ISSN 0027-1314, Moscow University Chemistry Bulletin, 2008, Vol. 63, No. 2, pp. 120–125. © Allerton Press, Inc., 2008.

Molecular Characterization of Bacteria Isolated from Waste Electrical Transformer Oil[1]

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Received November 23, 2007

Abstract—Electrical transformer oil (ETO) includes as dielectric fluids hazardous compounds such as PAHs and sometimes PCBs, which are highly toxic and resistant to degradation. Three species of bacteria, belonging to *Acinetobacter lwoffii, Bacillus amyloliquefaciens*, and *Bacillus pumilus*, were isolated from waste ETO. Phenotypic and molecular assays revealed a high potential of *A. lwoffii* for catabolism of phenanthrene and ETO as a sole carbon and energy source. This article reports for the first time the isolation, identification, and characterization of bacterial diversity hidden in ETO.

DOI: 10.3103/S0027131408020168

Electrical transformer oil (ETO) is a highly refined mineral oil that is stable at high temperatures and has excellent electrical insulating properties. Polychlorinated biphenyls (PCBs), synthetic chlorinated organic chemicals resistant to heat and chemically stable, are widely used as dielectric fluids and heat transfer agents in ETO. Nevertheless, PCBs are highly mutagenic and carcinogenic compounds belonging to the larger group of persistent organic pollutants (POPs) [1, 2]. In addition to being lipophilic (bioaccumulative) and hardly degraded by microorganisms, PCBs on incineration produce compounds such as dioxins and dibenzofurans, causing toxic effects on living beings and the environment [3, 4]. In Venezuela, there are currently about 2600 t of PCBs corresponding to the national electric and basic industries. Due to the incorporation of Venezuela into the Stockholm Convention, is necessary to safeguard the final disposal, management, and degradation of POPs such as PCBs [5, 6]. Bacteria have been shown to degrade PCB compounds directly and cometabolically under anaerobic and aerobic conditions [7]. The aim of this work is to isolate, identify, and characterize autochthonous cultivable bacterial strains from a waste ETO mixture in order to use them as biocatalysts in novel green processes for ETO biodegradation.

MATERIALS AND METHODS

Isolation of Bacteria from Waste ETO and Culture Conditions

A waste ETO sample was obtained from Electricity of Caracas (Venezuela). A standard enrichment technique was used to isolate cultivable bacteria from waste ETO. Erlenmeyer flasks (500 mL) containing a mixture of 100 mL Luria–Bertani (LB) medium [8] and 25 mL ETO were incubated at 30°C in a rotary shaker (200 rpm). Following five enrichment cycles, 1 mL of the culture was diluted and plated on basal salt medium (BSM) agar plates [9] supplemented with 25% (v/v) ETO as a sole carbon and energy source.

ETO Chemical Analysis

To determine the saturated and aromatic fraction in ETO samples, a method for separation of defined fractions from petroleum products and lubricants was used [10]. The PCB concentration was analyzed using analytical methods for Aroclors included in Environmental Protection Agency Method 8082 using a capillary column with GC/ECD [11–13].

Molecular Identification of Bacterial Strains from Waste ETO

Genomic DNA from each bacterial strain was extracted as described previously [14]. Two different DNA fingerprinting techniques were performed using the ERIC-PCR [15, 16] and GIRRN-LIRRN [17] sets of primers. PCR amplifications of the 16S rRNA gene were performed as described previously [18, 19]; purification and sequencing was carried out using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, California). DNA sequence analysis was performed using the Lasergene software package (DNASTAR, Inc., United Kingdom), BLASTN [20], and FASTA [21]. All other nucleic acid manipulations were carried out by standard methods [22].

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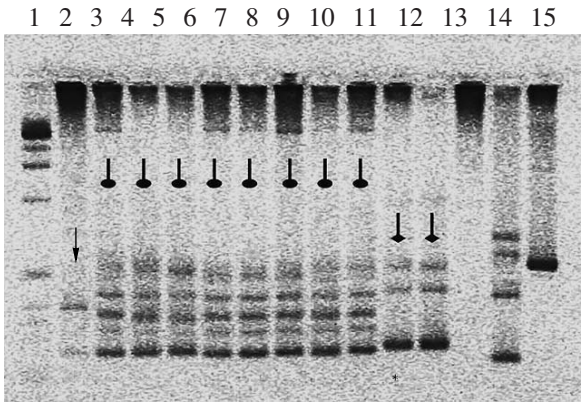

Fig. 1. DNA fingerprinting of the 11 bacterial strains isolated from waste ETO using GIRRN-LIRRN primer pairs. Note that the presences of the three different groups of strains are indicated with different symbols. 16S rRNA gene sequencing confirmed that the three defined groups belong to *Acinetobacter lwoffii* (one strain), Bacillus amyloliquefaciens (eight strains), and Bacillus pumilus (two strains). (*1*) 1-kb DNA ladder; (*2*) PCB1; (*3*) PCB2; (*4*) PCB3; (*5*) PCB4; (*6*) PCB5; (*7*) PCB6; (*8*) PCB7; (*9*) PCB8; (*10*) PCB9; (*11*) PCB10; (*12*) PCB11; (*13*) PCR reaction control; (*14*, *15*) *P. putida* 9816 and *Pseudomonas* sp. R1 used as controls, respectively.

Ability of Bacterial Strains to Use Polyaromatic Hydrocarbons (PAHs) as a Sole Carbon and Energy Source

The bacterial strains were tested for their ability to grow on BSM agar (2% w/v) plates supplemented with 5% (w/v) naphthalene or phenanthrene as a sole carbon and energy source and incubated at 30°C.

Molecular Detection of Genes Involved in PAH Degradation

The *nah*Ac gene, encoding the large subunit of naphthalene dioxygenase, was amplified by PCR using the following set of primers: FAc (5'-CCCYGGCGACTATGT-3r) and RAc (5'-CCTCRGGCATGTCTTTTTC-3') [23]. An 840-bp DNA fragment internal to the *nah*Ac gene from *Pseudomonas putida* R1 was used as a probe in the hybridization experiments.

Ability of Bacterial Strains to Use Waste ETO as a Sole Carbon Source

To study the ability to use ETO as a sole carbon and energy source, the bacterial strains were grown in dif-

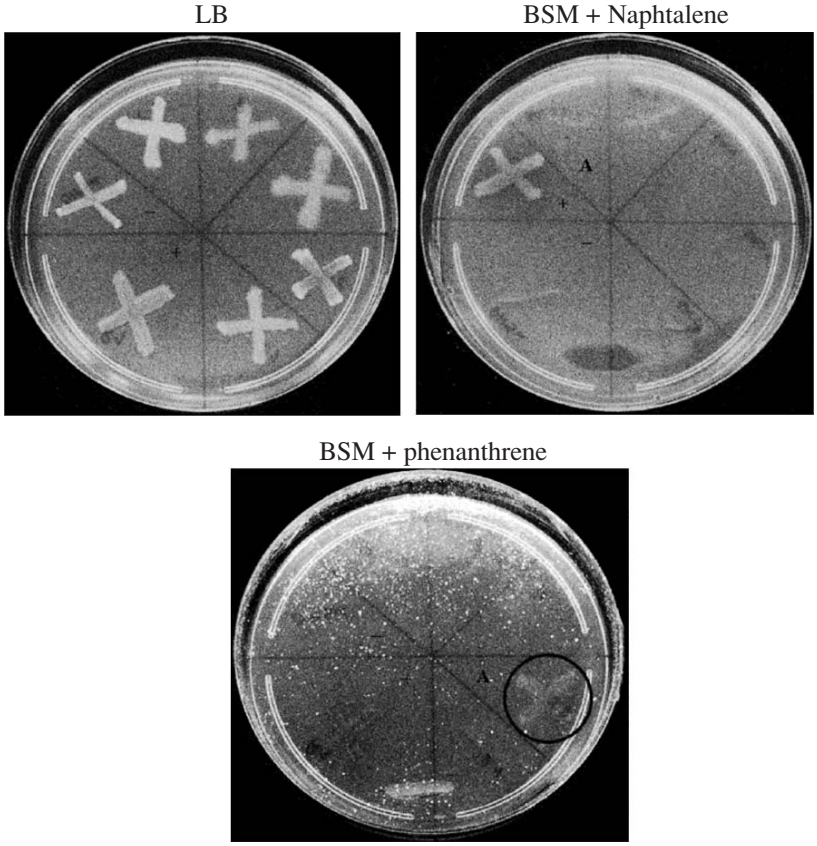

Fig. 2. Phenotypic characterization of bacterial strains according to their ability to grow with naphthalene or phenanthrene as a sole carbon and energy source. Note that all bacteria were incapable of growth with naphthalene or phenanthrene except *A. lwoffii*, which was able to grow with phenanthrene. PCB2.2: *A. lwoffii*; PCB2.1 and PCB11: *B. pumilus*; PCB4 and PCB7: *B. amyloliquefaciens*; R1 and DH5a: *Pseudomonas* sp. R1 and *E. coli* DH5± used as positive and negative controls, respectively.

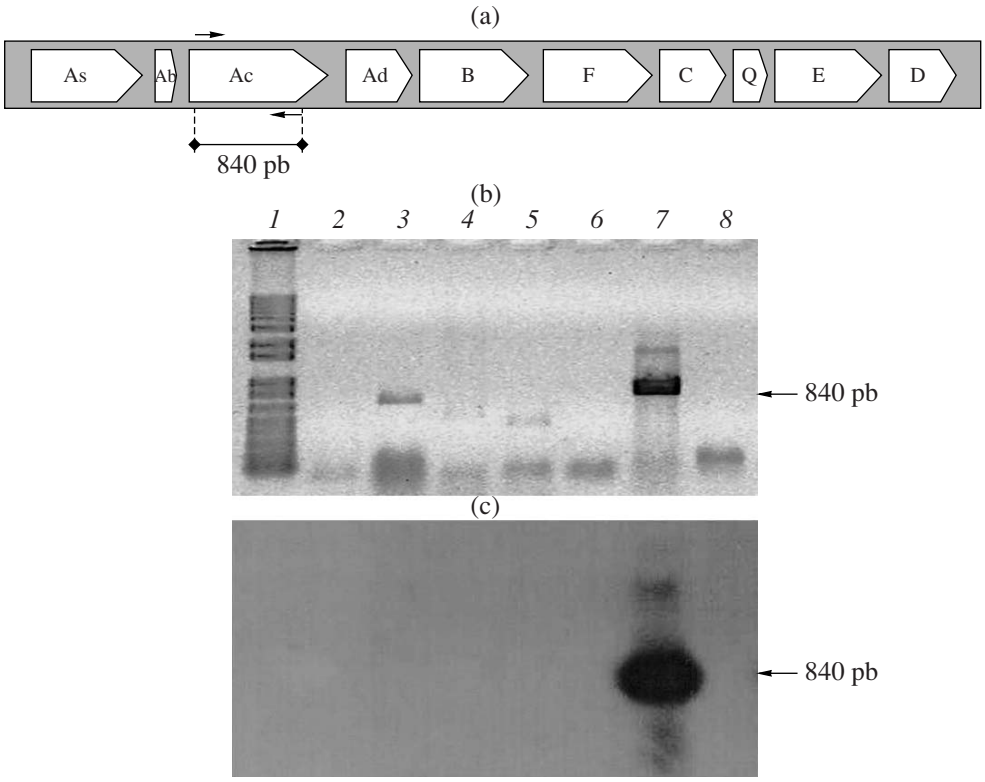

Fig. 3. (a) Naphthalene upper catabolic pathway genes of *Pseudomonas* strains named the *na*h operon. (b) PCR amplification of the *nah*Ac gene encoding naphthalene dioxygenase from the *na*h operon using genomic DNA from bacterial strains isolated from a waste ETO mixture and from *P. putida* R1 used as a positive control. (c) Southern hybridization using an 840-bp DNA fragment internal to the *nah*Ac gene from *P. putida* R1 as a probe. Note that the presence of the *nah*Ac gene was discarded in all bacteria studied. (*1*) 1-kb DNA ladder; (*2*) *B. pumilus* 11; (*3*) *A. lwoffii*; (*4*) *B. amyloliquefaciens* 4; (*5*) *B. amyloliquefaciens* 7; (*6*) *B. pumilus* 10; (*7*) *Pseudomonas* sp. R1 (positive control); (*8*) PCR reaction control.

ferent carbon sources: (i) BSM supplemented with 1% yeast peptone glucose medium (YPG), (ii) BSM supplemented with 1% YPG and 1% ETO, (iii) BSM supplemented with 1% ETO as a sole carbon source, and (iv) BSM without a carbon source (as a control). The Erlenmeyer flasks were inoculated in duplicate with 2% (v/v) inoculum of each bacterium (1×10^8 CFU/mL) and were incubated in a rotary shaker (200 rpm) at 30°C for 4 days. Three samples of 0.5 mL were collected every 3 h and the microbial growth was monitored at OD_{610} using UV WinLab version 2.85.04 2000 software for a PerkinElmer Lambda 35 UV/Vis spectrophotometer.

RESULTS AND DISCUSSION

Bacterial Strains Isolated from Waste ETO Only Included Species of the Genera Bacillus and Acinetobacter

There are a number of bacteria involved in biodegradation of PCBs [24–26], but the diversity of bacteria present in waste ETO and their potential for ETO biodegradation are unknown. In order to determine the genetic diversity present in ETO, bacterial strains were isolated by means of a standard enrichment technique and identified through several molecular methods. Eleven autochthonous bacterial strains were isolated and axenically cultivated. DNA fingerprinting using the ERIC-PCR [15] or GIRRN-LIRRN [17] primers was performed for identifying and differentiating among individual bacteria in the population. The GIRRN-LIRRN results (Fig. 1) showed the presence of three different groups, which were confirmed using ERIC-PCR [17] (data not shown). The three defined groups of strains were confirmed by 16S rRNA gene sequencing, belonging to three different species, *Acinetobacter lwoffii* (one strain), *Bacillus amyloliquefaciens* (eight strains), and *Bacillus pumilus* (two strains), suggesting a low genetic diversity present in waste ETO. The results indicated that both DNA fingerprinting methods used are useful for discriminating bacteria at the species level. This work reports for the first time the isolation, identification, and characterization of the bacterial diversity present in waste ETO.

A. lwoffii Is Able to Use Phenanthrene as a Sole Carbon Source via the Phthalate Pathway

In order to study the capacity of bacterial strains to catabolize PAHs, their ability to grow when naphtha-

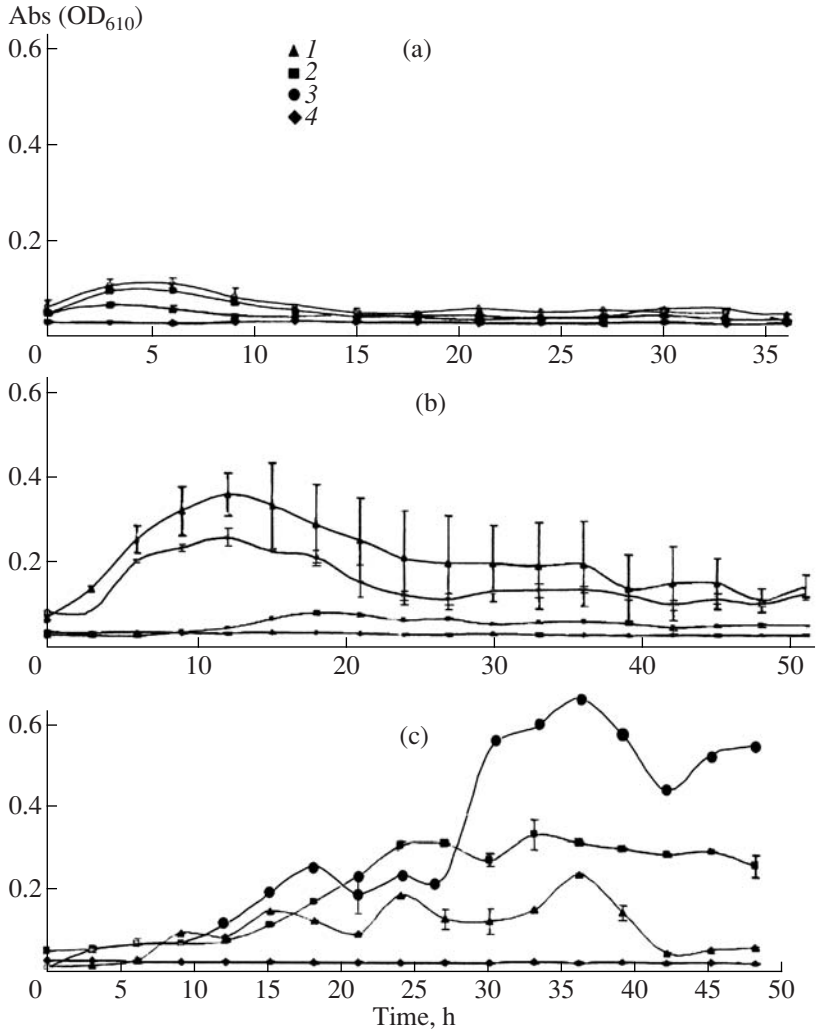

Fig. 4. Bacterial growth using different carbon sources: (*1*) BSM without a carbon source (negative control); (*2*) BSM + 1% ETO; (*3*) BSM + 1% YPG; (*4*) BSM + 1% YPG + 1% ETO. Bacterial growth of (a) *B. amyloliquefaciens*; (b) *B. pumilus*; and (c) *A. lwoffii*. Note that the growth of *A. lwoffii* using waste ETO as a sole carbon source was highly significant ($p < 0.01$) compared with both *Bacillus* strains, indicating its high potential for use as a biocatalyst in ETO biodegradation.

lene or phenanthrene was used as a sole carbon and energy source was tested. Phenotypic assays (Fig. 2) showed that *Bacillus* and *Acinetobacter* species were incapable of growth with naphthalene or phenanthrene, while *A. lwoffii* was able to grow with phenanthrene. These results are in accordance with the implication of *Acinetobacter* species in the biodegradation of diverse pollutants such as aromatic compounds and chlorinated biphenyls [27–29]. To detect the presence of genes involved in the degradation of PAHs in the bacterial strains, the *nah*Ac gene encoding the large subunit of naphthalene dioxygenase from the naphthalene upper catabolic pathway described in *Pseudomonas* strains [30] was amplified by PCR (Fig. 3a). A DNA fragment of 840 bp in *P. putida* R1 (positive control) was expected. The results showed that the *nah*Ac gene was not present in any bacteria studied, except for a DNA band of lesser size (about 800 bp) obtained in *A. lwoffii* (Fig. 3b). To determine if this band corresponded to the *nah*Ac gene, hybridization analysis was performed. The results (Fig. 3c) showed an 840-bp hybridization band in *P. putida* R1 (positive control), as expected. However, this band was not present in all bacteria studied, discarding the presence of the *nah*Ac gene, characteristic from the naphthalene catabolic pathway. Apparently, *A. lwoffii* has different genes for phenanthrene degradation from those reported in *Pseudomonas* strains. *Nocardioides* sp. KP7 (*phd* cluster genes) or *Alcaligenes faecalis* AFK2 (*phn* cluster genes), using the phthalate pathway for phenanthrene degradation, are unable to degrade naphthalene [30]. Both molecular and phenotypic results suggest that *A. lwoffii* is able to catabolize phenanthrene via the phthalate pathway.

A. lwoffii Is Capable of Efficiently Using Waste ETO as a Sole Carbon Source

ETO includes hazardous compounds such as PAHs and, sometimes, PCBs, which are highly toxic and difficult to degrade. Chemical analysis revealed that the ETO used in this work included, among other fractions, 65.7 and 6.6% of saturates and aromatics, respectively, with <0.1 ppm of Aroclors 1242, 1254, and 1260. To determine the potential of the bacterial strains in ETO biodegradation, their ability to grow using different carbon sources was tested. The main results showed that *B. amyloliquefaciens* was incapable of using ETO as a sole carbon source (compared with the control) even there was difference in absorbance 3 h ($p < 0.01$) after inoculation, suggesting its low potential for use as a biocatalyst in ETO biodegradation (Fig. 4a). *B. pumilus* and *A. lwoffii* showed significant differences at 18–27 h ($p < 0.05$) and 9–40 h ($p < 0.01$), respectively, using ETO as a sole carbon source compared with the control (Figs. 4b, 4c). However, the growth in *A. lwoffii* was highly significant at 15, 24, and 36 h ($p < 0.01$) compared with *B. pumilus.* In order to confirm the potential of *A. lwoffii* in ETO biodegradation, a large-scale assay (100 mL) using ETO as a sole carbon source was carried out as described in Materials and Methods. Interestingly, chemical analysis of biotreated ETO 15 days after inoculation revealed a decrease to 63.1 and 1.6% in the saturated and aromatic fractions, respectively. Compared with values for nonbiotreated ETO, degradation of 4 and 80% of the saturated and aromatic fractions, respectively, was obtained. These results suggest the ability of *A. lwoffii* to use initially low- and then high-molecular-weight fractions present in ETO. *Acinetobacter* species that can use various complex mixtures of alkanes, aromatic compounds, and chlorinated biphenyls as a carbon source are considered as efficient oil degraders [27] and important producers of biosurfactants, which enhance biodegradation of saturated and aromatic compounds [31, 32]. The results discussed in this article support the high potential of *A. lwoffii* in environmental and biotechnological applications such as ETO biodegradation.

ACKNOWLEDGMENTS

This work was supported by the IDEA Foundation, FONACIT (project no. S1-200500440), and the Ministry of Science and Technology of Venezuela. The authors thank M. González, A. Reyes, N. Lander, and L. Sena for technical assistance.

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