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Aerobic microbial metabolism of some alkylthiophenes found in petroleum

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Abstract

Six alkylthiophenes, 2-hexadecyl-5-methylthiophene (I), 2-methyl-5-tridecylthiophene (II) and 2-butyl-5-tridecylthiophene (III), 2-(3,7-dimethyloctyl)-5-methylthiophene (IV), 2-methyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene (V) and 2-ethyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene (VI) were synthesized and used as substrates in biodegradation studies. The products of their aerobic metabolism by pure bacterial cultures were identified. In most cases, the long alkyl chains of these thiophenes were preferentially attacked and in pure cultures of alkane-degrading bacteria, the major metabolites that accumulated in the medium were 5-methyl-2-thiopheneacetic acid from (I), 5-methyl-2-thiophenecarboxylic acid from (II) and occasionally from (V), 5-butyl-2-thiophenecarboxylic acid from (III) and 5-ethyl-2-thiopheneacetic acid from (VI). These transformations are consistent with the metabolism of the alkyl side chains via the beta-oxidation pathway. In contrast, 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid was produced from (IV). Because it was available in greatest supply, (I) was studied most thoroughly. It supported growth of the six *n*-alkane-degrading bacteria tested and (I) was degraded more quickly than pristane but not as quickly as *n*-hexadecane in mixtures of these three compounds. In the presence of Prudhoe Bay crude oil and a mixed culture of petroleum-degrading bacteria, the acid metabolites from (I), (II) and (III) underwent further biotransformations to products that were not detected by the analytical methods used. The addition of *n*-hexadecane to the mixed culture of petroleum-degrading bacteria also enhanced the further biotransformations of the metabolites from (I).

Introduction

A recent review (Fedorak 1990) demonstrated that relatively little is known about the microbial metabolism of organosulfur compounds in petroleum. Although hundreds of organic sulfur compounds have been identified in petroleum (Cyr et al. 1986; Payzant et al. 1986; Rall et al. 1972; Sinninghe Damsté et al. 1986, 1987), fewer than twenty of these have been subjected to biodegradation studies (Fedorak 1990) because so few petroleum-related organosulfur compounds are commercially available. Thus there is much to be learned about the fate of organosulfur compounds in petroleum-contaminated environments and about the biotransformations of these compounds in petroleum reservoirs. In addition, although biodesulfurization has been suggested for the specific removal of sulfur compounds from fossil fuels, only a few model compounds have been used in studies attempting to demonstrate the applicability of this process (Foght et al. 1990a).

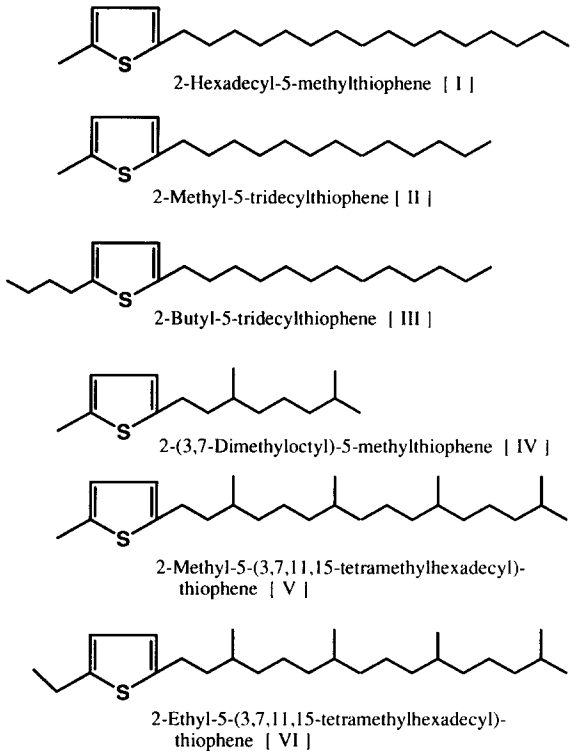

Fig. 1. Structures of the alkylthiophenes used in this study. The abbreviation for each compound is given in brackets.

Most studies on the biotransformations of individual petroleum-related organosulfur compounds have focussed on the condensed thiophenes including dibenzothiophene (Kargi & Robinson 1984; Kodama et al. 1973; Laborde & Gibson 1977; van Afferden et al. 1990), benzothiophene (Bohonos et al. 1977; Fedorak & Grbić-Galić 1991; Sagardía et al. 1975), and recently some alkylbenzothiophenes (Saftić et al. 1991).

Studies have shown that thiophene is resistant to biodegradation by aerobic microbial cultures whereas carboxythiophenes such as 2-thiophenecarboxylic, 3-thiophenecarboxylic, 2-thiopheneacetic and 5-methyl-2-thiophenecarboxylic acids are susceptible to microbial metabolism (Amphlett & Calley 1969; Cripps 1973; Evans & Venables 1990; Kanagawa & Kelly 1987). Based on oxygen uptake experiments, Sagardía et al. (1975) concluded that thiophene, 2-methylthiophene and 3-methylthiophene were degraded by a benzothiophene-adapted bacterial isolate. Fedorak et al. (1988) observed that the side chains of two *n*-alkyltetrahydrothiophenes (*n*-alkylthiolanes) were attacked by *n*-alkane-degrading bacteria and fungi.

A variety of alkylthiophenes have been observed recently in immature sediments, oil shales and crude oils (e.g. Sinninghe Damsté & de Leeuw 1990). These range from compounds with polycyclic and isoprenoidal to branched and linear carbon skeletons. Alkylthiophenes comprising a linear carbon skeleton, *n*-alkylthiophenes, have been observed in oil shales (Kohnen et al. 1990; Sinninghe Damsté et al. 1989) and crude oils (Sinninghe Damsté et al. 1989). Several classes have been identified extending from 2-alkyl-, 2-alkyl-5-methyl-, 2-alkyl-5-ethyl-, 2-alkyl-5-propyl- through to the so-called 'mid-chain' 2,5-dialkyl- thiophenes (Sinninghe Damsté et al. 1989).

Three *n*-alkylthiophenes and three isoprenoidal thiophenes (Fig. 1) were synthesized and used in the present study. These were added individually to pure cultures of *n*-alkane-degrading bacteria to determine whether they were biodegradable and the major metabolites were subsequently identified. In addition, some of the alkylthiophenes were added to a mixed culture of petroleum-degrading bacteria or to river water with Prudhoe Bay crude oil to assess whether these compounds were degraded in the presence of petroleum and to determine whether the metabolites underwent further biotransformations in a mixed culture.

Materials and methods

Chemicals and oil fractionation

The six alkylthiophenes used as substrates shown in Fig. 1 were synthesized using methods outlined by Peakman & Kock-van Dalen (1990). Compound I was available in the greatest quantity (700 mg) and therefore its biotransformation was studied most thoroughly. The amounts of the other alkyl thiophenes ranged from 58 to 380 mg.

5-Methylthiophene-2-carboxylic acid, heptanoic acid and Raney nickel (activity W-2) were all purchased from Aldrich (Milwaukee, WI). *n*-Hexade-

cane and *n*-tetradecane were purchased from Fisher (Fairlawn NJ) and Terrochem (Edmonton), respectively.

In some experiments, the saturated, aromatic and polar fractions of Prudhoe Bay crude oil were added to bacterial cultures. One-millilitre portions of crude oil were fractionated by silica gel chomatography as follows. A slurry of 35 g of silica gel (Fisher Scientific, 100–200 mesh, Type 150A, Grade 644, activated at 125 °C for 24 h) in methylene chloride was poured into a glass column (33 cm × 2.5 cm diameter) and the solvent was displaced with 250 mL of *n*-pentane. The crude oil was loaded onto the prepared column which was then developed with 25 mL of *n*-pentane, 25 mL of 20% methylene chloride in *n*-pentane, 125 mL of 50% methylene chloride in *n*-pentane and 150 mL of 50% methanol in benzene. The first 65-mL portion of column effluent was discarded because it contained no components from the crude oil. The next two 60-mL portions contained the saturated and aromatic fractions, respectively. The next 60 mL of effluent were discarded and the final 85 mL to elute contained the polar fraction.

Bacterial cultures and culture conditions

Six Gram-positive *n*-alkane-degrading bacteria isolated in our laboratory were used in this study. These strains were designated C1B, C1O, C1Y, C2, C3 (described by Fedorak at al. 1988) and SE (described Foght et al. 1990b). To prepare the inocula for liquid culture experiments, the isolates were grown on plate count agar (Difco, Detroit MI) at 28 °C for 2 or 3 days. Then the cell growth was aseptically washed from the plate with 3 mM phosphate buffer (pH 7.2) and the resulting cell suspension was used as inoculum.

All cultures were incubated for 14 days, unless otherwise noted, at 28 °C with shaking to provide aeration. During the initial studies with compound I, the isolates were inoculated into 125-mL Erlenmeyer flasks containing 50 mL of liquid mineral medium (Fedorak at al. 1988) supplemented with trace metals solution (Fedorak & Grbić-Galić 1991) and 5 μL of the *n*-alkylthiophene as the sole carbon and energy source. In pure-culture studies with compounds II and III, 10 μL of the *n*-alkylthiophene were added to 50 mL of medium.

Because of the small amounts of isoprenoidal thiophenes available, growth experiments used a mixture of *n*-tetradecane and the isoprenoidal thiophene of interest. The *n*-alkane served as the primary carbon and energy source in these cultures when transformations of compounds IV, V and VI were studied. The isoprenoidal thiophenes were dissolved in ether and portions of this solution were added to sterile 500-mL Erlenmeyer flasks. After the ether had evaporated, leaving about 5 μg of thiophene, 200 mL of sterile mineral medium supplemented with trace metals and 10 μL of *n*-tetradecane were added to each flask. These were inoculated with isolate SE or C2 and incubated for 14 days at 28 °C with shaking.

The susceptibility of compound I to microbial attack was compared to the susceptibilities of *n*-hexadecane and pristane. Isolates C2 or SE were inoculated into several 500-mL Erlenmeyer flasks that each contained 2 mg of *n*-hexadecane, 2 mg of pristane (2,6,10,14-tetramethylpentadecane), 2 mg of compound I and 200 mL of mineral medium supplemented with trace metals. After various times of incubation at 28 °C under shake-flask conditions, one of the replicate cultures was analyzed to determine the proportions of *n*-hexadecane, pristane and compound I remaining compared to appropriate sterile controls.

A mixed bacterial culture, designated SLPB, was also used. It was enriched from fuel-contaminated beach material from Shell Lake, Northwest Territories, Canada. This culture has been maintained since 1983 by monthly transfers to fresh medium containing Prudhoe Bay crude oil as the sole carbon source.

Two hundred millilitres of mineral medium supplemented with trace metals solution in 500-mL Erlenmeyer flasks containing 0.2-mL portions of Prudhoe Bay crude oil were inoculated with 20 mL of the mixed bacterial culture SLPB. Duplicate cultures were supplemented with 2 μL of compound I and two others were supplemented with 1 mg of 5-methyl-2-thiophenecarboxylic acid. A third pair of cultures received 2 μL of compound I

with no crude oil supplement. Appropriate sterile controls were incubated with the active cultures. The ability of the mixed culture SLPB to degrade compounds II and III in the absence or presence of Prudhoe Bay crude oil was also determined. In these experiments the cultures received 2 μL of either of these compounds in place of compound I.

To determine which components of the crude oil stimulated the biotransformation of the acidic metabolites of compounds I and II, Prudhoe Bay crude oil was fractionated into saturated, aromatic and polar fractions. Portions of these individual fractions, equivalent to the amounts of the corresponding fraction in 0.2 mL of crude oil, and combinations of these fractions were added to medium containing 2 μL of compound I and inoculated with the mixed culture SLPB.

From the results with the oil fractions, it was speculated that the addition of *n*-hexadecane may stimulate the biotransformation of the acidic metabolites of compound I. To test this hypothesis, eight flasks were prepared, each containing 200 mL of medium and 100 μL of *n*-hexadecane. Four of these received 2 μL of compound I and each of the remaining flasks received 1 mg of 5-methyl-2-thiophenecarboxylic acid. One-half of these flasks were inoculated with the mixed culture SLPB and the others remained as sterile controls. The cultures and controls were acidified and extracted after 15 and 28 days incubation. Just prior to extraction, 1 mg of benzoic acid was added to each flask as an internal standard.

North Saskatchewan River water was collected in Edmonton, upstream of the sewage treatment plant outfall but downstream of several storm sewer outfalls. Two hundred millilitre-portions of the river water were placed in 500-mL Erlenmeyer flasks and these were supplemented with 2 mL of NP solution (Fedorak & Westlake 1981), 2 μL of compound I and 0.2 mL of Prudhoe Bay crude oil. These cultures and suitable sterile controls were incubated at 28 °C with shaking. Each week for 4 weeks, one culture and a sterile control were acidified and extracted to recover the residual oil for GC analysis.

Analytical methods

After appropriate incubation times, the liquid cultures that received pure *n*-alkylthiophenes were acidified with sulfuric acid to pH <2 and extracted four times with 15 mL of methylene chloride to recover residual substrate and any metabolites that formed.

For the cultures that received crude oil and a *n*-alkylthiophene, an extraction method was designed to separate the acidic products from the oil. To do this, the liquid cultures were adjusted to pH 12 with a NaOH solution and 50 μL of a benzoic acid solution in methylene chloride was added to give 1 mg of benzoic acid as an internal standard in each culture. Each culture was extracted five times with 15 mL of methylene chloride and these extracts were pooled and analyzed by GC to determine the extent of the petroleum degradation. The aqueous phase was adjusted to pH <2 with sulfuric acid and this was extracted four times with 15 mL of methylene chloride. These extracts, which contained the internal standard and acid metabolites, were combined and analyzed by GC.

To ensure that the extraction method could recover sulfur-containing acid products in the presence of biodegraded oil, the medium with the mixed bacterial culture SLPB and Prudhoe Bay crude oil was incubated for 14 days to allow for oil degradation. The culture was then spiked with 1 mg of 5-methyl-2-thiophenecarboxylic acid just before the beginning of the extraction procedure. There was no difficulty quantitatively recovering the 5-methyl-2-thiophenecarboxylic acid.

For routine monitoring for sulfur-containing metabolites, culture extracts were analyzed by capillary GC (Fedorak & Grbić-Galić 1991) using an instrument fitted with a flame ionization detector (FID) and a sulfur-selective, flame photometric detector (FPD) (Fedorak & Westlake 1983a). The DB-5 capillary column effluent was split so that the sample was analyzed by both detectors simultaneously. The oven temperature program was 90 °C for 2 min, 4 °/min to 250 ° for 16 min. Selected extracts with sulfur-containing metabolites were analyzed by electron impact GC-MS (Fedorak & Westlake 1986) at the Mass Spectrometry Labora-

tory in the Chemistry Department at the University of Alberta. Methyl esters of the metabolites were prepared as outlined by Fedorak & Westlake (1983b).

Raney nickel desulfurization and reduction

Raney nickel was washed with distilled water, 95% ethanol and anhydrous ethanol (Furniss et al. 1989). Culture extracts, free of methylene chloride, were refluxed for 4 h with 0.1 g of Raney nickel in anhydrous ethanol. After cooling, the nickel was extracted with dilute alkali (Moziono et al. 1943). Then the solution was made acidic with sulfuric acid and extracted with methylene chloride. The GC retention time of the carboxylic acid resulting from the Raney nickel reaction was matched with that of the appropriate authentic standard.

Results

Metabolism of 2-hexadecyl-5-methylthiophene (compound I) by pure cultures

Initial studies focussed on the Gram-positive isolate C2 grown on compound I. GC analysis of the extract from a 28-day-old culture showed three sulfur-containing compounds. The third peak to elute from the column was residual compound I. The first sulfur-containing peak to elute had the same retention time as a sample of the commercially available compound 5-methyl-2-thiophenecarboxylic acid. In addition, the mass spectrum of the metabolite (Fig. 2a) was virtually identical to that of 5-methyl-2-thiophenecarboxylic acid.

The second sulfur-containing peak to elute from the GC column was the more abundant metabolite. Its mass spectrum showed a molecular ion at *m/z* 156 (Fig. 2b). The base peak was *m/z* 111 which corresponds to a loss of -COOH. A base peak at *m/z* 111 has been observed previously in the electron impact mass spectra of 2-alkyl-5-methylthiophenes resulting from the cleavage of the alkyl side chain beta to the thiophene ring (Payzant et al.

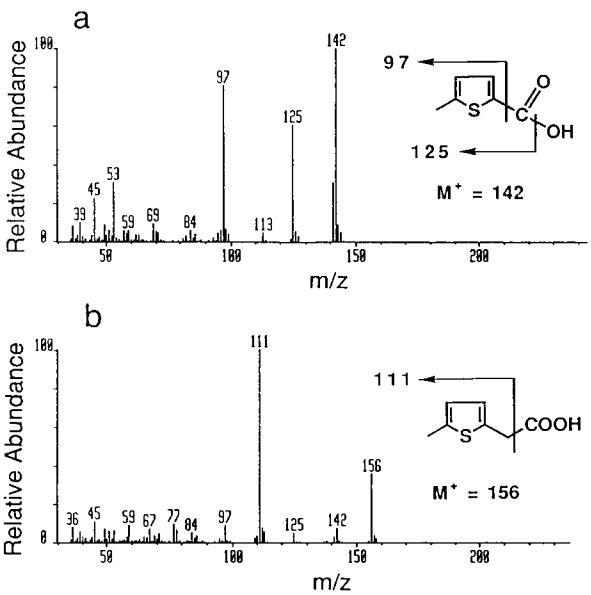

Fig. 2. Mass spectra obtained from the GC-MS analysis of the extract from isolate C2 grown on compound I. (a) Minor metabolite, 5-methyl-2-thiophenecarboxylic acid; (b) major metabolite, 5-methyl-2-thiopheneacetic acid.

1988; Sinninghe Damsté et al. 1986). These results suggested that the metabolite was 5-methyl-2-thiopheneacetic acid.

A portion of the culture extract from isolate C2 was treated to produce methyl esters of the carboxylic acids present. In addition to the residual substrate, two sulfur-containing compounds were observed upon GC analysis. The first had the same GC retention time and mass spectrum as the methyl ester made from authentic 5-methyl-2-thiophenecarboxylic acid. GC-MS analysis showed that the methyl ester of the second metabolite produced a parent ion at *m/z* 170 with a base peak at *m/z* 111. These results were consistent with the latter metabolite being 5-methyl-2-thiopheneacetic acid.

To verify that the more abundant metabolite was 5-methyl-2-thiopheneacetic acid, combined extracts from cultures of isolate SE grown on compound I were desulfurized and reduced with Raney nickel. This reaction was expected to yield heptanoic acid from 5-methyl-2-thiopheneacetic acid. Indeed, the product obtained from the Raney nick-

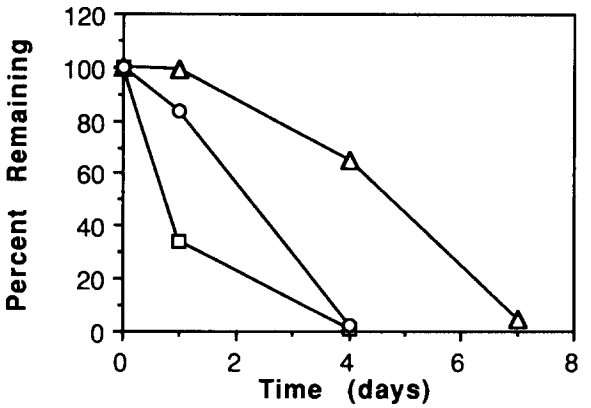

Fig. 3. Comparison of the relative susceptibilities of hexadecane □), pristane (Δ), and compound I (O) to biodegradation by isolate C2.

el reaction had the identical GC retention time as authentic heptanoic acid.

The other five *n*-alkane-degrading bacterial isolates were also able to use compound I as their sole carbon and energy source. Each isolate produced two sulfur-containing metabolites with the same GC retention times as those produced by isolate C2. GC-MS analyses of the extracts from isolates C1Y and SE showed that these metabolites were also 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid. The latter metabolite was more abundant than the former metabolite in culture extracts.

Figure 3 shows the results of the time course study with isolate C2 and the three different substrates, *n*-hexadecane, pristane and compound I. As expected from the literature (Blumer & Sass 1972; Fedorak & Westlake 1981; Jobson et al. 1974; Pirnik et al. 1974), *n*-hexadecane was degraded before pristane. *n*-Hexadecane was also degraded more quickly than compound I. However, compound I was consumed more quickly than pristane. Experiments with isolate SE also showed that compound I was consumed more quickly than pristane.

Metabolism of 2-methyl-5-tridecylthiophene (compound II) by pure cultures

Isolates SE and C2 were individually incubated for 14 days in liquid medium containing compound II

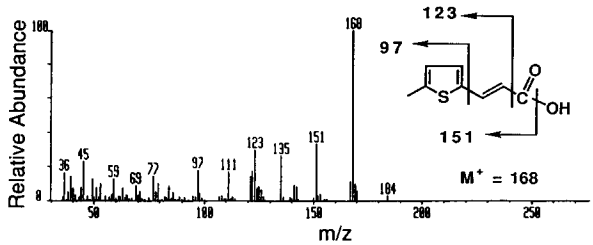

Fig. 4. Mass spectrum of one of the metabolites observed in the GC-MS analysis of the extract from isolate C2 grown on compound II. Identified as 5-methyl-2-thiophenepropenoic acid.

as the sole carbon and energy source. GC analysis of the extract from isolate SE showed that it metabolized virtually all of the substrate leaving one predominant sulfur-containing metabolite. GC-MS analysis and comparison with an authentic standard showed that the product was 5-methyl-2-thiophenecarboxylic acid. There was also a trace of another metabolite that was 5-methyl-2-thiopheneacetic acid.

Analysis of the extract from isolate C2 showed two major and one minor sulfur-containing metabolites. GC-MS analysis demonstrated that one of the major metabolites was 5-methyl-2-thiophenecarboxylic acid and that the minor metabolite was 5-methyl-2-thiopheneacetic acid. The parent peak of the other major metabolite was the molecular ion at *m/z* 168 (Fig. 4). Other observed ions and some proposed lost fragments were at *m/z* 151 (-OH), 123 (-COOH), and 97 (-CH=CHCOOH). The relative intensities of each of these ions were <40%. These results suggest that the metabolite was 5-methyl-2-thiophenepropenoic acid. The ion at *m/z* 111 was not abundant. This ion, which results from cleavage beta to the thiophene ring during electron impact mass spectrometry, is usually only abundant when a saturated alkyl chain is substituted onto the methylthiophene moiety.

The extract from isolate C2 grown on compound II was treated to produce the methyl esters of the carboxylic acid metabolites. GC-MS analysis showed the presence of a compound with a molecular ion *m/z* 182 which was consistent with the metabolite being 5-methyl-2-thiophenepropenoic acid. The base peak was at *m/z* 151 which corresponded to the loss of -OCH_3. The next most abun-

dant ion was *m/z* 123 corresponding to the loss of $-COOCH_3$. Again the fragment ion at *m/z* 111 was rather weak which would be expected because of the position of the double bond in this metabolite.

Metabolism of 2-butyl-5-tridecylthiophene (compound III) by pure cultures

Isolates SE and C2 grew on compound III. GC analysis of the extract from isolate SE showed two sulfur-containing metabolites and the mass spectra of these metabolites are shown in Fig. 5. The more abundant metabolite had a molecular ion at *m/z* 184 (Fig. 5a) suggesting that the compound was 5-butyl-2-thiophenecarboxylic acid. The commonly observed cleavage beta to the thiophene ring that occurs during mass spectrometric analysis of alkyl-substituted thiophenes would give the loss of $-CH_2CH_2CH_3$ and hence the ion at *m/z* 141 which can subsequently lose CO_2 yielding the ion at *m/z* 97.

The less abundant metabolite showed a molecular ion at *m/z* 198 (Fig. 5b) suggesting that the compound was 5-butyl-2-thiopheneacetic acid. Loss of $-CH_2CH_2CH_3$ via cleavage beta to the thiophene ring would give rise to the base peak at *m/z* 155 which can subsequently lose CO_2 to give the fragment at *m/z* 111. The fragment ion *m/z* 141 may have resulted from the loss of the entire butyl side chain by alpha cleavage.

The extract from isolate SE grown on compound III was reacted with methanol and sulfuric acid to yield the methyl esters of the carboxylic acid metabolites. GC-MS analysis showed that the more abundant compound had a molecular ion at *m/z* 198 and a base peak at *m/z* 155. The former ion is consistent with the addition of a methyl group to 5-butyl-2-thiophenecarboxylic acid and the latter ion would arise from the loss of $-CH_2CH_2CH_3$ from the methyl ester via cleavage beta to the thiophene ring. The less abundant compound had a molecular ion at *m/z* 212, a base peak at *m/z* 169 and a major ion at *m/z* 153. The molecular ion is consistent with the addition of a methyl group to 5-butyl-2-thiopheneacetic acid and the base peak would arise from the loss of $-CH_2CH_2CH_3$ from the methyl

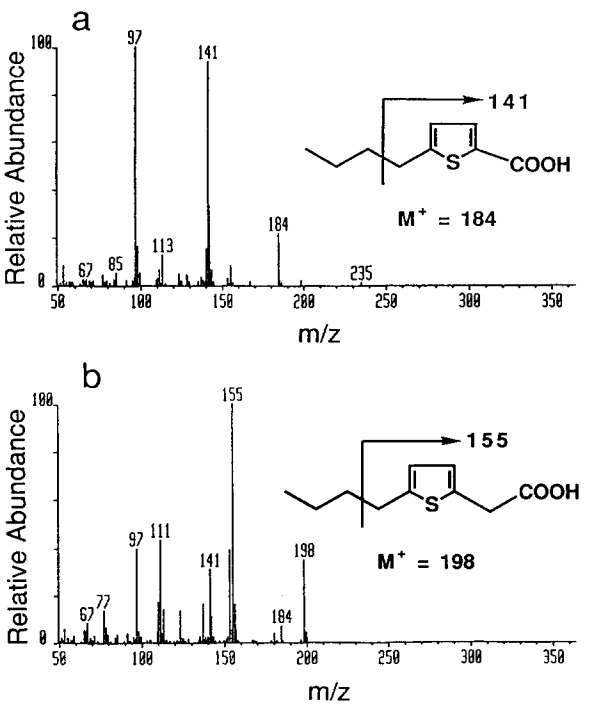

Fig. 5. Mass spectra obtained from the GC-MS analysis of the extract from isolate SE grown on compound III. (a) Major metabolite, 5-butyl-2-thiophenecarboxylic acid; (b) minor metabolite, 5-butyl-2-thiopheneacetic acid.

ester via a cleavage beta to the thiophene ring. The other major ion would result from the loss of $-COOCH_3$.

Metabolism of 2-(3,7-dimethyloctyl)-5-methyl-thiophene (compound IV) by pure cultures

GC analysis of the extract from isolate SE grown on *n*-tetradecane in the presence of compound IV showed complete removal of the substituted thiophene and the presence of one sulfur-containing metabolite which had a longer retention time than compound IV. Fig. 6 shows the mass spectrum of this metabolite with a weak molecular ion at *m/z* 268. This suggests that the metabolite is 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid. Cleavage beta to the thiophene ring via a McClafferty rearrangement (McClafferty 1980) would give the base peak of *m/z* 142 which can subsequently lose CO_2 to give the ion at *m/z* 98. In competition with

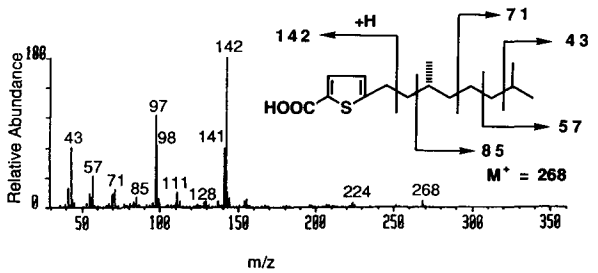

Fig. 6. Mass spectrum of the metabolite observed in the GC-MS analysis of the extract from isolate SE grown on *n*-tetradecane in the presence of compound IV. Identified as 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid.

this fragmentation pathway is the normal beta-cleavage to give *m/z* 141 which can also lose CO_2 to give, in this case, *m/z* 97 which is the second most abundant ion in the mass spectrum. Fragmentation of the branched alkyl chain would produce the ions *m/z* 43, 57, 71 and 85 (Silverstein & Bassler 1967). These correspond to the fragments C_3H_7, C_4H_9, C_5H_{11}, and C_6H_{13}, respectively.

The methyl ester of the metabolite formed by isolate SE grown on *n*-tetradecane in the presence of compound IV was synthesized and analyzed by GC-MS. The mass spectrum of the methyl ester was consistent with the metabolite being 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid. For example, the molecular ion and the parent peak were *m/z* 182 and 156, respectively. The latter ion would result from cleavage beta to the thiophene ring via a McClafferty rearrangement. These two ions were each 14 mass units greater than those observed for the free acid providing more evidence that the carboxyl group was on the thiophene ring. The second most abundant ion was *m/z* 97 which would arise from the loss of $-COOCH_3$ from the parent peak. In addition, peaks at *m/z* 43, 57 and 71 were observed resulting from the fragmentation of the branched alkyl chain.

Metabolism of 2-methyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene (compound V) by pure cultures

In several experiments, cultures were incubated for 14 days in the presence of *n*-tetradecane and compound V. GC analyses of the culture-extracts showed little or no compound V remained and no sulfur-containing metabolites were detected. However, in one experiment, the extract from a culture of SE yielded a sulfur-containing metabolite with the same GC retention time as 5-methyl-2-thiophenecarboxylic acid.

Metabolism of 2-ethyl-5-(3,7,11,15-tetramethylhexadecyl)thiophene (compound VI) by pure cultures

GC analysis of the extract from isolate SE grown on *n*-tetradecane in the presence of compound VI showed virtually complete removal of the substituted thiophene and the presence of one sulfur-containing metabolite. The mass spectrum of the metabolite is shown in Fig. 7 and it suggests that the compound is 5-ethyl-2-thiopheneacetic acid. The molecular ion is *m/z* 170 and the base peak at *m/z* 125 would result from the loss of -COOH due to cleavage beta to the thiophene ring. The base peak of compound VI is also *m/z* 125 (data not shown). These results show that the 20-carbon branched side chain of compound VI was attacked by isolate SE yielding 5-ethyl-2-thiopheneacetic acid which accumulated in the medium in this pure culture study.

Biotransformation of n*-alkylthiophenes by the mixed culture SLPB*

When the mixed culture SLPB was grown on compound I in the absence of crude oil, a small amount of 5-methyl-2-thiophenecarboxylic acid and a larger amount of 5-methyl-2-thiopheneacetic acid remained in the medium after 14 days incubation. After a further 14 days of incubation, there was no detectable decrease in the amount of the more abundant metabolite.

Figure 8 shows the FID chromatograms of Prudhoe Bay crude oil supplemented with compound I extracted from a sterile control and a 14-day-old culture of SLPB. The mixed culture degraded the *n*-alkanes and isoprenoids, pristane and phytane,

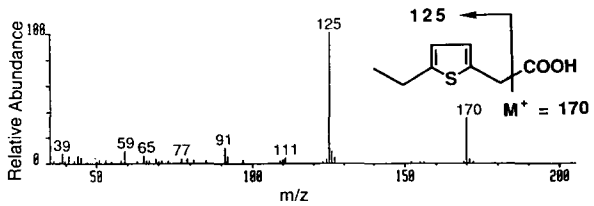

Fig. 7. Mass spectrum of the metabolite observed in the GC-MS analysis of the extract from isolate SE grown on *n*-tetradecane in the presence of compound VI. Identified as 5-ethyl-2-thiopheneacetic acid.

and compound I. The GC chromatograms obtained from sulfur-selective FPD analysis of the extract from the SLPB culture that contained oil and compound I are shown in Fig. 9. The SLPB culture clearly removed all of compound I during the 14-day incubation period and there was no evidence for the accumulation of any sulfur-containing metabolites such as 5-methyl-2-thiophenecarboxylic acid or 5-methyl-2-thiopheneacetic acid which were observed to accumulate in the pure cultures. In addition, many dibenzothiophenes were evident in the extract from the sterile control but were absent in the extract from the oil-degrading mixed culture.

The extracts from 14-day-old SLPB cultures that originally contained oil and 5-methyl-2-thiophenecarboxylic acid had none of the latter compound. Sterile controls that contained oil and 1 mg of 5-methyl-2-thiophenecarboxylic acid were also prepared and incubated with the test cultures. The amounts of 5-methyl-2-thiophenecarboxylic acid recovered from sterile controls extracted at the time of inoculation and those extracted after 14 days incubation were virtually identical, thereby verifying the reliability of the extraction method and demonstrating no evaporative loss of this acid under the incubation conditions used.

These experiments demonstrated that in the presence of crude oil, the mixed bacterial culture could degrade compound I and transform the resulting metabolites, 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid. Clearly, some component of the crude oil was required to produce further transformations of the metabolites of compound I.

The ability of the mixed culture SLPB to degrade

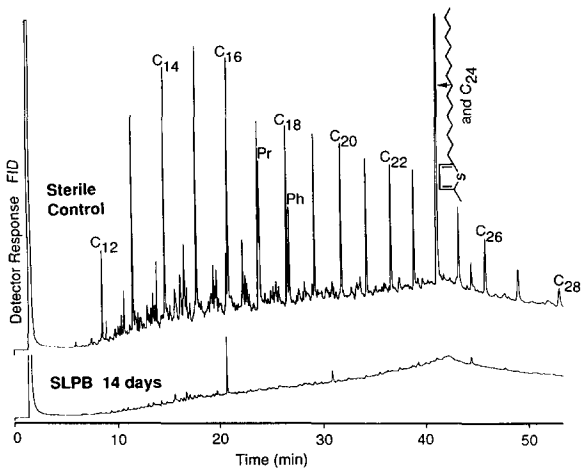

Fig. 8. FID chromatograms of the extracts from cultures after 14 days incubation with Prudhoe Bay crude oil supplemented with Compound I. Pr = pristane, Ph = phytane.

compounds II and III in the presence of Prudhoe Bay crude oil was also investigated. The results obtained with compound II were essentially the same as those obtained with compound I. That is, when crude oil was present in the culture, no metabolites were detected in the extract. However, when no oil was present, 5-methyl-2-thiophenecarboxylic acid was found in the culture extract. In contrast, no metabolites from compound III were observed in the extracts from the SLPB culture grown in the presence or absence of Prudhoe Bay crude oil.

To determine which components of the crude oil stimulated the transformation of the acidic metabolites of compound I, various fractions of the oil were supplemented to cultures containing SLPB and the alkylthiophene. Although small amounts of 2-methyl-5-thiopheneacetic acid were found in the cultures supplemented with the saturated or aromatic fractions, alone or in combination, no 2-methyl-5-thiophenecarboxylic acid was found in these cultures. Thus it appeared that the presence of these fractions stimulated the further biotransformation of the acid metabolites.

Because the addition of the saturated fraction of Prudhoe Bay crude oil stimulated the biotransformation of the acid products from compound I by the mixed culture SLPB, it was speculated that the

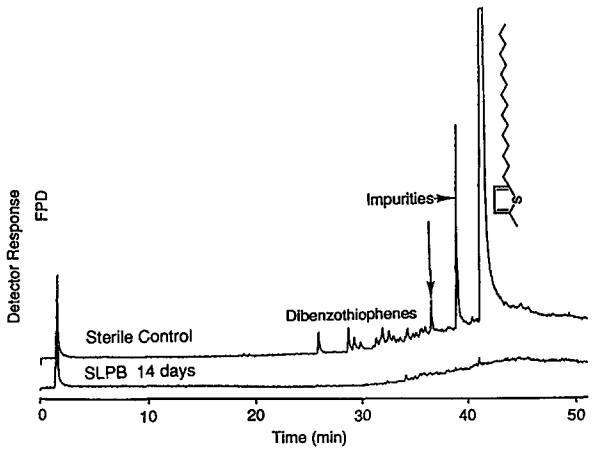

Fig. 9. FPD chromatograms of the extracts from cultures after 14 days incubation with Prudhoe Bay crude oil supplemented with Compound I.

addition of *n*-hexadecane may have the same effect. GC analysis of a culture extract showed that after 15 days incubation, all of the *n*-hexadecane had been consumed by a culture that originally contained the *n*-alkane and 1 mg of 2-methyl-5-thiopheneacetic acid. This culture had only 35% as much of this acid as the 15-day-old sterile control. By day 28, only 18% as much 5-methyl-2-thiophenecarboxylic acid remained in the active culture as was found in the corresponding sterile control.

GC analysis of the extract from a 15-day-old culture, that initially contained *n*-hexadecane and compound I, detected neither of these compounds. No 5-methyl-2-thiopheneacetic acid was detected in this culture and only a trace amount of 5-methyl-2-thiophenecarboxylic acid was found. By day 28, no 5-methyl-2-thiophenecarboxylic acid was detected. These results demonstrate that *n*-hexadecane stimulated the further biotransformation of the products remaining after the digestion of the hexadecyl side chain.

Biotransformation of compound I by microbes in river water incubated with prudhoe bay crude oil

After 7 days incubation, the N and P-supplemented river water inoculum had degraded virtually all the *n*-alkanes from the Prudhoe Bay crude oil leaving the isoprenoids, pristane and phytane, as the most abundant peaks in the chromatogram. Also by this time, all of compound I had been removed and the predominant sulfur-containing compound was 5-methyl-2-thiopheneacetic acid. By day 14, pristane and phytane were still detected in the chromatogram but 5-methyl-2-thiopheneacetic acid was not. Clearly, the mixed microbial population in the North Saskatchewan River was able to degrade compound I and its metabolite, 5-methyl-2-thiopheneacetic acid in the presence of Prudhoe Bay crude oil. In addition, these data showed that compound I was more susceptible to biotransformation than the isoprenoids, pristane and phytane. This observation was consistent with that obtained with isolate C2 using a mixture of *n*-hexadecane, pristane and compound I (Fig. 3).

Discussion

Metabolism of alkylthiophenes by pure cultures

Table 1 summarizes the identities of the metabolites found in the extracts of pure cultures grown with the seven alkylthiophenes shown in Fig. 1. The initial survey with compound I showed that each of the six *n*-alkane-degrading bacterial isolates grew on this *n*-alkylthiophene. However, because there were only small amounts of compounds II and III available, only two of the isolates, SE and C2, were tested on these compounds. Both biodegraded these *n*-alkylthiophenes.

The results demonstrated that the long side chains of the *n*-alkylthiophenes were preferentially attacked and metabolized, presumably, via a series of beta-oxidations. Metabolism of compound I, with a C_{16} side chain, yielded predominantly 5-methyl-2-thiopheneacetic acid which would result from the removal of seven acetate units by a series of beta-oxidations. Metabolism of compounds II and III, with C_{13} side chains, yielded predominantly 5-methyl-2-thiophenecarboxylic acid and 5-butyl-2-thiophenecarboxylic acid, respectively. These would result from six beta-oxidations of the *n*-alkylthiophenes thereby shortening the side chains by twelve carbon atoms. During the beta-

oxidation process, the action of an acyl dehydrogenase oxidizes a saturated intermediate yielding an unsaturated intermediate (Mahler & Cordes 1966). The detection of 5-methyl-2-thiophenepropenoic acid in the extract of isolate C2 grown on compound II is therefore consistent with the metabolism of this *n*-alkylthiophene via beta-oxidations. The enzymatic addition of water across the double bond in 5-methyl-2-thiophenepropenoic acid and subsequent removal of an acetate unit would yield 5-methyl-2-thiophenecarboxylic acid.

Interestingly, the butyl side chain of compound III remained unaltered indicating that it was too short to be attacked by these pure cultures of *n*-alkane-degrading bacteria. In studies with various *n*-alkylbenzenes (Fedorak & Westlake 1986), minimum side chain lengths of C_4, C_8 and C_9 were required for various *n*-alkane-degrading fungi to metabolize these hydrocarbons.

Small amounts of 5-methyl-2-thiophenecarboxylic acid and 5-butyl-2-thiopheneacetic acid were detected in cultures grown on compounds I and III, respectively. These products would not arise via beta-oxidations of the substrates but they might result from the alpha-oxidation (oxidative decarboxylation) (Ratledge 1978) of one of the predominant metabolites. Alternatively, these may be the products of the beta-oxidations of trace contaminants in the preparations of compounds I and III. For example, GC-MS analysis of the preparation of compound I showed the presence of a contaminant with a molecular ion at *m/z* 308 and a base peak at *m/z* 111. These data suggest that the contaminant was 2-methyl-5-pentadecylthiophene which would yield 5-methyl-2-thiophenecarboxylic acid after a series of seven beta-oxidations.

The accumulations of carboxylic acids in the culture media observed during this study were similar to results of other investigations of the microbial metabolism of cyclic compounds with long alkyl side chains. For example, Davis & Raymond (1961) found that phenylacetic acid accumulated in cultures of various strains of *Nocardia* sp. grown on dodecylbenzene. Similarly, studies with *n*-alkane-degrading bacteria and fungi showed that the predominant metabolites from 2-undecyltetrahydrothiophene and 2-dodecyltetrahydrothiophene were 2-tetrahydrothiophenecarboxylic acid and 2-tetrahydrothiopheneacetic acid, respectively, and that these metabolites persisted in the culture media (Fedorak et al. 1988). Other studies have shown that the resulting carboxylic acids were transient intermediates. These include work with *Nocardia salmonicolor* (Sariaslani et al. 1974) and several fungi (Fedorak & Westlake 1986) grown on dodecylbenzene. In each of the above mentioned studies, the initial microbial attack was on the alkyl side chain rather than on the cyclic moiety.

As was observed with the *n*-alkylthiophenes, the long side chains of two of the isoprenoidal thiophenes, compounds V and VI, were metabolized by pure cultures to give 5-methyl-2-thiophenecarboxylic acid and 5-ethyl-2-thiopheneacetic acid, respectively. This likely occurred via a terminal ox-

Table 1. Summary of metabolites identified in pure cultures of bacteria grown with various alkylthiophenes.

Alkylthiophene[a]	Metabolites	
	Major	Minor
I	5-Methyl-2-thiopheneacetic acid	5-Methyl-2-thiophenecarboxylic acid
II	5-Methyl-2-thiophenecarboxylic acid 5-Methyl-2-thiophenepropenoic acid[b]	5-Methyl-2-thiopheneacetic acid
III	5-Butyl-2-thiophenecarboxylic acid	5-Butyl-2-thiopheneacetic acid
IV	5-(3,7-Dimethyloctyl)-2-thiophenecarboxylic acid	ND[c]
V	ND	5-Methyl-2-thiophenecarboxylic acid
VI	5-Ethyl-2-thiophenecarboxylic acid	ND

[a] Structures are shown in Fig. 1.
[b] Major metabolite in one culture, not found in others.
[c] None detected.

idation of the tetramethylhexadecyl moiety and subsequent beta-oxidations removing C_3- (propionyl-SCoA) and C_2-units (acetyl-SCoA) as was observed during the bacterial metabolism of pristane (McKenna & Kallio 1971; Pirnik et al. 1974). Omega-oxidation yielding dicarboxylic acid intermediates plays a key role in the metabolism of the isoprenoid pristane (Pirnik et al. 1974). However, the formation of 5-ethyl-2-thiopheneacetic acid from compound VI indicates that removal of the isoprenoidal side chain can occur without omega-oxidation. The inability to find metabolites from compound V in several of our experiments may indicate that omega-oxidation plays a role in its biotransformation. Dicarboxylic acids arising from this type of oxidation would be too polar to be detected by our analytical methods. Further investigations are required to determine whether any dicarboxylic acids were produced.

The oxidation of the methyl group of compound IV by strain SE giving 5-(3,7-dimethyloctyl)-2-thiophenecarboxylic acid was unique among the compounds examined in this study. With each of the other alkyl thiophenes, the longer side chain was oxidized and the methyl group was unaltered. The results with compound IV suggest that its dimethyloctyl side chain was quite resistant to microbial attack.

Biotransformation of n*-alkylthiophenes by mixed cultures*

When added to Prudhoe Bay crude oil, each of the three *n*-alkylthiophenes was metabolized by the oil-degrading mixed culture SLPB. Indeed, the presence of the crude oil enhanced the biotransformation of compounds I and II because no carboxylic acid intermediates were detected in the extracts from the mixed culture supplemented with crude oil. In addition, when 5-methyl-2-thiophenecarboxylic acid was added to oil-containing medium with the SLPB culture, this acid could not be detected after 14 days incubation. However, the mixed culture did not transform the metabolites from compounds I and II in the absence of crude oil in the medium. In contrast, the SLPB culture did transform 5-butyl-2-thiophenecarboxylic acid produced from compound III when there was no crude oil in the medium. Supplementing the SLBP culture with the saturated and/or aromatic fractions of Prudhoe Bay crude oil or with *n*-hexadecane stimulated the further biotransformation of 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid.

The biotransformation products of the carboxylic acid intermediates of the alkylthiophenes have not been determined. However, several studies have shown that bacterial isolates can grow on various thiophene carboxylic acids. For example, Cripps (1973) isolated a bacterium that could use 2-thiophenecarboxylic acid as its sole carbon, sulfur and energy source and Kanagawa & Kelly (1987) isolated strains of *Rhodococcus* that used 2-thiophenecarboxylic acid, 5-methyl-2-thiophenecarboxylic acid and 2-thiopheneacetic acid as sole carbon and energy sources. Similarly, Evans & Venables (1990) isolated a *Vibrio* species that could grow on 2-thiophenecarboxylic acid and 2-thiopheneacetic acid. Oxygen uptake experiments showed that resting cells of the *Vibrio* species were able to oxidize several thiophene derivatives including 2-methylthiophene, 3-thiophenecarboxylic acid, and 3-thiopheneacetic acid. Whether the carboxylic acid intermediates of the alkylthiophenes served as growth substrates for some of the bacteria in the SLPB mixed culture or whether these intermediates were cometabolized to products that were not detected by our analytical method remains to be determined.

This work showed that five of the six model alkylthiophenes studied were readily metabolized by *n*-alkane-degrading bacteria that removed the side chains leaving carboxylic acid intermediates. The exception was compound IV. In this case, the methyl group, rather than the longer alkyl group was oxidized. When added to a crude oil, the *n*-alkylthiophenes were also metabolized by a mixed culture of petroleum-degrading microorganisms and the carboxylic acid intermediates were further transformed to unidentified products. Although these alkylthiophenes are usually minor components of petroleum, they are quite susceptible to microbial metabolism and therefore should not ac-

cumulate in petroleum-contaminated environments.

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