987) used enrichment culture methods to isolate two strains of *Rhodococcus* that grew on 5-methyl-2-thiophenecarboxylic acid. They observed that a decrease in the pH of the medium accompanied the growth on this substrate, but they did not report on the identities of any metabolites, so it is not known whether the *Rhodococcus* spp. oxidized the methyl group to give 2,5-thiophenedicarboxylic acid.

The onset of 2,5-thiophenedicarboxylic acid degradation was delayed when this acid was present at 100 mg/L (0.58 mM). Kanagawa & Kelly (1987) routinely grew their *Rhodococcus* isolates on 200 mg of 2-thiophenecarboxylic acid/L (1.6 mM), and they observed a delay in growth when higher concentrations of the acid were used in the medium. Similarly, Abdulrashid & Clark (1987) observed that their thiophene-degrading mutants of *Escherichia coli* were sensitive to elevated concentrations of 2-thiophenecarboxylic acid. For example, one strain grew weakly on succinate in the presence of 500 mg of 2-thiophenecarboxylic acid/L (3.9 mM) but it did not grow in the presence of 1,000 mg of the thiophene-containing acid/L (7.8 mM). Evans & Venables (1990) typically grew their *Vibrio* YC1 on 4 to 6 mM 2-thiophenecarboxylic acid, and found that this substrate was strongly inhibitory

at 9 mM. Our SLPB mixed culture was more sensitive to elevated 2,5-thiophenedicarboxylic acid concentrations than the pure cultures of other investigators grown on 2-thiophenecarboxylic acid.

2,5-Thiophenedicarboxylic acid served as a growth substrate for the mixed culture SLPB. However, the final OD_{600} values of our cultures were low, and these were limited by the inhibitory nature of the dicarboxylic acid. At the maximum concentration studied in a batch culture (0.58 mM), the final OD was measured to be 0.07. Using these data, the ratio of the final OD to initial substrate concentration is 0.12 OD units/mM. From their studies with 2-thiophenecarboxylic acid, Kanagawa & Kelly (1987) and Evans & Venables (1990) presented growth as OD readings, allowing calculations of final OD to initial substrate concentration ratios, for comparison with our results with the dicarboxylic acid. From an initial concentration of 4.5 mM 2-thiophenecarboxylic acid, Kanagawa & Kelly (1987) observed a final OD of 0.3, giving a ratio of 0.07 OD units/mM. Evans & Venables (1990) reported final OD readings of about 0.8 when their pure culture grew in medium containing 4 to 6 mM 2-thiophenecarboxylic acid. These data give a range of ratios between 0.13 and 0.2 OD units/mM. Based on these comparisons, the growth yield of the mixed culture SLPB on 2,5-thiophenedicarboxylic acid is similar to the growth yields of pure cultures on 2-thiophenecarboxylic acid.

Four metabolites were detected from 2-(3,7-dimethyloctyl)-5-methylthiophene (compounds II to V, Table 2), and seven metabolites were detected from 2,5-diundecylthiophene (compounds IV, and VI to XI, Table 2). However, this was not an exhaustive study to detect all of the metabolites from the oxidation of these two 2,5-dialkylthiophenes, rather several metabolites were detected and identified to ascertain the mode of attack on the alkyl groups. Dicarboxylic acids that contained the thiophene ring were detected from these two substrates, indicating that both alkyl groups in each molecule were susceptible to oxidation. The formation of compound I (Figure 4) indicates that in some cases, both terminal methyl groups in 2-(3,7-dimethyloctyl)-5-methylthiophene are oxidized prior to the removal of any of the alkyl carbon atoms. However, the detection of compounds II and III (Figure 5) shows that in some cases, removal of alkyl carbon atoms from the isoprenoidal group preceded oxidation of the methyl group. During the metabolism of 2,5-diundecylthiophene, both side-chains were degraded simultaneously, as indicated by the detection of seven dicarboxylic acids (Table 2 and Figure 5). Only trace amounts of 2,5-thiophenedicarboxylic acid were found from 2,5-diundecylthiophene, suggesting that it was rapidly metabolized by the mixed culture SLPB.

Hutton & Steinberg (1973) studied the degradation pathway for the isoprenoidal compound, phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), in rat liver. They demonstrated the release of [^{14}C]propionate from [U-^{14}C]phytanic acid, verifying that the isoprenoidal degradation occurred by successive beta-oxidative cleavages with alternate release of C_3 and C_2 fragments. In our study, the release of a C_3 fragment during the microbial metabolism of the isoprenoidal thiophene, 2-(3,7-dimethyloctyl)-5-methylthiophene, was also observed. The molecular weights of two metabolites, 4-(5-carboxy-2-thienyl)-2-methylbutanoic and 5-carboxy-2-thiopheneacetic acids (shown in Figure 5 as their methyl esters, compounds V and IV, respectively), differ by 42 mass units, equivalent to three methylene groups. This is consistent with the removal of propionic acid from 4-(5-carboxy-2-thienyl)-2-methylbutanoic acid and the oxidation of its gamma-carbon atom to a carboxylic acid, yielding 5-carboxy-2-thiopheneacetic acid. Pirnik et al. (1974) demonstrated the alternate release of C_3 and C_2 fragments from pristane by *Brevibacterium erythrogenes*.

The removal of C_2 units during the degradation of the *n*-alkyl side chains of 2,5-diundecylthiophene was apparent among some of the metabolites observed. For example, there is a difference of 28 mass units between 5-carboxyethyl-2-thiophenepropenoic and 5-carboxy-2-thiophenepropenoic acids (shown in Figure 5 as their methyl esters, compounds IX and VII, respectively). Double this difference (56 mass units) is observed between 5-carboxyethyl-2-thiophenepentanoic and 5-carboxy-2-thiophenepropanoic acids (shown in Figure 5 as their methyl esters, compounds X and VI, respectively), consistent with two beta-oxidations during side chain degradation. It is not known whether the *n*-alkyl side chains of 2,5-diundecylthiophene were initially attacked by terminal or subterminal oxidations (Atlas & Bartha 1992). In either case, carboxylic acids containing the thiophene ring would be produced, and these would be metabolized further by beta- oxidation.

The mixed culture SLPB contains a diverse bacterial population that is capable of metabolizing a wide variety of compounds in petroleum including *n*-alkanes (Figure 6), di- and tricyclic aromatic hydrocarbons (Fedorak & Coy, unpublished data) and dibenzothiophenes (Fedorak & Peakman 1992). It is hypothesized that alkane-degrading bacteria in the mixed culture remove the alkyl side

chains from 2,5-diundecylthiophene, leaving 2,5-thiophenedicarboxylic acid or some of its homologues to be consumed by other bacteria. This hypothesis is consistent with the pure culture studies of Fedorak & Peakman (1992) that showed the accumulation of 5-methyl-2-thiophenecarboxylic and 5-methyl-2-thiopheneacetic acids from 2-hexadecyl-5-methylthiophene. The current study provides evidence that the alkane-degrading isolate SE could not metabolize 2,5-thiophen-dicarboxylic acid over a 28-day incubation time. Further work to test this hypothesis awaits the isolation of a bacterium that uses 2,5-thiophenedicarboxylic acid as its growth substrate.

The methyl group on the thiophene ring in 2-(3,7-dimethyloctyl)-5-methylthiophene appears to be more difficult to oxidize than the longer side chain, because some monocarboxylic acids were detected as metabolites of the isoprenoidal thiophene. Similarly, the methyl group in 5-methyl-2-thiophenecarboxylic acid was quite resistant to oxidation. However, its oxidation yielding 2,5-thiophenedicarboxylic acid in a mixed bacterial culture was demonstrated.

Kanagawa & Kelly (1987) observed stoichiometric production of sulfate from 2-thiophenecarboxylic acid. Similarly, growing cultures of Organism R1 (Cripps 1973) converted about 90% of the sulfur in 2-thiophenecarboxylic acid to sulfate, whereas resting cell suspensions of this bacterium effected quantitative conversion. Cripps (1973) provided evidence that the sulfur atom was released as sulfide, which was subsequently oxidized to thiosulfate and sulfate by Organism R1.

Stoichiometric formation of sulfate was not observed in the mixed culture experiments with 2,5-thiophenedicarboxylic acid or 2,5-diundecylthiophene. Typically about 50% of the sulfur in the dicarboxylic acid was detected as sulfate (Table 1) cultures of SLPB, whereas only 37% of the sulfur from 2,5-diundecylthiophene was detected as sulfate. The fate of the remaining sulfur is unknown, but the detection of increased sulfate concentrations in the medium clearly demonstrated that cleavage of the thiophene ring occurred in the mixed cultures of SLPB.

The specific removal of the sulfur atom from dibenzothiophene has been studied extensively as a model for the biodesulfurization of fossil fuels (Monticello 1993; Krawiec 1990). For example, van Afferden et al. (1990) reported that a *Brevibacterium* sp., growing on dibenzothiophene as its sole source of carbon, sulfur and energy, released sulfite in a stoichiometric amount, and this ion was further oxidized to sulfate. In contrast, a *Corynebacterium* sp. metabolized dibenzothiophene to its sulfoxide, sulfone and 2-hydroxybiphenyl, but the amount of sulfate detected was only about 15% of the stoichiometric amount (Omori et al. 1992). Those authors concluded that the small amount of sulfate released from dibenzothiophene indicated that it was effectively utilized by their bacterial strain.

The mechanism by which the ring of 2,5-thiophenedicarboxylic acid is attacked by the bacteria in the mixed culture SLPB is unknown at this time. Cripps (1973) demonstrated that 2-thiophenecarboxylic acid was cleaved between C-5 and the sulfur atom, which was released as sulfide, yielding 2-oxoglutarate. Alternatively, it is possible the sulfur atom is oxidized before ring cleavage as was observed for dibenzothiophene (van Afferden et al. 1990; Omori et al. 1992).

This investigation showed that both of the alkyl groups in two 2,5-dialkylthiophenes were oxidized to dicarboxylic acids that are too polar to be extracted by the method used by Fedorak & Peakman (1992), thereby explaining why these metabolites were not detected in that earlier study. In addition, these finding suggest that 2,5-dialkylthiophenes which may be present in petroleum-contaminated environments should be quite susceptible to biodegradation with the release of some of the sulfur from the thiophene ring as sulfate.

Acknowledgments

Funding for these studies was provided by Environment Canada, through the Groundwater and Soil Remediation Program, contract KA168-2-2191; by the Canadian Petroleum Products Institute; and by the Natural Sciences and Engineering Research Council of Canada. We thank L. Harrower and K.G. Kropp for GC-MS analyses and S. Kenefick for ion chromatography analyses.

References

Abdulrashid N & Clark DP (1987) Isolation and genetic analysis of mutations allowing the degradation of furans and thiophenes by *Escherichia coli*. J. Bacteriol. 169: 1267–1271

Andersson JT & Bobinger S (1992) Polycyclic aromatic sulfur heterocycles. II. Photochemical oxidation of benzo[b]thiophene in aqueous solution. Chemosphere. 24: 383–388

APHA (1989) Standard Methods for the Examination of Water and Wastewater, 17th Edition. American Public Health Association, Washington, DC

Atlas RM & Bartha R (1992) Microbial Ecology: Fundamentals and Applications, 3rd Edition. Benjamin/Cummings Publishing Company, Inc. Redwood City, CA

Cripps RE (1973) The microbial metabolism of thiophen–2- carboxylate. Biochem. J. 134: 353–366

Evans JS & Venables WA (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

Fedorak PM & Grbić-Galić D (1991) Aerobic microbial cometabolism of benzothiophene and 3- methylbenzothiophene. Appl. Environ. Microbiol. 57: 932–940

Fedorak PM & Peakman TM (1992) Aerobic microbial degradation of some alkylthiophenes found in petroleum. Biodegradation. 2: 223–236

Fedorak PM & Westlake DWS (1983) Selective degradation of biphenyl and methylbiphenyls in crude oil by two strains of marine bacteria. Can. J. Microbiol. 29: 497–503

Fedorak PM & Westlake DWS (1984) Degradation of sulfur heterocycles in Prudhoe Bay crude oil by soil enrichments. Water, Air Soil Pollut. 21: 225–230

Fedorak PM & Westlake DWS (1986) Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. 51: 435–437

Foght JM, Fedorak PM & Westlake DWS (1990) Mineralization of ^{14}C-hexadecane and ^{14}C-phenanthrene in crude oil: Specificity among bacterial isolates. Can. J. Microbiol. 36: 169–175

Hutton D & Steinberg D (1973) Identification of propionate as a degradation product of phytanic acid oxidation in rat and human tissue. J. Biol. Chem. 248: 6871–6875

Kanagawa T & Kelly DP (1987) Degradation of substituted thiophenes by bacteria isolated from activated sludge. Microb. Ecol. 13: 47–57

Krawiec S (1990) Bacterial desulfurization of thiophenes: screening techniques and some speculations regarding the biochemical and genetic basis. Dev. Ind. Microbiol. 31: 103–114

Mahler HR & Cordes EH (1966) Biological Chemistry. Harper and Row, New York, NY

Monticello DJ (1993) Biocatalytic desulfurization of petroleum and middle distillates. Environ. Prog. 12: 1–4

Omori T, Monna L, Saiki Y & Kodama T (1992) Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1. Appl. Environ. Microbiol. 58: 911–915

Peakman TM & Kock-van Dalen AC (1990) Identification of alkylthiophenes occurring in the geosphere by synthesis of authentic standards. In: Orr WL & White CM (Eds.) Geochemistry of Sulfur in Fossil Fuels (pp 397–416). ACS Books, Washington, DC

Pirnik MP, Atlas RM & Bartha R (1974) Hydrocarbon metabolism by *Brevibacterium erythrogenes*: Normal and branched alkanes. J. Bacteriol. 119: 868–878

Sinninghe Damsté JS, ten Haven HL, de Leeuw JW & Schenck PA (1986) Organic geochemical studies of a Messinian evaporitic basin, northern Apennines (Italy). II. Isoprenoid and *n*- alkyl thiophenes and thiolanes. Org. Geochem. 10: 791–805

Sinninghe Damsté JS, de Leeuw JW, Kock-van Dalen AC, de Zeeuw MA, de Lange F, Rijpstra WIC & Schenck PA (1987) The occurrence and identification of series of organic sulphur compounds in oils and sediments. I. A study of Rozel Point oil (U.S.A.) Geochim. Cosmochim. Acta 51: 2369–2391

Sinninghe Damsté JS & de Leeuw JW (1989) Analysis, structure and geochemical significance of organically-bound sulphur in the geosphere: State of the art and future research. Org. Geochem. 16: 1077–1101.

Sinninghe Damsté JS, Rijpstra WIC, de Leeuw JW & Schenck PA (1989) The occurrence and identification of series of organic sulphur compounds in oils and sediment extracts. II. Their presence in the samples from hypersaline and non-hypersaline depositional environments and possible application as source, maturity and palaeoenvironmental indicators. Geochim. Cosmochim. Acta. 53: 1323–1341

Tanaka H, Maeda H, Suzuki H, Kamibayashi A & Tonomura K (1982) Metabolism of thiophene–2-carboxylate by a photosynthetic bacterium. Agric. Biol. Chem. 46: 1429–1438

van Afferden M, Schacht S, Klein J & Trüper HG (1990) Degradation of dibenzothiophene by *Brevibacterium* sp. DO. Arch. Microbiol. 153: 324–328