Looking For Polycyclic Aromatic Hydrocarbon Metabolizing Microorganisms In The Oral Cavity of Smokers

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Abstract

Certain soil microbes resist and metabolize polycyclic aromatic hydrocarbons (PAHs). The same is true for certain skin microbes. Oral microbes have the potential to oxidize tobacco PAHs to increase their ability to cause cancer. We hypothesized that oral microbes that resist high levels of PAH in smokers exist and can be identified based on their resistance to PAHs. We isolated bacteria and fungi that survived long term in minimal media with PAHs as the sole carbon source from the oral cavity in 11 of 14 smokers and only 1 of 6 nonsmokers. Of bacteria genera that included species that survived harsh PAH exposure *in vitro*, all were found at trace levels on the oral mucosa, except for *Staphylococcus* and *Actinomyces*. Two PAH-resistant strains of *Candida albicans (C. albicans)* were isolated from smokers. *C. albicans* is found orally at high levels in tobacco users and some *Candida* species can metabolize PAHs. The two *C. albicans* strains were tested for metabolism of two model PAH substrates, pyrene and phenanthrene. The result showed that the PAH-resistant *C. albicans* strains did not metabolize the two PAHs. In conclusion, evidence for large scale oral microbial metabolism of tobacco PAHs by common oral microbes remains lacking.

Introduction

Years ago, it was recognized that exposure to tobacco smoke has both short- and long-term effects on the oral cavity. These effects have been best described for the periodontia where, in long time smokers, there is an increase in inflammation at the tooth mucosal border which precedes a loss in tissue[1]. There are also changes in bacteria that exist in the sulci that surround the teeth. Some of the changes are directly related to tobacco smoke exposure[2–4]. Similarly, the remaining oral mucosa that lines the oral cavity also show changes with tobacco smoke exposure[5]. Long-term mucosal changes include hypertrophy and hyperkeratosis initially. These changes can be followed by more serious changes including dysplasia, which can precede malignancy. Certain sites thought to be most heavily exposed to cigarette smoke include frequent sites of oral squamous cell carcinoma (OSCC), the tongue, floor of mouth (FOM) and, to a lesser degree, gingiva. These surfaces also are coated with bacteria though the exact makeup of the population depends on the site[6–8]. Other microorganisms, such as the yeast *C. albicans*, can also become a large part of the oral microbiome in certain individuals. The microbes in the saliva in turn provide a window to the oral microbiome that exists on the oral surfaces, including the teeth, the periodontal sulci, and the remaining oral mucosa[9].

There has been much research on how combusted tobacco products provide a stream of reactive chemicals to the oral tissue[10,11]. These include PAHs and nitrosamines, which have received the most attention because they are pro-carcinogens. Given that studies have revealed the existence of environmental microorganisms that have the ability to metabolize PAHs,[12–14, 15 ,16–22] and that bacteria with similar qualities exist on human skin[21,23], we looked for evidence of the same in the oral cavity. We used the same method for selection of environmental microbes, obtained for example from petroleum waste sites, that have the ability to survive long term in minimal media with PAHs as the sole carbon

source[15,17,21,24]. In the current work, microorganisms were harvested from the oral mucosa surfaces and then exposed over weeks *in vitro* to a cocktail of PAHs with no other carbon source to determine if smokers preferentially harbored microbes tolerant of tobacco smoke PAHs. We then examined the microbiome on two oral mucosa surfaces, lateral border of the tongue, and the attached gingiva, in tobacco users to determine if long-term exposure to cigarette smoke had similar effects *in vivo* on the mucosal microbiome. Most of these taxa were found, at most, at trace levels in the oral cavity of the studied population. *C. albicans* was identified from two different tobacco users. As a yeast known to be at relatively high levels in the oral cavity of tobacco users, an examination of these isolates' ability to metabolize model PAHs was undertaken in order to examine a role for this species in the conversion of these PAHs to more reactive carcinogens.

Methods

Subjects

Subjects were patients of Dental Clinics at the University of Illinois College of Dentistry. All subjects provided written informed consent to participate in accordance with guidelines of the Office for the Protection of Research Subjects of the University of Illinois at Chicago, with formal approval of the study protocol, 2012-1030, by the Institutional Review Board 1 of the University of Illinois Chicago. This study was done in full accordance with the principles of the Declaration of Helsinki. Samles were taken only from sites that appeared normal in appearance in the clinic.

Mucosal sample collection and selection

For the PAH selection procedure swab samples were collected from the lateral border of the tongue, buccal, attached gingiva and oral pharynx. These were combined and placed in 3 mL Bushnell-Haas Broth (BHB) without glycerol. One milliliter culture was used to inoculate 9 mL BHB with 10 µg/mL each of benzo[a]pyrene, chrysene, fluoranthrene, naphthalene, phenanthrene, and pyrene. The culture was incubated with aeration for 3 weeks at 37°C. One hundred microliters were collected and plated on BHI agar plate and incubated for 48 h at 37°C in aerobic conditions. Samples used directly for 16S rRNA analysis were from gingiva or tongue and were immediately frozen in TE.

PAH degradation by microbes in liquid culture

C. albicans was grown overnight in YPD broth at 30°C with aeration. The positive control, *Mycobacterium rutilum* was prepared in BHI medium and incubated overnight at 30°C with aeration[15]. The overnight cultures of *C. albicans* and *Mycobacterium rutilum* were centrifuged, washed 3 times with BHB medium, and resuspended in BHB. Each culture was divided in 9 large glass test tubes, each containing 10 mL culture in BHB medium. Pyrene or phenanthrene was added (triplicates) to a final concentration of 20 µg/mL. BHB medium with microbial cells without PAH (triplicates) and BHB medium only served as controls. Tubes were incubated with aeration for 14 days in the dark at 30°C.

Identification of pyrene and phenanthrene metabolites

After the PAHs were incubated with the microbes, they were stored unopened at -80°C until analyzed by GC/MS. Once the samples thawed, the cultures were spiked with 20 µg of phenanthrene d-10 or pyrene d-10 prior to extraction to allow estimation of the recovery of the unreacted pyrene and phenanthrene. After spiking with the deuterated standard, the samples were vortexed and stored overnight in a refrigerator. Each of the conical glass vials containing samples were acidified to a pH of 2 and extracted three times with 3 mL of MTBE (methyl-tert butyl ether, a total of 9 mL). The MTBE/aqueous mixtures were centrifuged at 900 g for 10 minutes to separate the aqueous and MTBE layers. The MTBE extracts were combined and allowed to evaporate overnight in a standard hood and the following morning the remaining MTBE was evaporated under nitrogen. The test tubes containing the residual solid were rinsed (vortexed) with two, 400 µl aliquots of MTBE. The MTBE was transferred to an autosampler vial (with a borosilicate glass insert) and evaporated to dryness in a speed vac. This concentration and transfer step was repeated twice. Analytical procedures were designed to analyze oxidized (hydroxylated) phenanthrene and pyrene metabolites.

The samples were then derivatized (silylated) with *N,O*-Bis(trimethylsilyl)- trifluoroacetamide (BSTFA) with 1% trimethylsilyl chloride (TMSC) to convert the hydroxyl groups to trimethyl silyl groups. Extracts in the vials mentioned above were [10 µl BSTFA/TMCS in 200 µl CHCl3]. These samples were diluted 1/20 and 1/50 in chloroform and analyzed by GC/MS for the purpose of quantifying the unreacted PAHs. The most concentrated, undiluted samples were run as well for the purpose of identifying metabolites.

Derivatized extracts were analyzed using an Agilent 5977 Mass selective detector interfaced to an Agilent 7800 GC. A 30-meter Agilent DB5-MS column was used for the separation. The mobile phase was helium at a flow rate of 1 mL/min. The injector temperature was 240°C. A 20-min GC gradient was used for the separation with a helium flow rate of 1 mL/min. The initial temperature was held at 60°C for two minutes and then increased to 320°C at a rate of 20°/minute and held at 320°C for 10 min. Full scans were acquired for all analyses.

DNA extraction

Genomic DNA was extracted from swabs using the MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, WI, USA) according to manufacturer's instructions. For samples harvested from in vitro incubations sample DNA was extracted using alkaline lysis and Express Matrix purification (MP Biomedical, Santa Ana, CA, USA). Identification of clones was done after PCR amplification using consensus 16S rRNA gene specific primers, F1 and R1 or in the case of *C. albicans* 28S rRNA fungal specific primers NL-1 and NL-4.

Characterization of microbial community structure

Microbial community structure was characterized using high-throughput sequencing of PCR amplicons generated from the V1-V3 variable regions of bacterial 16S ribosomal RNA (rRNA) genes. Briefly, the widely used primer sets 27F/534R, targeting the V1-V3 variable region of the 16S rRNA gene of bacteria, were used for amplification as done earlier with slight modifications[53].

A two-stage PCR or "targeted amplicon sequencing" (TAS) approach was performed to generate amplicon libraries, as described previously[54,55]. In the first of the TAS procedure, the templates were amplified (28 cycles) using primers containing 27F and 534R 16S rRNA gene sequence containing 5' linkers CS1 and CS2 linkers, as described previously35. PCR were performed in 10 µl reaction volumes using the KAPA HiFi HotStart PCR Kit. The PCR conditions were as follows: 5 min initial denaturation at 95°C, followed by 28 cycles of: 95°C for 30", 50°C for 30", 72°C for 60". Subsequently, a second PCR reaction was established, with 1 µl of amplification product from the first stage used as input to the second reaction.

The primers for the second stage amplifications were the AccessArray barcoding system primers (Fluidigm, San Francisco, CA, USA), containing Illumina sequencing adapters, sample-specific barcodes, and CS1 and CS2 linkers. PCR conditions for the second reaction were as follows: 5 min initial denaturation at 95°C, followed by 8 cycles of: 95°C for 30", 60°C for 30", 72°C for 60". Samples were pooled in equimolar ratio and quantified using a Qubit 2.0 fluorometer.

Sequencing was performed on an Illumina MiSeq sequencer using standard V3 chemistry with paired-end, 300 base reads. Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. Demultiplexing of reads was performed on instrument. Library preparation was performed at the DNA Services Facility at the University of Illinois at Chicago.

Bioinformatics Analysis

Raw paired-end FASTQ files were merged using the Paired-End reAd merger (PEAR) algorithm[56]. Merged data were then quality trimmed (Q20), and sequences shorter than 450 bases were removed. The remaining sequences were exported as FASTA and processed through the software package QIIME (v1.8.0)[57]. Sequences were screened for chimeras using the USEARCH61 algorithm and putative chimeric sequences were removed from the data set[58]. Chimera-free samples were then pooled, and clustered into operational taxonomic units (OTU) at 97% similarity using USEARCH assigned to taxonomic levels from phylum to species.

Statistical Analysis

BIOMs were used to identify taxa which were significantly differentially abundant between a priori defined groups. Differences in microbiota taxonomic abundance between the groups were tested using Welch's t-test using the software package STAMP[59]. Significance was set at $P < 0.05$. The Student t-test was used to compare number of distinct taxa isolated from smokers and nonsmokers, which takes into account that several smokers produced multiple taxa in the assay

Results

Survival of sampled oral mucosal bacteria after long-term PAH exposure

Studies of environmental and skin microorganisms have revealed bacteria that survive in high levels of PAHs can use these chemicals as a nutrient source. We took advantage of that knowledge to investigate functional changes in the oral mucosa microbiome that occur with exposure to tobacco smoke PAHs. First, we took swab samples from the oral mucosa of 6 nonsmokers and 14 smokers. The washed microorganisms were than inoculated into minimal broth and allowed to incubate for 3 weeks in the presence of a cocktail of PAHs as the only carbon source. At the end of the incubation time, the cells were plated on rich medium agar plates (BHI) for 48 h to allow the detection of surviving bacteria. We found that PAH selection indeed allowed the survival and then growth of several bacteria that were identified by 16S rRNA gene sequencing (Figure 1). There were microbes in the oral cavity that could survive these harsh selective conditions where PAH was the only carbon source, and cigarette smokers showed higher numbers of these microbes (Fisher's Exact Test, $p < 0.018$). In addition, multiple isolates revealed the presence of the yeast *C. albicans*, which was identified by 28S rRNA gene sequencing.

Differences in oral mucosal bacteria with long-term tobacco usage

Having identified oral bacteria on the oral mucosa of smokers as resistant to PAH exposure, the question remained whether these bacteria comprised a significant level of the mucosal microbiome and if they were indeed elevated on oral mucosal surfaces in smokers. Mucosal surface samples were taken from two sites, the gingiva and lateral border of the tongue. These sites were chosen because they are common sites of tobacco-associated OSCC. A total of 37 subjects, 15 smokers (average age 53.6, 9 Male, 6 Female) and 22 nonsmokers (average age 42.1, 12 Male, 10 Female) were selected. The bar graph reveals the major bacterial taxa in this population. In fact, of the 8 genera identified in the earlier assay only *Staphylococcus* and *Actinomyces* were present at higher than 0.01% of oral bacteria genera at the two mucosal sites (Table 1 and 2). Identification at the genus level does not assure identification at the species or strain level.

PAH metabolites produced by oral microorganism in vitro

Of the candidate microorganisms that showed ability to survive PAH exposure in the *in vitro* test *C. albicans* was the most commonly identified in the assay and is well known to be at appreciable level in the oral mucosa of a subset of both smokers and nonsmokers (Figure 1)[25,26]. Two separate isolates of *C. albicans* obtained from two different tobacco users were incubated with PAHs as sole carbon source. In this experiment the PAHs tested were phenanthrene and pyrene. These chemicals were chosen because they can be found at relatively high levels in the oral cavity in tobacco users[25,26] and they are readily digested by many environment microbes[13–22]. A soil-derived strain, *Mycobacterium rutilum*, capable of

metabolizing pyrene or phenanthrene as sole carbon source was used as a positive control. Comparative metabolism studies clearly show that *C. albicans* does not oxidize PAHs to a significant extent while mycobacteria do readily. These results are summarized in the Figure 2 bar graph. The first two columns on the left in the figure are the negative controls and represent the recoveries of phenanthrene (PhYe, 83 ± 7%) and pyrene (PyYe, 84 ± 11%) from the incubation matrix. The two bars in the center represent the amount of PAH recovered from *C. albicans* after incubation (PhYe and PyYe). The two bars on the right represent the amount of each PAH recovered from the mycobacteria incubations (PhMy and PyMy). The data indicates that the metabolism of the phenanthrene in the mycobacterium is nearly complete. Only 1.3± 1.6% of the phenanthrene remains after incubation. Pyrene was more difficult for the mycobacterium to digest. Here 29± 6% of the starting concentration remained after two weeks of incubation. The recoveries of the PAHs from the *C. albicans* (76 ± 10% and 91 ± 3 %) are like those from the un-incubated matrices (83 ± 7% and 84 ± 11%). The data suggests that *C. albicans* does not degrade PAH significantly when compared to the mycobacteria.

Characterization of Metabolic Products

The most abundant potential phenanthrene and pyrene metabolites formed in *C. albicans* and *Mycobacterium rutilum* incubations were assumed to have hydroxyl groups[13,19]. Therefore, extracts were silylated to enhance the metabolite response in the gas chromatography/mass spectrometry (GC/MS) analysis.

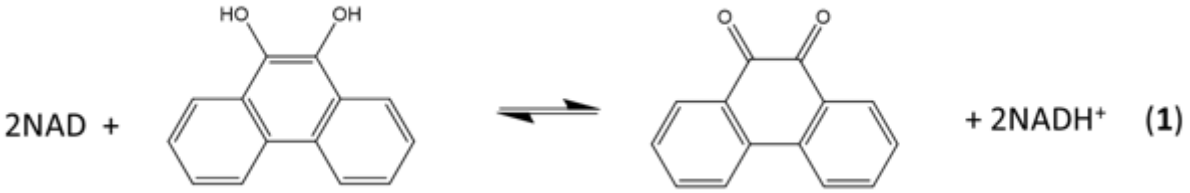
Phenanthrene

The major metabolites found in the analysis of the positive control *Mycobacterium rutilum* incubation of phenanthrene were 9,10-dihydro-9,10-phenanthrenediol, 9,10-phenanthrenedione, and dihydroxyphenanthrene. A mass spectrum of the trimethylsilyl derivative of the 9,10-dihydrophenanthrene diol generated by the mycobacterium is shown in Figure 3. Analysis of chromatograms derived from extracts from oral yeast *C. albicans* and phenanthrene co-incubation failed to detect similar, or any, metabolites.

The molecule ion is m/z 356. Characteristic fragment ions include a loss of CH_3 (m/z 341) and an ion at m/z 117 [$(CH_3)_3SiCH_2CH_3^+$]. We also observed a compound with a fragmentation pattern similar to the diol in Figure 3 eluting at 9.95 min with a molecular mass two daltons less. The mass spectra suggested that this compound was the 9,10-dihydroxypyrene (Figure 4).

The molecule ion is observed at m/z 354 and the ions at m/z 339 and m/z 117 were formed in the same way as in the spectra of the derivatized diol in Figure 3. The 9,10-dihroxypyrene was detected in the study of mycobacteria metabolism by GC/MS as a dimethyl derivative (m/z 238)[15]. Figure 4 mass spectra also shows characteristic ions at m/z 264 formed by the loss of $(CH_3)_3SiOH$ as well. In human hepatic liver[27,28] and yeast[29] cells, 9,10-dihydroxyphenanthrene is in a redox equilibrium with 9,10-phenanthrenedione (Equation 1). Under aerobic conditions formation of the dione is favored[27]. Therefore, if we find the 9,10-dihydroxyphenanthrene we should observe the dione as well. The molecular mass of

the 9,10-phenanthrene dione is 208 daltons. The m/z 208 ion was observed in the mycobacterium sample (Figure 5A) but not in the *C. albicans* (Figure 5B) shown below. This observation provides more evidence that *C. albicans* was not oxidizing PAHs.



Pyrene

Shown are spectra from products produced by positive control *Mycobacterium* digestion of pyrene. No similar products, nor any other products, were detectable after pyrene incubation with *C. albicans*. Pyrene formed both mono- and dihydroxy metabolites in the mycobacterium incubations. Two silylated dihydroxy pyrene metabolites with molecule ions at m/z 380 were observed with retention times of 10.29 and 10.50 min, respectively (Figure 6). The mass spectra of the two compounds were similar (Supplemental Figure 1). These two compounds were most likely the silylated trans (Rt 10.29) and cis (Rt 10.50) 4,5-dihydroxy-4,5-dihydropyrene. Heitkamp et al. used HPLC, mass spectrometry, and NMR to determine the structures of the two most abundant *Mycobacterium* pyrene degradation products[30]. The trans isomer was found to be more abundant and have a shorter retention time than the cis isomer

Two mono-hydroxypyrene metabolites were detected in the mycobacterium incubation as well. The most abundant was determined to be 1-hydroxypyrene (Supplemental Figure 2A) through co-chromatography with a standard compound. 1-Hydroxypyrene is a human urinary metabolite of pyrene that is frequently monitored to assess PAH exposure in humans[31,32]. The most abundant peak in the Supplemental Figure 2 mass spectra was the molecule ion at m/z 290. We also observed a compound eluting at Rt 10.84, whose mass spectra was similar to that of the silylated 1-hydroxypyrene (Supplemental Figure 2A). There are three possible mono-hydroxy isomers. To our knowledge, the 2- and 4-hydroxy isomers have not been observed as metabolites in any previous study of pyrene metabolism. The abundance of the mono hydroxyl isomer in Supplemental Figure 2B is about 1% of the 1-hydroxypyrene shown in Supplemental Figure 2A.

Discussion

In vitro selection of bacteria that survive in minimal media in the presence of either a single PAH or a mixture of PAHs is a common assay to select for potential PAH-degrading environmental bacteria[18,20,21,24]. Surprisingly, when applied to oral isolates, taxa not commonly associated with the oral cavity, such as *Bacillus pumilus, Bacillus subtilis, Rhodococcus and Agrobacterium tumefaciens*, were identified. To verify this result, a second sample was taken from one subject weeks after the first with the

selection isolating the same species, *Agrobacterium tumefaciens*. While these bacteria may come from the environment, and Al-Hebshi have shown the presence of soil taxa in the oral cavity[33], it is important to note that *Bacillus pumilus, Bacillus subtilis, Acinetobacter*, and *Staphylococcus epiderimis* have been shown to be present in cigarette tobacco[34-36]. Overall, this may lead one to conjecture that the reason why cigarette smokers had more of these bacteria in the *in vitro* assay is that they are being contaminated on a continuing basis through constant handling of cigarettes.

Bacterial species typically found in soil*, Acinetobacter baumannii, Acinetobacter Junii, Bacillus subtilis, Bacillus pumilus, Kocuria rhizophila* have all been shown to be able to metabolize pyrene, naphthalene, and/or phenanthrene in related assays[37-40]. The same is true for some species of *Rhodococcus* but it is not clear how many species of this genus have that property[41,42]. With rare exceptions, they are maintained at low levels in the oral cavity. *Acinetobacter* and *Bacillus* genera were detectable at approximately 0.02% of the gingival and lingual biofilms. *Rhodococcus* and *Micrococcus* genera were easily detectable in a small minority of subjects at levels approaching 0.1%. *Staphylococcus epidermis* was selected due to survival in PAH in oral samples from 2 different subjects. Some species of *Staphylococcus*, such as *Staphylococcus epidermidis,* have been shown to be capable of metabolizing PAH[43,44]. *Staphylococcus* as a genus was shown to be enriched 6x to 0.3% of the bacteria genera on gingival mucosa of cigarette smokers, suggesting their resistance to PAH toxicity may select for them *in vivo* (Table 1). However, the level of the specific species would be lower. *Actinomyces* was also selected in the assay, but it is unclear which species was present. While this work is consistent with the conclusion that smokers have bacteria on their oral mucosal capable of metabolizing PAH, most taxa are probably at too low a level to cause much damage.

This work stands in contrast to that of Sawada *et al*. who used a similar approach to study skin microbes[21]. Their 4-week assay of skin microbes with PAH(s) as sole carbon source revealed multiple common core species from the skin of each human subject were able to thrive. Furthermore, most were capable of completely metabolizing Benzo[a]pyrene and about half able to proliferate when grown on phenanthrene. Differences in the assay may contribute to the findings as Sawada et al. used Benzo[a]pyrene as the sole carbon source in the selection, while in this study a mixture of 5 PAHs was used. Nevertheless, it is apparent that PAH metabolizers are more ready isolatable from skin than the oral cavity, suggesting a low level of PAH metabolizing microbes in the oral environment [45]. One might speculate that oral niches for bacteria are less hospitable to PAH metabolizing microbes than those of the skin.

The *in vitro* assay identified oral microorganism that survived high levels of PAH as a sole carbon source. These PAH-metabolizing microorganisms are found at substantial levels in the oral microbiome. The yeast, *C. albicans*, was also found to survive the PAH assay, and was more frequently isolated from smokers than nonsmokers. Members of the *Candida* genus, including *Candida tropicalis, Candida maltosalike, Candida viswanathii,* isolated from environmental sites have been shown to be capable of metabolizing pyrene, phenanthrene, benzo[a]pyrene, napthalene, etc.[18,22,46] though it was not known if *C.*

albicans had that ability (see Figure 6). Because of the high level of *C. albicans* in the mouths of many people, an in-depth analysis of oral *C. albicans* strains' ability to metabolize PAHs was undertaken.

Preliminary studies revealed of benzo[a]pyrene, chrysene, fluoranthrene, phenanthrene, and pyrene assayed separately with *C. albicans* isolated from the oral cavity of a tobacco user, none produced obvious products (data not shown). To allow higher resolution in the assay the focus was put on two model PAHs, known to be metabolized by other *Candida* species[14,18,37,42,47]. In four repetitions of this experiment minimal loss of starting material occurred when pyrene or phenanthrene PAHs were incubated over 14 days with *C. albican*s isolated from smokers. Any losses of material were similar to that seen with negative controls that lacked microbes but were similarly incubated and isolated. This contrasts with the *Mycobacterium rutilum*, which metabolized 98% of the phenathrene and 79% of the pyrene.

An initial rough scan revealed no obvious metabolites from pyrene or phenanthrene incubated with *C. albicans*. For the positive control, microbe incubation with phenanthrene detected products included 9,10-phenanthrene quinone, dihydroxyphenanthrene, and phenanthrene diol, all detectable when phenanthrene are metabolized by *Mycobacterium rutilum* and other environmental bacteria. And for pyrene, incubation with *Mycobacterium rutilum* produced 4,5-dihydro-4,5-pyrenediol, 1,2-dihydro-1,2-pyrenediol, 2-Hydroxypyrene and 4-Hydroxypyrene. Unlike the *Mycobacterium rutilum* incubation with phenathrene little quinone was formed. Analysis done at higher resolving power to look for possible oxidation products of the two PAHs by the oral *C. albicans* revealed none.

The *C. albicans* isolates studied were from the mucosa of tobacco users, assumed to have continual exposure to PAHs, and were subjected to an additional selection for 3 weeks in a mixture of PAHs including these two substrates. Finally, they were given two weeks incubation with either pyrene or phenanthrene to allow detection of the PAH metabolizing activity. The simplest conclusion is that despite their resistance to PAH incubation (see Figure 1) there is a lack of PAH metabolizing activity in the *C. albicans* strains tested. A limit of this study is that maybe under different assay conditions *C. albicans* strains isolated would show PAH metabolism. It is possible that assays with mixtures of oral microbes might show easier to detect PAH metabolism[13,48,49]. At times, incubation of a second carbon containing molecule, such as lactose can stimulate PAH metabolism[13,50-52]. However, the standard approach to identify PAH-metabolizing microbes has shown great facility in identifying soil metabolizing skin microbes. This approach has failed to do the same in the oral cavity for the phenanthrene and pyrene. *Staphylococcus epidermidis* and *Actinomyces* remain as candidates to be PAH metabolizers of the oral mucosa. However, if *C. albicans* can indeed metabolize PAH it has lower activity than the positive control, *Mycobacteria Rutilum* and, likely, skin bacteria[15,21,23].

Declarations

Data availability

The sequencing data from this study are available on request.

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Author Contributions

LT, JLS, MPC, and GRA, designed the research; MPC, JLS, BS, GRA, SIP, SJG and JVD, conducted the research; GRA, SJG and MPC analyzed the data; and GRA, MPC, and LT wrote the manuscript. GRA, LT, and MPC had primary responsibility for the final content. All authors read and approved the final manuscript.

Competing Interests

There are no competing interests to declare.

Additional information

Supplementary information is available for this paper.

References

1. Brook, I. The impact of smoking on oral and nasopharyngeal bacterial flora. *J Dent Res*, **90**, 704–710 https://doi.org/10.1177/0022034510391794 (2011).
2. Kumar, P. S., Matthews, C. R., Joshi, V., de Jager, M. & Aspiras, M. Tobacco smoking affects bacterial acquisition and colonization in oral biofilms. *Infect Immun*, **79**, 4730–4738 https://doi.org/10.1128/IAI.05371-11 (2011).
3. van Winkelhoff, A. J., Bosch-Tijhof, C. J., Winkel, E. G. & van der Reijden, W. A. Smoking affects the subgingival microflora in periodontitis. *J Periodontol*, **72**, 666–671 https://doi.org/10.1902/jop.2001.72.5.666 (2001).
4. Zambon, J. J. *et al.* Cigarette Smoking Increases the Risk for Subgingival Infection With Periodontal Pathogens. *J Periodontol*, **67 Suppl 10S**, 1050–1054 https://doi.org/10.1902/jop.1996.67.10s.1050 (1996).
5. Taybos, G. Oral changes associated with tobacco use. *Am J Med Sci*, **326**, 179–182 https://doi.org/10.1097/00000441-200310000-00005 (2003).

6. Segata, N. *et al.* Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol* **13**, R42, doi:10.1186/gb-2012-13-6-r42 (2012).
7. Thomas, A. M. *et al.* Alcohol and tobacco consumption affects bacterial richness in oral cavity mucosa biofilms. *BMC Microbiol*, **14**, 250 https://doi.org/10.1186/s12866-014-0250-2 (2014).
8. Yu, G. *et al.* The effect of cigarette smoking on the oral and nasal microbiota. *Microbiome*, **5**, 3 https://doi.org/10.1186/s40168-016-0226-6 (2017).
9. Wu, J. *et al.* Cigarette smoking and the oral microbiome in a large study of American adults. *ISME J*, **10**, 2435–2446 https://doi.org/10.1038/ismej.2016.37 (2016).
10. Ding, Y. S., Trommel, J. S., Yan, X. J., Ashley, D. & Watson, C. H. Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes. *Environ Sci Technol*, **39**, 471–478 https://doi.org/10.1021/es048690k (2005).
11. Rodgman, A., Smith, C. J. & Perfetti, T. A. The composition of cigarette smoke: a retrospective, with emphasis on polycyclic components. *Hum Exp Toxicol*, **19**, 573–595 https://doi.org/10.1191/096032700701546514 (2000).
12. Habe, H. & Omori, T. Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci Biotechnol Biochem*, **67**, 225–243 https://doi.org/10.1271/bbb.67.225 (2003).
13. Ghosal, D., Ghosh, S., Dutta, T. K. & Ahn, Y. Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Front Microbiol*, **7**, 1369 https://doi.org/10.3389/fmicb.2016.01369 (2016).
14. Hadibarata, T., Khudhair, A. B., Kristanti, R. A. & Kamyab, H. Biodegradation of pyrene by Candida sp. S1 under high salinity conditions. *Bioprocess Biosyst Eng*, **40**, 1411–1418 https://doi.org/10.1007/s00449-017-1798-7 (2017).
15. Hennessee, C. T. & Li, Q. X. Effects of Polycyclic Aromatic Hydrocarbon Mixtures on Degradation, Gene Expression, and Metabolite Production in Four Mycobacterium Species. *Appl Environ Microbiol*, **82**, 3357–3369 https://doi.org/10.1128/AEM.00100-16 (2016).
16. Hesham, A. E., Alamri, S. A., Khan, S., Mahmoud, M. E. & Mahmoud, H. M. Isoaltion and molecualr genetic characterization of a yeast strain able to degreade petroleum polycyclic aromatic hydrocarbons. *African J. Biotech*, **8**, 6 (2009).
17. Husain, S. Literature overview: Microbial metabolism of high molecular weight polycyclic aromatic hydrocarbons. *Remediation*, **18**, 31 https://doi.org/10.1002/rem.20165 (2008).
18. MacGillivray, A. R. & Shiaris, M. P. Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments. *Appl Environ Microbiol*, **59**, 1613–1618 https://doi.org/10.1128/aem.59.5.1613-1618.1993 (1993).
19. Mallick, S., Chakraborty, J. & Dutta, T. K. Role of oxygenases in guiding diverse metabolic pathways in the bacterial degradation of low-molecular-weight polycyclic aromatic hydrocarbons: a review. *Crit Rev Microbiol*, **37**, 64–90 https://doi.org/10.3109/1040841x.2010.512268 (2011).

20. Mbachu, A. E., Chukwura, E. I. & Mbachu, N. A. Isoation and Characterization of Hydrocarbon Degrading Fungi from Used (Spent) Engine Oil Polluted Soil and Their Use for Polycyclic Aromatic Hydrocarbons (PAHs) Degradation. Universial J of Microbiology Research 4, 4, doi:DOI: 10:13189/ujmr 2016.040105 (2016).
21. Sowada, J., Schmalenberger, A., Ebner, I., Luch, A. & Tralau, T. Degradation of benzo[a]pyrene by bacterial isolates from human skin. *FEMS Microbiol Ecol*, **88**, 129–139 https://doi.org/10.1111/1574-6941.12276 (2014).
22. Sutherland, J. B. Detoxification of polycyclic aromatic hydrocarbons by fungi. *J Ind Microbiol*, **9**, 53–61 https://doi.org/10.1007/BF01576368 (1992).
23. Sowada, J. *et al.* Toxification of polycyclic aromatic hydrocarbons by commensal bacteria from human skin. *Arch Toxicol*, **91**, 2331–2341 https://doi.org/10.1007/s00204-017-1964-3 (2017).
24. Juhasz, A. L., Stanley, G. A. & Britz, M. L. Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by Stenotrophomonas maltophilia strain VUN 10,003. *Lett Appl Microbiol*, **30**, 396–401 https://doi.org/10.1046/j.1472-765x.2000.00733.x (2000).
25. Darwazeh, A. M., Al-Dwairi, Z. N. & Al-Zwairi, A. A. The relationship between tobacco smoking and oral colonization with Candida species. *J Contemp Dent Pract*, **11**, 017–024 (2010).
26. Sheth, C. C. *et al.* Alcohol and tobacco consumption affect the oral carriage of Candida albicans and mutans streptococci. *Lett Appl Microbiol*, **63**, 254–259 https://doi.org/10.1111/lam.12620 (2016).
27. Huang, M. *et al.* Metabolism of a representative oxygenated polycyclic aromatic hydrocarbon (PAH) phenanthrene-9,10-quinone in human hepatoma (HepG2) cells. *Chem Res Toxicol*, **27**, 852–863 https://doi.org/10.1021/tx500031p (2014).
28. Matsunaga, T. *et al.* Involvement of an aldo-keto reductase (AKR1C3) in redox cycling of 9,10-phenanthrenequinone leading to apoptosis in human endothelial cells. *Chem Biol Interact*, **181**, 52–60 https://doi.org/10.1016/j.cbi.2009.05.005 (2009).
29. Rodriguez, C. E., Sobol, Z. & Schiestl, R. H. 9,10-Phenanthrenequinone induces DNA deletions and forward mutations via oxidative mechanisms in the yeast Saccharomyces cerevisiae. *Toxicol In Vitro*, **22**, 296–300 https://doi.org/10.1016/j.tiv.2007.09.001 (2008).
30. Heitkamp, M. A., Freeman, J. P., Miller, D. W. & Cerniglia, C. E. Pyrene degradation by a Mycobacterium sp.: identification of ring oxidation and ring fission products. *Appl Environ Microbiol*, **54**, 2556–2565 https://doi.org/10.1128/aem.54.10.2556-2565.1988 (1988).
31. Flores-Ramirez, R. *et al.* Exposure to polycyclic aromatic hydrocarbon mixtures and early kidney damage in Mexican indigenous population. *Environ Sci Pollut Res Int*, **28**, 23060–23072 https://doi.org/10.1007/s11356-021-12388-w (2021).
32. Jain, R. B. Concentrations of selected monohydroxy polycyclic aromatic hydrocarbons across various stages of glomerular function. *Environ Sci Pollut Res Int*, **28**, 23220–23234 https://doi.org/10.1007/s11356-021-12376-0 (2021).
33. Al-Hebshi, N. N., Nasher, A. T., Idris, A. M. & Chen, T. Robust species taxonomy assignment algorithm for 16S rRNA NGS reads: application to oral carcinoma samples. *J Oral Microbiol*, **7**, 28934

https://doi.org/10.3402/jom.v7.28934 (2015).

34. Chopyk, J. *et al.* Temporal Variations in Cigarette Tobacco Bacterial Community Composition and Tobacco-Specific Nitrosamine Content Are Influenced by Brand and Storage Conditions. *Front Microbiol*, **8**, 358 https://doi.org/10.3389/fmicb.2017.00358 (2017).
35. Rooney, A. P., Swezey, J. L., Wicklow, D. T. & McAtee, M. J. Bacterial species diversity in cigarettes linked to an investigation of severe pneumonitis in U.S. Military personnel deployed in operation iraqi freedom. *Curr Microbiol*, **51**, 46–52 https://doi.org/10.1007/s00284-005-4491-z (2005).
36. Sapkota, A. R., Berger, S. & Vogel, T. M. Human pathogens abundant in the bacterial metagenome of cigarettes. *Environ Health Perspect*, **118**, 351–356 https://doi.org/10.1289/ehp.0901201 (2010).
37. Ali, N., Eliyas, M., Al-Sarawi, H. & Radwan, S. S. Hydrocarbon-utilizing microorganisms naturally associated with sawdust., **83**, 1268–1272 https://doi.org/10.1016/j.chemosphere.2011.03.052 (2011).
38. Toledo, F. L., Calvo, C., Rodelas, B. & Gonzalez-Lopez, J. Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. *Syst Appl Microbiol*, **29**, 244–252 https://doi.org/10.1016/j.syapm.2005.09.003 (2006).
39. Chen, Y. *et al.* Microcalorimetric investigation of the toxic action of pyrene on the growth of PAH-degrading bacteria Acinetobacter junii. *J Environ Sci Health A Tox Hazard Subst Environ Eng*, **45**, 668–673 https://doi.org/10.1080/10934521003648826 (2010).
40. Hunter, R. D., Ekunwe, S. I., Dodor, D. E., Hwang, H. M. & Ekunwe, L. Bacillus subtilis is a potential degrader of pyrene and benzo[a]pyrene. *Int J Environ Res Public Health*, **2**, 267–271 https://doi.org/10.3390/ijerph2005020010 (2005).
41. Di Gennaro, P. *et al.* Genome Sequence of Rhodococcus opacus Strain R7, a Biodegrader of Mono- and Polycyclic Aromatic Hydrocarbons. *Genome Announc*, **2**, https://doi.org/10.1128/genomeA.00827-14 (2014).
42. Muangchinda, C. *et al.* Biodegradation of high concentrations of mixed polycyclic aromatic hydrocarbons by indigenous bacteria from a river sediment: a microcosm study and bacterial community analysis. *Environ Sci Pollut Res Int*, **24**, 4591–4602 https://doi.org/10.1007/s11356-016-8185-9 (2017).
43. Moscoso, F., Teijiz, I., Deive, F. J. & Sanroman, M. A. Efficient PAHs biodegradation by a bacterial consortium at flask and bioreactor scale. *Bioresour Technol*, **119**, 270–276 https://doi.org/10.1016/j.biortech.2012.05.095 (2012).
44. Ye, J., Yin, H., Peng, H., Bai, J. & Li, Y. Pyrene removal and transformation by joint application of alfalfa and exogenous microorganisms and their influence on soil microbial community. *Ecotoxicol Environ Saf*, **110**, 129–135 https://doi.org/10.1016/j.ecoenv.2014.08.031 (2014).
45. Lemoine, L. *et al.* Microbially competent 3D skin: a test system that reveals insight into host-microbe interactions and their potential toxicological impact. *Arch Toxicol*, **94**, 3487–3502 https://doi.org/10.1007/s00204-020-02841-z (2020).

46. Deng, Y., Zhang, Y., Hesham Ael, L., Liu, R. & Yang, M. Cell surface properties of five polycyclic aromatic compound-degrading yeast strains. *Appl Microbiol Biotechnol*, **86**, 1933–1939 https://doi.org/10.1007/s00253-010-2477-7 (2010).
47. Wang, X., Gong, Z., Li, P. & Zhang, L. Degradation of pyrene in soils by free and immobilized yeasts, Candida tropicals. *Bull Environ Contam Toxicol*, **78**, 522–526 https://doi.org/10.1007/s00128-007-9156-0 (2007).
48. Boonchan, S., Britz, M. L. & Stanley, G. A. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Appl Environ Microbiol*, **66**, 1007–1019 https://doi.org/10.1128/AEM.66.3.1007-1019.2000 (2000).
49. Suja, F. *et al.* Effects of local microbial bioaugmentation and biostimulaiton on the bioremediation of toal petroleum hydrocarbons in crude oail contaminated soil based on laboratory and field observations. *Internatinal Biodeterioration & Biodegradation*, **90**, 8 https://doi.org/10.1016/j.ibiod.2014.03.006 (2014).
50. Atagana, H. I., Haynes, R. J. & Wallis, F. M. Optimization of soil physical and chemical conditions for the bioremediation of creosote-contaminated soil., **14**, 297–307 https://doi.org/10.1023/a:1024730722751 (2003).
51. Feng, T., Lin, H., Tang, J. & Feng, Y. Characterization of polycyclic aromatic hydrocarbons degradation and aresnate reduction by a versatile Psuedomonas isolate. *Int. Biodeteriation & Biodegradation*, **90**, 8 https://doi.org/10.1016/j.ibiod.2014.01.015 (2014).
52. Rafin, C., Veignie, E., Fayeulle, A. & Surpateanu, G. Benzo[a]pyrene degradation using simultaneously combined chemical oxidation, biotreatment with Fusarium solani and cyclodextrins. *Bioresour Technol*, **100**, 3157–3160 https://doi.org/10.1016/j.biortech.2009.01.012 (2009).
53. Yildirim, S. *et al.* Characterization of the fecal microbiome from non-human wild primates reveals species specific microbial communities. *PLoS One*, **5**, e13963 https://doi.org/10.1371/journal.pone.0013963 (2010).
54. Bybee, S. M. *et al.* Targeted amplicon sequencing (TAS): a scalable next-gen approach to multilocus, multitaxa phylogenetics. *Genome Biol Evol*, **3**, 1312–1323 https://doi.org/10.1093/gbe/evr106 (2011).
55. Green, S. J., Venkatramanan, R. & Naqib, A. Deconstructing the polymerase chain reaction: understanding and correcting bias associated with primer degeneracies and primer-template mismatches. *PLoS One*, **10**, e0128122 https://doi.org/10.1371/journal.pone.0128122 (2015).
56. Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR., **30**, 614–620 https://doi.org/10.1093/bioinformatics/btt593 (2014).
57. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*, **7**, 335–336 https://doi.org/10.1038/nmeth.f.303 (2010).
58. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST., **26**, 2460–2461 https://doi.org/10.1093/bioinformatics/btq461 (2010).

59. Parks, D. H., Tyson, G. W., Hugenholtz, P. & Beiko, R. G. STAMP: statistical analysis of taxonomic and functional profiles., **30**, 3123–3124 https://doi.org/10.1093/bioinformatics/btu494 (2014).

Tables

Table 1 Gingiva levels of PAH-selected bacteria

Genus	Percentage Level, Never smoker	Percentage Level, Smoker	Dectected[1]	Probability[2]
Acinetobacter	0.0238+ 0.0658	0.0042+0.0103	9/35	0.256
Agrobacterium	0	0	0/35	
Actinomyces	1.25+2.45	5.08+4.86	35/35	0.0187
Bacillus	0.0723+0.180	0.00393+.000778	12/35	0.148
Kocuria	0	0	2/35	
Rhodococcus	0.00288+0.052	0.000216+0.000780	5/35	0.0586
Staphylococcus	0.0377+0.039	0.219+0.283	32/35	0.038

[1] *Number of subjects with the genus at detectable levels*

[2] Probability of difference in never smoker vs. smoker levels using Welch's t-test

Table 2 Tongue levels of PAH-selected bacteria

Genus	Percentage Level, Never smoker	Percentage Level, Smoker	Dectected[1]	Probability[2]
Acinetobacter	0	0	0/41	
Agrobacterium	0	0	0/41	
Actinomyces	2.31+3.62	5.29+5.76	41/41	0.092
Bacillus	0.00456+0.0054	0.00798+0.0272	7/41	0.639
Kocuria	0	0	0/41	
Rhodococcus	0	0	0/41	
Staphylococcus	0.07738+0.0807	0.182+0.214	40/41	0.078

[1] *Number of subjects with the genus at detectable levels*

[2] Probability of difference in never smoker vs. smoker levels using Welch's t-test

Figures

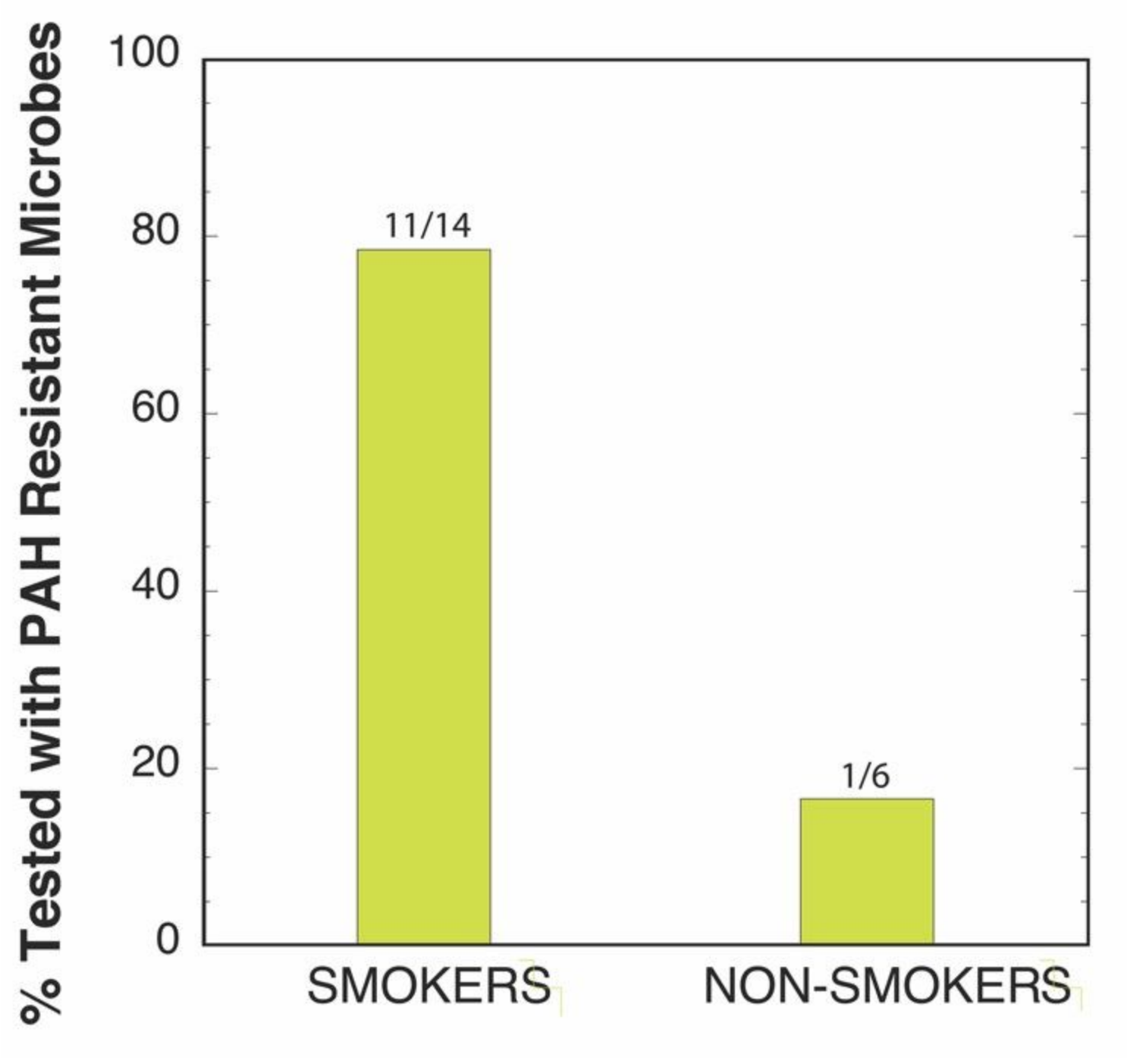

Figure 1

Oral mucosal taxa that survived three-week selection with PAHs as sole carbon source. Smokers (11 of 14): C. albicans, Acinetobacter junii, Acinetobacter baumannii, Agrobactrium tumerfaciens, Actinomyces, Bacillus pumillus, Bacillus subtilis, Kocuria rhizophila, Rhodococcus, Staphylococcus epidermidis, Staphylococcus NonSmokers (1 of 6): Rhodococcus.

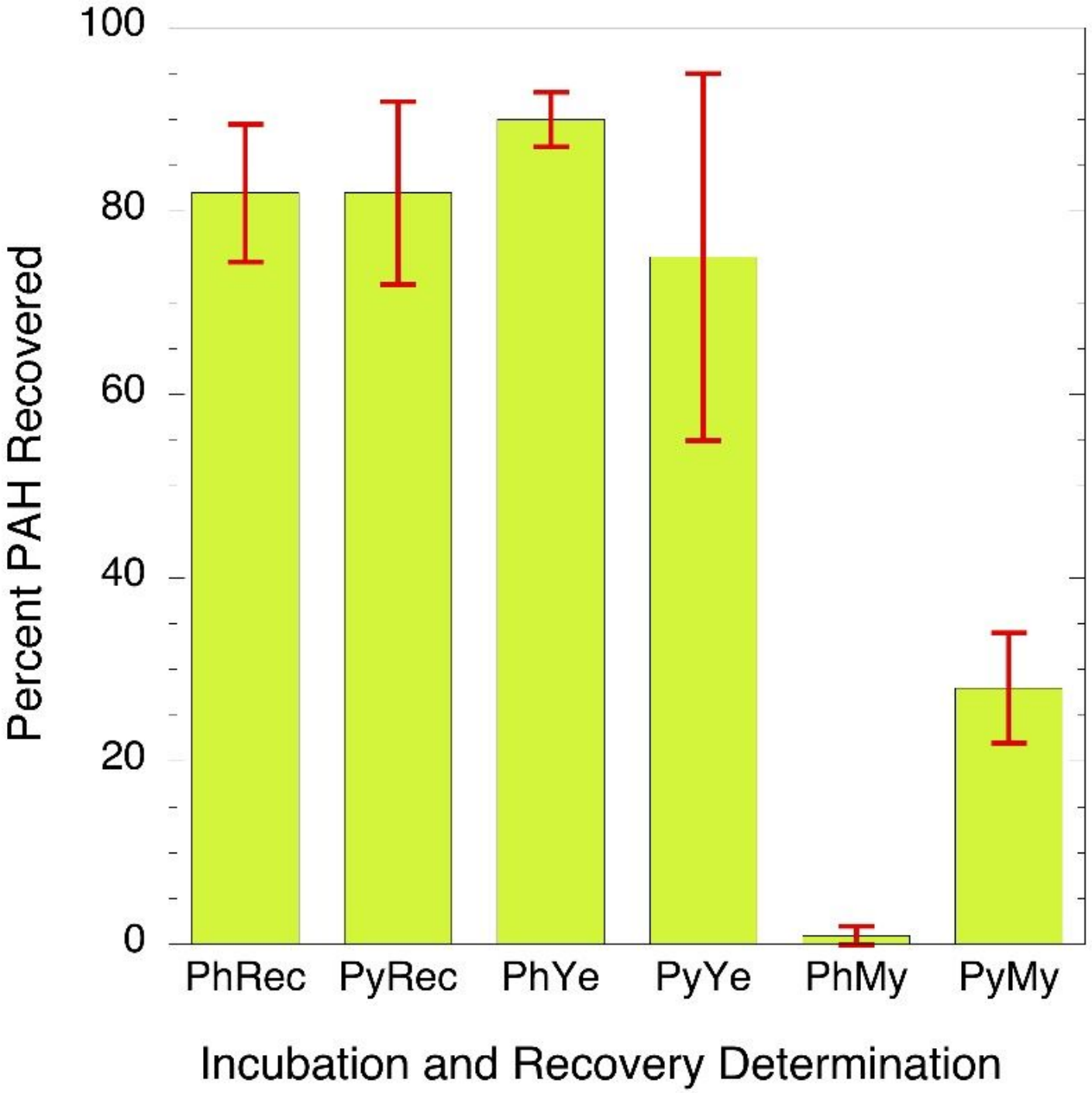

Figure 2

Bar graph showing percent of parent PAH extracted from mixtures of microorganisms and incubation broth. PhRec and PyRec = Phenanthrene and Pyrene recovery from unincubated samples; PhYe and PyYe = phenanthrene and pyrene recovered from incubated yeast samples; PhMy and PyMy = Phenanthrene and Pyrene recovery from incubated mycobacterium samples.

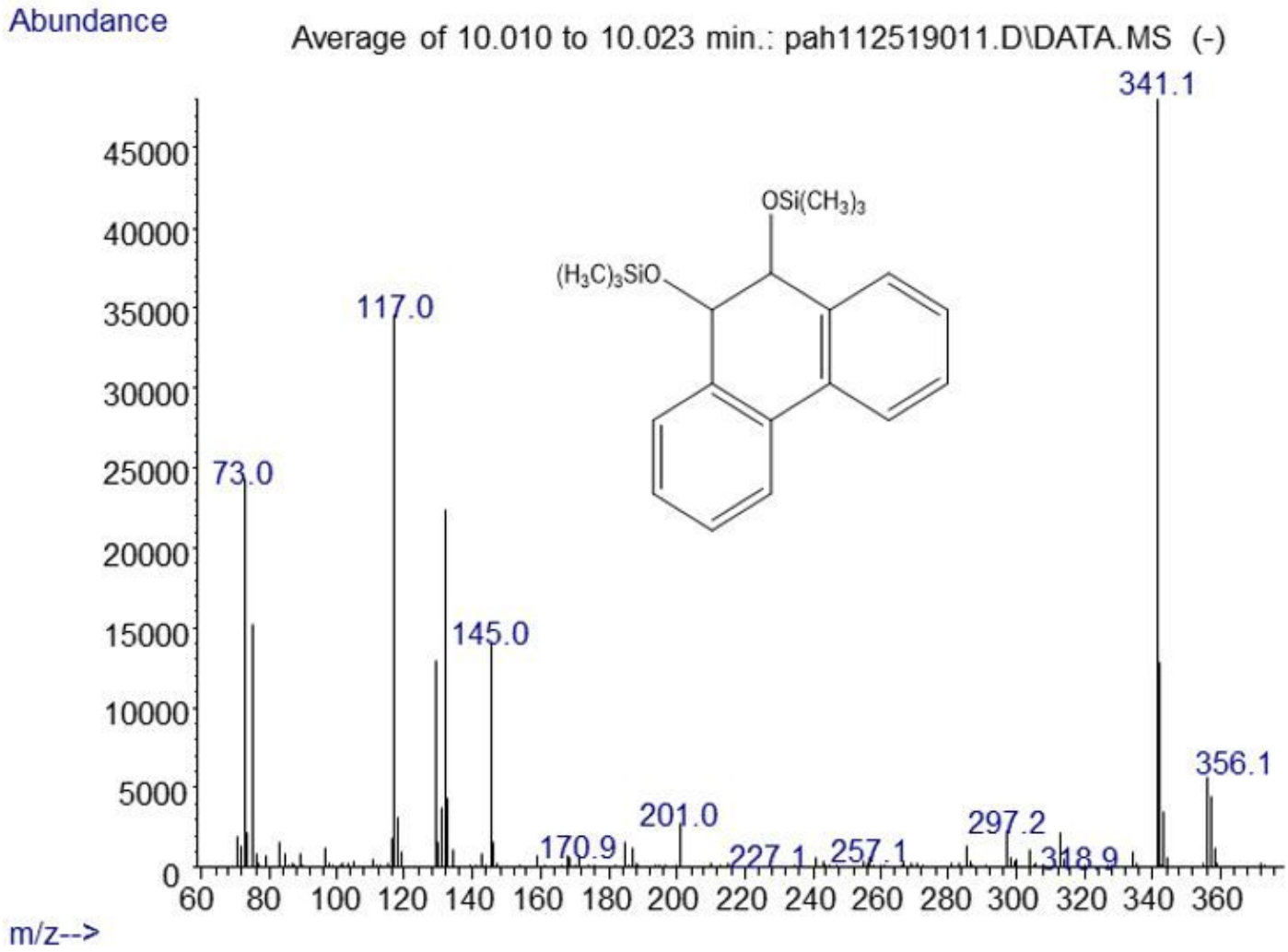

Figure 3

Electron ionization mass spectrum of compound eluting at 10 min suggested a silylated phenanthrene diol from incubation of Mycobacterium rutilum with phenanthrene.

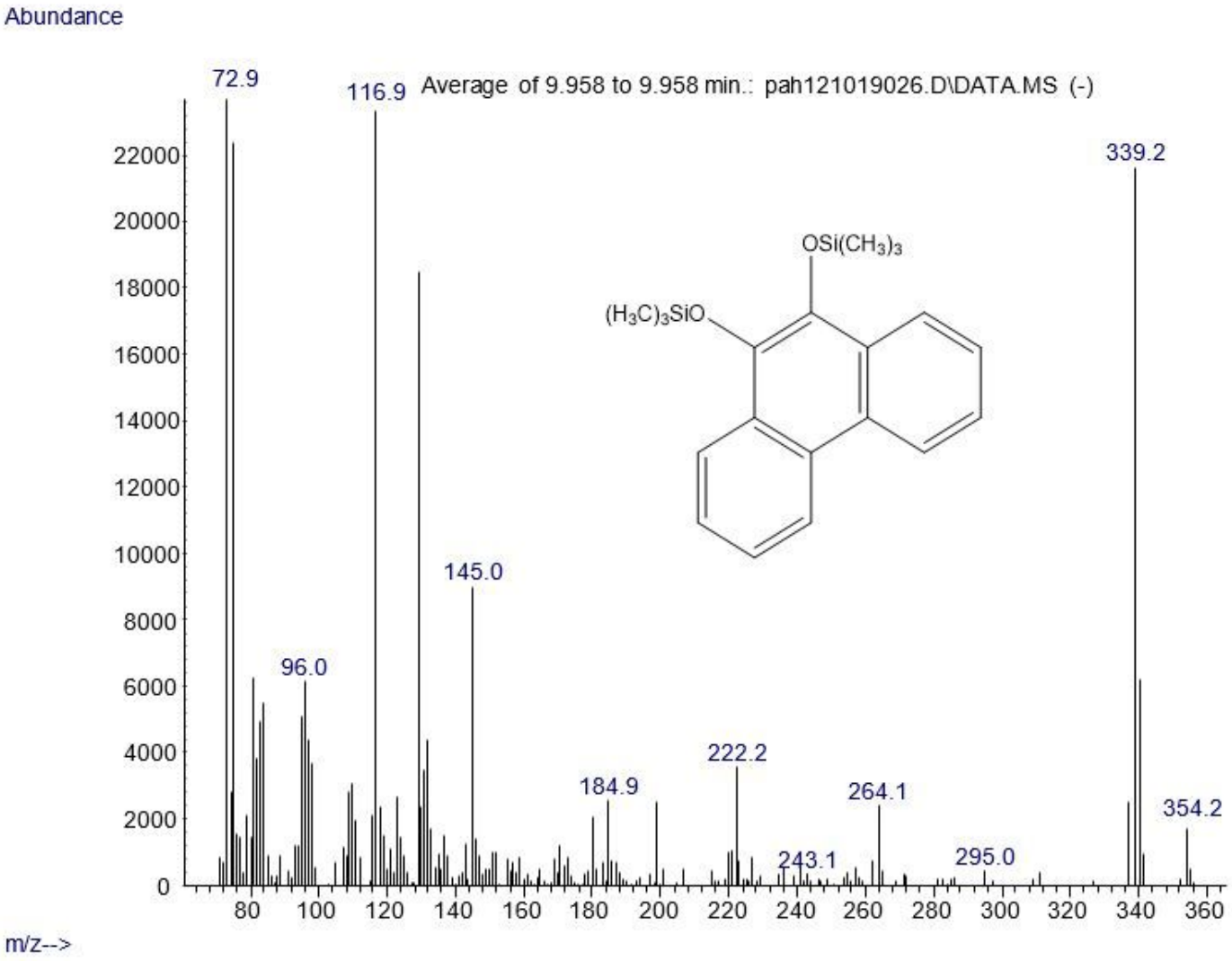

Figure 4

Electron ionization mass spectrum of compound eluting at 9.95 minutes suggested to be silylated 9,10-dihydroxyphenanthrene from incubation of Mycobacterium rutilum with phenanthrene.

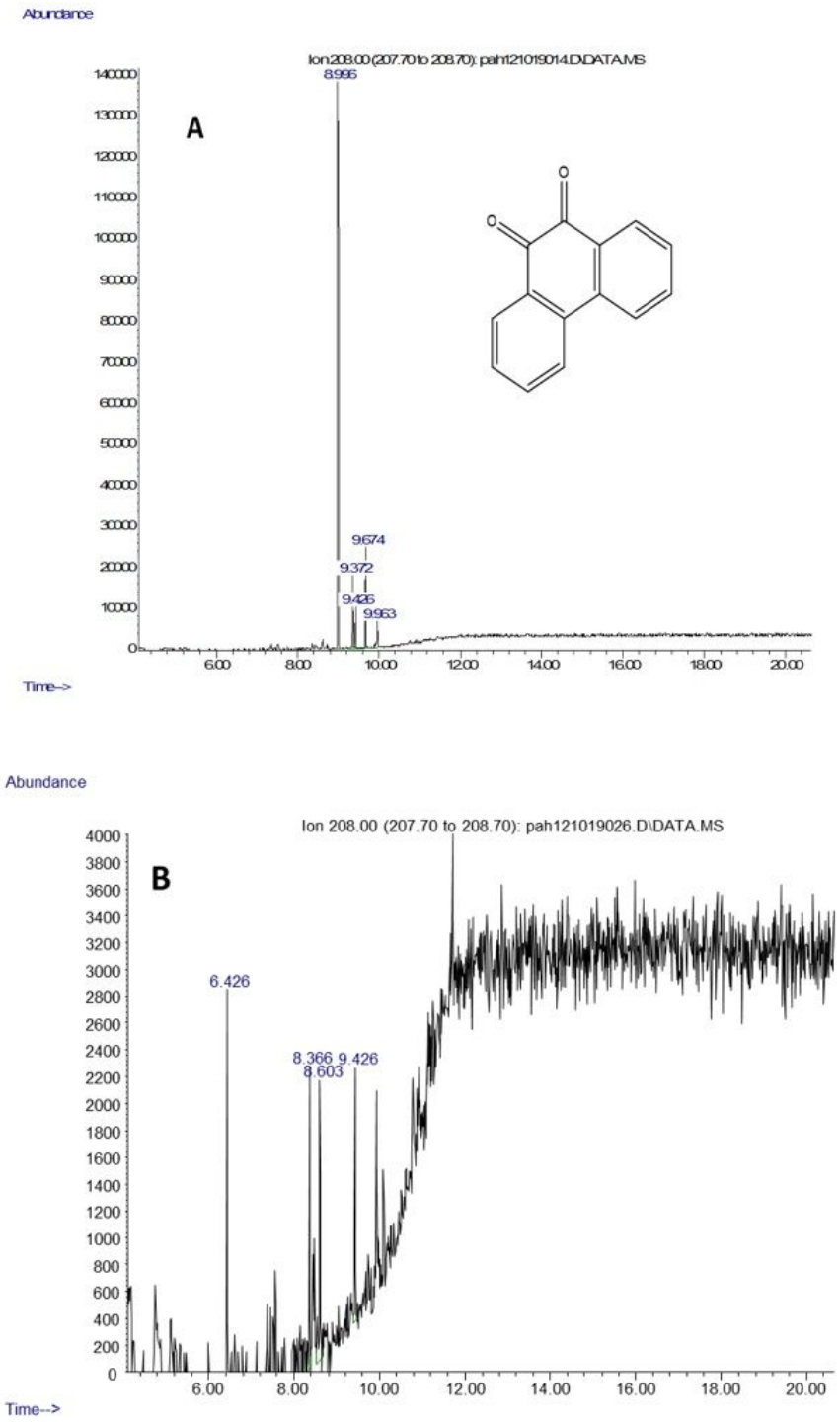

Figure 5

Extracted ion chromatograms of m/z 208 (the molecule ion of 9,10-phenanthrene quinone) derived from the GC/MS analysis of phenanthrene incubated with A) Mycobacterium rutilum and B) C. albicans.

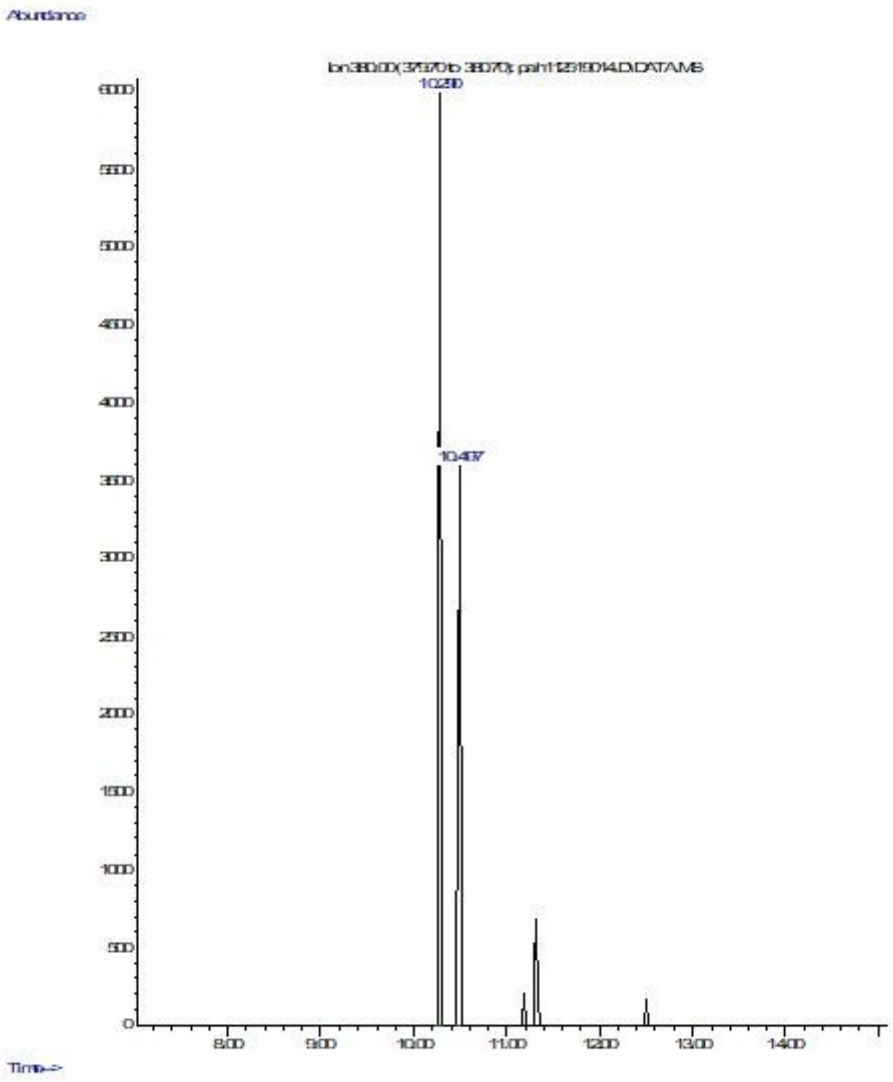

Figure 6

Extracted Ion Chromatogram of m/z 380 derived from the GC/MS analysis of pyrene incubated with Mycobacterium rutilum.

Supplementary Files

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- SupplementalDataPAHAdami.pdf