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Linkage Between Biodegradation of Polycyclic Aromatic Hydrocarbons and Phospholipid Profiles in Soil Isolates

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Abstract A bacterial consortium capable of utilizing a variety of polycyclic aromatic hydrocarbons has been isolated from a former manufactured gas plant site. The consortium consisted of four members including *Arthrobacter* sp., *Burkholderia* sp., *Ochrobacterium* sp., and *Alcaligenes* sp., which were identified and characterized by the patterns of fatty acid methyl esters (FAME analysis) and carbon source utilization (BIOLOG system). With the individual members, the biodegradation characteristics of aromatic hydrocarbons depending on different growth substrates were determined. FAME analyses demonstrated that microbial fatty acid profiles changed to significant extents in response to different carbon sources, and hence, such shift profiles may be informative to characterize the biodegradation potential of a bacterium or microbial community.

Key words: Polycyclic aromatic hydrocarbons, fatty acid methyl ester (FAME), phospholipid, MIDI, BIOLOG

Polycyclic aromatic hydrocarbons (PAHs) are a major class of contaminants in the environment and many of them are carcinogenic or mutagenic [29]. Nonionic hydrophobicity of the organic compounds is sorbed more easily and even sequestered in soil, sediment, and aquifer solids, and eventually the compounds become more persistent in nature [2]. At the same time, biological degradation takes place in the environment as a major loss mechanism of PAHs [1]. Many bacteria capable of degrading PAHs have been isolated so far, and their genetic characteristics in relation to degradation are an important subject of study in this field. However, such information has been concentrated on the naphthalene degradation pathway [31] and only a few on phenanthrene [17] and pyrene [6].

In addition to genetic information, it also seems possible to use fatty acid profiles of the cell membrane in identifying and characterizing bacterial species [10] and their metabolic activities [4, 12]. Microbial fatty acids in cell membrane change in response to environmental conditions such as available carbon sources, the presence of toxic chemicals, and so on. The possible adaptive or resistant mechanisms are i) an increase of the acyl chain length of phospholipids, ii) saturation of unsaturated fatty acids in membrane, and iii) isomerization of *cis*- to *trans*-unsaturated fatty acids [26]. All are related to the decrease in the fluidity or flexibility of cell membrane so that surrounding toxic chemicals cannot easily penetrate through the membrane. A good example can be found in some *Pseudomonas putida* strains capable of resisting or metabolizing toluene. *P. putida* IH-2000 [15] and *P. putida* PpG1 [23] could tolerate up to 50% of toluene without metabolizing the compound, while *P. putida* Idaho [7] and *P. putida* S12 [30] could utilize high concentrations (i.e., more than 50% to supersaturated amounts) of toluene.

In the present study, we have isolated an active consortium degrading a variety of PAHs from a former manufactured gas plant (MGP) site, and biodegradation characteristics of each member of the consortium were studied. We determined the effect of different growth substrates on the degradation characteristics, and the results were related to microbial fatty acid profiles determined by FAME analysis. Most studies so far dealt with the fatty acid changes of cell membrane in the aspect of a microbial resistance mechanism to toxic chemicals [14]. In this study, these changes in response to growth substrates have been investigated to distinguish the degradation property of a specific bacterial species.

MATERIALS AND METHODS

Isolation of a Consortium Degrading PAHs

Enrichment culture technique was used to isolate PAHs-degrading bacteria from a coal tar-contaminated soil. Two-

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to five-gram samples of MGP soil collected from the depth of 0–2 m below surface were incubated with a mixture of PAHs in 100 ml of an inorganic salts solution (0.10 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3$, 0.10 g $MgSO_4 \cdot 7H_2O$, 0.10 g NH_4NO_3, 0.20 g KH_2PO_4, and 0.80 g K_2HPO_4/l of dH_2O; pH 7.0) at 30°C for two weeks. PAHs including naphthalene (NAP), phenanthrene (PHE), anthracene (ANT), pyrene (PYR), chrysene (CHR), and benzo(a)pyrene (BaP) were dissolved in methanol (10 mg/ml for the first three compounds and 1 mg/ml for the others), and the PAHs-methanol solution was used as substrates for the enrichment. All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). After two weeks of incubation, 10 ml of the supernatant was collected and incubated for two more weeks as described above. By this procedure, a consortium capable of degrading a variety of PAHs was obtained, and the consortium was maintained in 50 ml of inorganic salts solution containing 50 mg each of NAP and PHE as carbon sources.

Identification and Characterization

An aliquot of the consortium grown in a Trypticase Soy Broth (TSB) was spread on a Trypticase Soy Agar (TSA) plate, and different types of colonies were observed. The colonies were picked and grown in TSB for identification as a phospholipid and for BIOLOG analysis. A total of four members were found and they were named KP2, KP3, KP5, and KP8. Phospholipid ester-linked fatty acids (PLFA) that existed in each isolate were analyzed in the form of fatty acid methyl esters (FAME) by using the MIDI system (Microbial Insights, Inc., Newark, DE, U.S.A.). The protocol provided by the manufacturer was used with some modifications. Briefly, cells harvested following the 24 h of growth on TSA were heated to 100°C with NaOH-methanol to saponify cellular lipids, and the released fatty acids were methylated by heating with HCl-methanol at 80°C. Fatty acid methyl esters were solvent-extracted, and analyzed by gas chromatography/mass spectrometry (GC/MS). Fatty acid methyl esters were identified by comparing their retention times and mass spectra with those of authentic standards provided by the MIDI database.

The pattern of carbon source utilization by each isolate was determined using GP2 (for Gram (+)) or GN2 (for Gram (−)) MicroPlate (BIOLOG, Hayward, CA, U.S.A.). A single colony grown on a TSA plate was streaked onto a BUG (BIOLOG Universal Growth) agar medium containing 5% sheep blood, and the plate was incubated at 30°C for 16 h. The grown cells were suspended in saline (0.15% NaCl) and the inoculum density was adjusted to reduce the specified turbidity to the range recommended by the manufacturer. Each well (96 wells in total) plate of GP2 or GN2 was inoculated with 150 µl of the inoculum suspension. After 24 h of incubation, the utilization pattern of carbon source in each well was read by using the BIOLOG-automated MicroStation. Detailed procedures were followed as per the BIOLOG manual. Identification by 16S rDNA sequencing, when necessary, was performed as described elsewhere [16].

Biodegradation of PAHs

Biodegradation capacity of the isolated consortium was determined in a soil spiked with various PAHs, including NAP, PHE, ANT, PYR, CHR, and BaP. Ten grams of a silt loam passed through a 2-mm sieve were suspended with 100 ml of inorganic salts solution in a 250-ml flask, to which 100 µl of the PAHs-methanol solution used for enrichment was spiked. This accounted for 1 mg each of NAP, PHE, and ANT and 100 µg each of PYR and BaP in 10 g soil. The PAHs-spiked soil suspension was shaken (150 rpm) for 1 h at room temperature, and the consortium washed twice with the inorganic salts solution was inoculated at the level of 10^7 cells/g soil. Biodegradation was performed at room temperature with shaking (150 rpm) for 4 weeks. A total of 12 flasks were made and three flasks were taken every week for the determination of PAHs remained in soil. The PAHs in soil were solvent-extracted and analyzed by GC/FID [20].

Effect of Carbon Source on Mineralization

Each of the four isolates was grown in inorganic salts solution containing NAP or PHE as a sole carbon source at 30°C. After a substantial growth was observed (usually, 4 days), the pregrown cells adapted to NAP or PHE were washed twice with inorganic salts solution and used for mineralization experiments. The cells were subjected to mineralize various PAH compounds and the mineralization extents were determined by measuring $^{14}CO_2$ evolved. For this purpose, radiolabeled PAHs including NAP ([UL-^{14}C]; specific activity, 31.3 mCi/mmol), PHE ([9-^{14}C]; specific activity, 14.0 mCi/mmol), ANT ([1,2,3,4,4A,9A-^{14}C]; specific activity, 20.6 mCi/mmol), and PYR ([4,5,9,10-^{14}C]; specific activity, 58.7 mCi/mmol) were used. Approximately 10^5 dpm of radiolabeled compound was added to 10 ml inorganic salts solution, and a desired amount was adjusted with unlabeled compound. The concentration of each PAH was 1 µg and the inoculum density was 10^7 cells per milliliter of inorganic salts solution. Each flask was then sealed with a Teflon-wrapped silicon stopper, in which was placed an 18-gauge needle and a 16-gauge steel cannula. From the cannula was suspended a small vial containing 1.5 ml of 0.5 N NaOH to trap $^{14}CO_2$ released from mineralization. The NaOH solution was periodically removed and replaced with fresh solution, and the amount of evolved $^{14}CO_2$ was determined by a liquid scintillation counter (Model LS 5000 TD; Beckman Instruments, Inc., Irvine, CA, U.S.A.). The flasks were incubated at room temperature for 10 days on a rotary shaker (120 rpm).

Determination of Changes in Phospholipids

The shifts in PLFA of the four isolates from the different carbon sources were determined. Four isolates separately

grown on TSB were collected and washed twice in potassium phosphate buffer (pH 7.0). The washed isolates were individually grown in 100 ml of inorganic salts solution containing 1 mg/ml of NAP or PHE as a sole carbon source. After 4 days of incubation at 30°C, the composition of FAME was analyzed by using the MIDI system.

According to the IUPAC (International Union of Pure and Applied Chemistry) system, fatty acids are designated as total number of carbon atoms:number of double bonds, with the position of the double bond nearest to the aliphatic (ω) end of the molecule indicated with the geometry 'c' for *cis* and 't' for *trans*. The prefixes 'ISO' and 'ANTE' represent a methyl group occurring at the second and the third to the aliphatic end in the chain, respectively. Cyclopropyl fatty acids are designated as 'CYCLO', with the ring position relative to the aliphatic end.

RESULTS AND DISCUSSION

Characterization of a Consortium

A consortium capable of degrading a variety of PAHs was isolated from a MGP site. The consortium was composed of four members and they were designated as KP2, KP3, KP5, and KP8. KP2 was identified as a Gram (+) bacterium *Arthrobacter* sp. by MIDI analysis, with a similarity index of 0.547, but BIOLOG could not identify the isolate. An 16S rDNA sequence analysis confirmed that the isolate was an *Arthrobacter* species. The other three isolates (KP3, KP5, and KP8) were Gram (-) bacteria, and the results from MIDI and BIOLOG analyses were identical, but the similarity indices were much higher in MIDI analysis. Similarity index (SI) is a numerical value which expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains in the MIDI library. An exact match of the fatty acid composition of the unknown and the mean of the library entry would result in an SI of 1.000, and a strain with an SI of 0.600 falls three standard deviations from the mean in Gaussian distribution of the fatty acid composition. In general, strains with an SI of 0.500 or higher with a separation of 0.100 between the first and second choice by the MIDI system are considered a good library comparison. KP3 was identified as a *Burkholderia* sp. with an SI of 0.603 and KP8 as a *Alcaligenes* sp. with an SI of 0.884. The isolate designated as KP5 was identified as an *Ochrobacterium* sp. with an SI of 0.885, which has not been previously reported as a xenobiotics-degrading bacterium.

Biodegradation of a Mixture of PAHs

The capacity of the consortium to degrade various PAHs was determined in soil (Fig. 1). NAP and PHE were immediately removed from the spiked soil probably with some amounts of volatilized NAP. The loss by volatilization

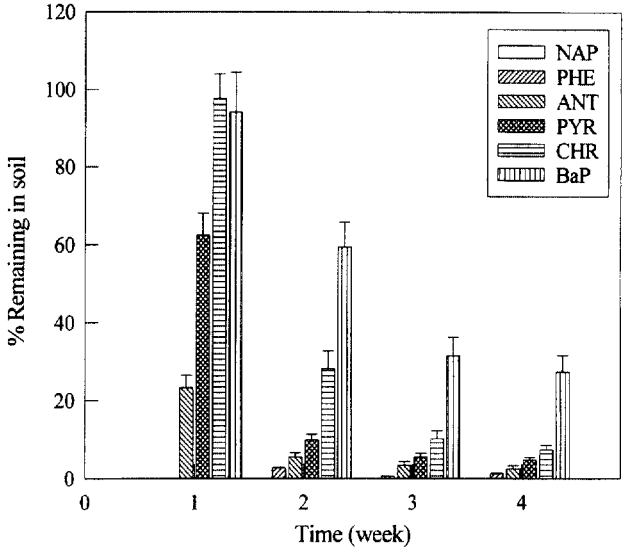

Fig. 1. Biodegradation of various PAHs by a consortium in a silt loam.
NAPs were not detected from all samples and PHEs were not from the first week sample. Bars represent the mean values of triplicate determinations, and standard deviations are shown.

was not determined since that was beyond the scope of this study. ANT and PYR were readily degraded, leaving less than 10% of initial amounts after 2 weeks of biodegradation. CHR and BaP were resistant for the first week, but thereafter they were gradually biodegraded: Only about 7% and 27% remained after 4 weeks, respectively. During the experiments, a set of abiotic controls was always run in parallel with the biodegradation samples to account for possible losses from volatilization, abiotic degradation, and so on. In general, for the PAHs except NAP, volatilization is not a major loss mechanism from soil due to their high hydrophobicity and octanol-water partitioning coefficients [18, 27].

Effect of Different Carbon Sources on the Mineralization of PAHs

The four isolates were separately grown in inorganic salts solutions containing either NAP or PHE as a sole carbon source, and mineralization patterns of various PAH compounds were determined using the substrate-adapted cells. As shown in Fig. 2, the patterns of mineralization of NAP, PHE, and ANT were very similar between *Burkholderia* sp. KP3 and *Ochrobacterium* sp. KP5. NAP-grown cells mineralized less NAP and PHE than PHE-grown cells, and showed 5–6 days of lag period for the onset of PHE mineralization. In addition, PHE-grown cells could degrade ANT to about 30%, but NAP-grown cells could not. These isolates were not able to degrade pyrene as a sole carbon source. When a nutrient medium (i.e., TSB) was used as a carbon source, the resulting cells could not utilize ANT as NAP-grown cells. They were able to mineralize NAP and

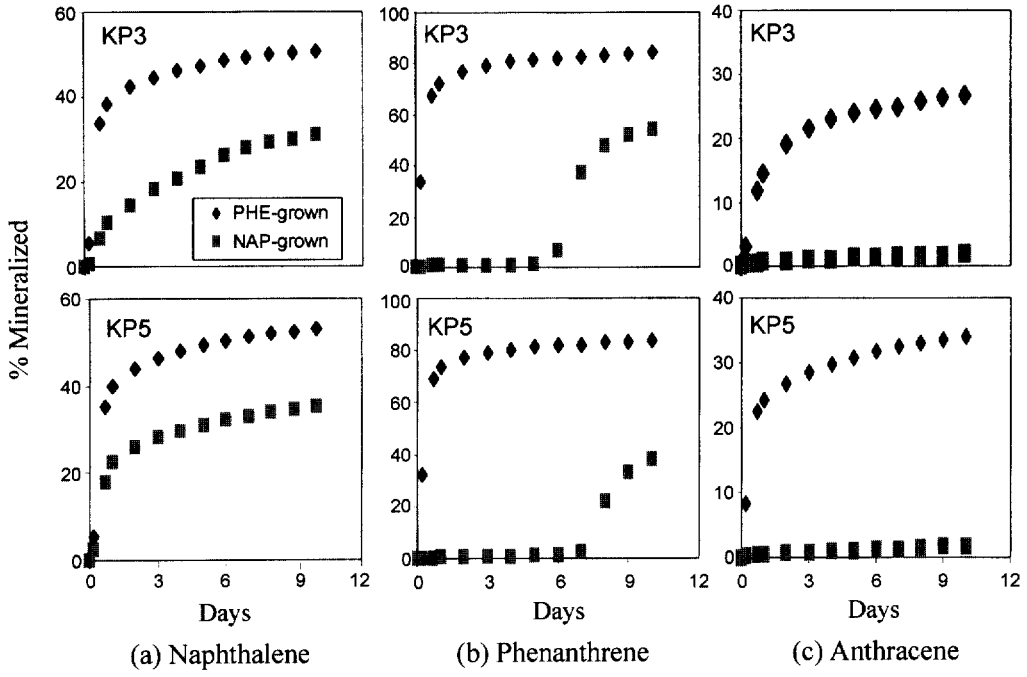

Fig. 2. Biodegradation of NAP, PHE, and ANT by *Arthrobacter* sp. KP2, *Burkholderia* sp. KP3, and *Ochrobacterium* sp. KP5 grown on different carbon sources.
The three isolates could not degrade PYR. In the figure, diamonds and squares represent phenanthrene-grown and naphthalene-grown cells, respectively, and points are the mean values of triplicate determinations.

PHE, but the extents were less than a half compared to PHE-grown cells (data not shown). *Arthrobacter* sp. KP2, a Gram (+) bacterium, showed very similar patterns of mineralization for the three PAHs. Most bacteria degrading xenobiotics are known as Gram (-) with some Gram (+) bacteria: Among these are *Arthrobacter* sp. KP2 degrading pesticides [13, 21], chlorinated phenol [28], and fluorene [5]; and *Mycobacterium* sp. degrading pyrene and benzo(a)pyrene [22]. *Alcaligenes* sp. KP8 also showed similar mineralization patterns for NAP and PHE. However, the isolate could not utilize ANT regardless of the growth substrates and did mineralize PYR when it was grown on PHE but not on NAP (Fig. 3). For higher molecular weight PAHs, the four isolates could not degrade CHR and BaP while their consortium did.

Changes in Fatty Acid Profiles

The MIDI data demonstrated the changes of fatty acid composition in the isolates depending on the carbon sources used for growth by the isolates (Table 1). A Gram (+) bacterium *Arthrobacter* sp. KP2, when grown on TSB, showed a very different FAME composition from the same bacterium grown on NAP or PHE as a sole carbon source. When grown on TSB, branched odd number fatty acids consisting of 15:0 ISO, 15:0 ANTE, and 17:0 ANTE were dominant in *Arthrobacter* sp. KP2, which was also observed with other Gram (+) bacteria such as *Bacillus* species [8]. However, when the bacterium was grown on NAP or PHE, a saturated fatty acid 16:0 and two cyclopropyls such as 17:0 CYCLO and 19:0 CYCLO ω8c became dominated while the branched odd number fatty acids disappeared. Similarly, the abundance of 16:0 and the large increase of cyclopropyls in response to NAP and PHE were observed with the other three Gram (-) isolates. On the other hand, the amounts of unsaturated fatty acids such as 16:1 ω7c and 18:0 ω7c decreased, and they were negligible when PHE was used as a carbon source, explaining the increase of cyclopropyls described above. Cyclopropyl rings can be formed by a transmethylation reaction across the double bond of an esterified monounsaturated fatty acid already incorporated into the membrane as phospholipid [9]. The observed changes (i.e., the increase in saturated or branched fatty acids) seem to increase membrane ordering and hence decrease the membrane fluidity, which has been suggested as a bacterial defense mechanism [26]. The *Ochrobacterium* sp. KP5 provides a good example in that regard: An unsaturated fatty acid 18:1 ω7c decreased from 81.3% to a tracer level and a saturated fatty acid 16:0 increased from 2.9 to 45.9% in response to PHE. The ratio of unsaturated to saturated fatty acids decreased as the growth substrate changed from nutrients (i.e., TSB) to harmful chemicals (i.e., NAP or PHE).

Our data demonstrated that odd number fatty acids such as 17:0 and 19:0, each with a cyclopropyl ring, increased when the tested bacterial species were exposed to NAP

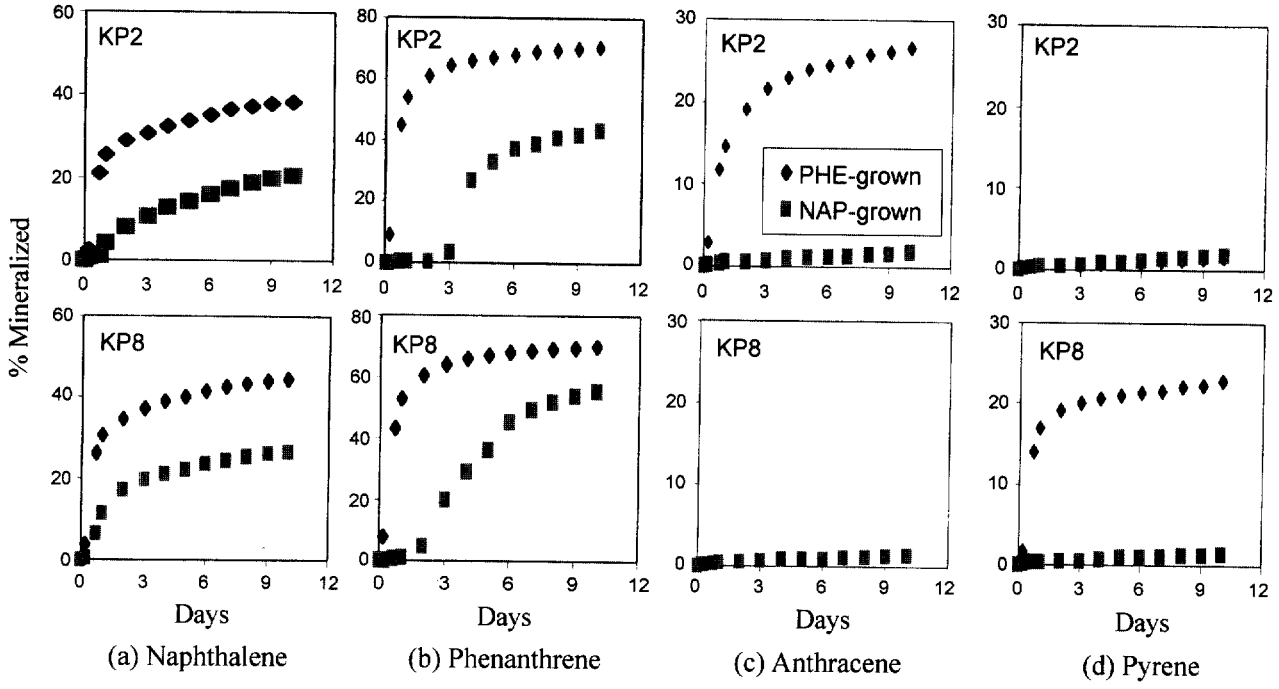

Fig. 3. Biodegradation of NAP, PHE, ANT, and PYR by *Alcaligenes* sp. KP8 grown on either NAP or PHE.
In the figure, diamonds and squares represent phenanthrene-grown and naphthalene-grown cells, respectively, and points are the mean values of triplicate determinations.

or PHE. Although not with aromatic hydrocarbons, a few studies exist showing the similar changes of PLFA composition in hydrocarbon-degrading bacteria when they were exposed to *n*-alkanes. A significant increase in the amounts of odd-numbered straight chain fatty acids such as 15:0 and 17:0 was observed with *Micrococcus cerificans* when grown on undecane, tridecane, pentadecane, and heptadecane [19], and with *Mycrobacterium* sp., *Brevibacterium* sp., and *Norcardia* sp. when grown on propane [8]. Recently, Aries *et al.* [3] reported a similar result with a consortium reconstituted from marine hydrocarbon-degrading bacteria. They observed an appreciable increase in saturated PLFA, and moreover, the increase mainly resulted from the odd-numbered straight chains, not from the even-numbered chains. Interestingly, a significant amount of branched saturated fatty acids newly appeared following the exposure, which is not consistent with our study. They exposed the consortium to petroleum hydrocarbons and observed a total disappearance of *n*-alkanes, but their aromatic hydrocarbon fraction remained unchanged, whereas the carbon source we used were aromatic hydrocarbons. Nevertheless, the study demonstrated the effect of carbon sources on the PLFA composition, which was consistently observed in our study.

This study clearly showed two points: By the type of available carbon sources, biodegradation capacity of a bacterium is influenced, and such change may be related to the fatty acid composition of the cell membrane. Our results

Table 1. Changes in the composition of fatty acids by different growth substrates.

FAME	*Arthrobacter* sp. (KP2)			*Burkholderia* sp. (KP3)			*Ochrobacterium* sp. (KP5)			*Alcaligenes* sp. (KP8)		
	TSB	NAP	PHE	TSB	NAP	PHE	TSB	NAP	PHE	TSB	NAP	PHE
15:0 ISO	12.3											
15:0 ANTE	52.2											
16:0	5.0	31.0	37.9	24.4	40.5	28.1	2.9	35.5	45.9	35.0	35.8	32.0
16:1 ω7c	5.1	10.6		24.6	20.8		0.6	17.8		28.8	21.7	
17:0 ANTE	11.1											
17:0 CYCLO	0.3	13.5	26.5	3.3	6.1	18.1		9.1	16.7	6.8	9.5	21.5
18:1 ω7c				27.5	14.8	7.8	81.3	9.0		7.7	6.2	
19:0 CYCLO ω8c		9.3	26.1	1.2		18.8	5.2	3.4	18.5	0.3	6.1	21.1
unsat./sat.	0.06	0.20	0	1.80	0.76	0.10	10.1	0.56	0	0.77	0.54	0

are meaningful in that consistent changes in the fatty acid composition were observed in response to the exposure of the test isolates to NAP or PHE, and the bacteria seem to recognize xenobiotics as stressors although they can utilize the compounds. When cells are exposed to hydrophobic toxic chemicals, the chemicals partition into the cell membranes and accumulate between acyl chains inside the membranes, which results in the swelling and increased fluidity of the membranes. This alters the structural integrity of cell membranes and causes functional disorder [24, 25]. Therefore, defense mechanisms of bacteria to toxic chemicals will occur in such a way as to decrease the partitioning of the hydrophobic chemicals. In our system, since the isolates were able to utilize NAP or PHE as a sole carbon source and the chemicals were the only carbon sources provided, it is also possible that the isolates might have changed their membrane structure (i.e., fatty acid composition) to expedite the uptake of the hydrophobic chemicals. Indeed, a recent report attempted to find potential biomarkers of hydrocarbon-degrading bacterial activities [3]. However, the point cannot be further verified from the current data. Considering that biodegradation potential is a genetic characteristic, not a membrane-related property, our data would be more informative by accompanying a genetic study. We have conducted a more detailed study comprising genetic work with another isolate.

Acknowledgments

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