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NOTE

Phylogenetic Analysis of *Mycobacterium* sp. C2-3 Degrading Polycyclic Aromatic Hydrocarbons

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Mycobacterium **sp. C2-3 was isolated from petroleum-contaminated soil around an oil reservoir and identified by analysis of its 16S rRNA gene sequence. Strain C2-3 was able to use fluorene, phenanthrene, fluoranthene and pyrene as sole sources of carbon and energy, yet unable to degrade naphthalene. The strain was also able to use n-alkanes, such as hexadecane and heptadecane, and phenanthrene and pyrene, in particular, were degraded rapidly. The phylogenetic data suggested that the isolate C2-3 is a thermosensitive, fast-growing strain of** ***Mycobacterium*** **sp.**

Key words: polycyclic aromatic hydrocarbon, pyrene, *Mycobacterium* sp.

Polycyclic aromatic hydrocarbons (PAHs) are fused-ring aromatic compounds whose presence in contaminated soils and sediments poses a significant risk to the environment. They also have cytotoxic, mutagenic and, in some cases, carcinogenic effects on human tissues (9).

While two- to three-ring PAH degradation by microorganisms is already well understood and documented, the microbial degradation of larger-ring PAHs has not been studied extensively. Bacteria have been shown to be unable to use four-ring compounds as a sole source of carbon and energy. Heitkamp and Cerniglia (7) reported on the isolation of a *Mycobacterium* strain, PYR-1, from a drainage pond chronically exposed to petrogenic chemicals, downstream of an oil tank farm near Port Aransas, Texas (7), which mineralizes pyrene and fluoranthene more rapidly than naphthalene and phenanthrene. Grosser *et al.* (1991) described a *Mycobacterium* strain, RJGII-135, isolated from an abandoned coal gasification plant in southern Illinois (5), which was later compared with strain PYR-1 (4). The isolation and characterization of *Mycobacterium* sp. CH1, which is capable of mineralizing phenanthrene, pyrene, and fluoranthene, was recently reported (1). In addition to PAHs, this strain can also use branched alkanes and *n*-alkanes as the sole carbon and energy source.

Members of the genus *Mycobacterium* are widespread in nature, and ranged from harmless inhabitants of water and soil to agents of such devastating diseases as tuberculosis and leprosy. It is well known that many species of mycobacteria can degrade various pollutants in the natural ecosystem (11, 17). These *Mycobacterium* spp. have been shown to be non-pathogenic saprophytes by phylogenetic analysis.

The current study reports on the isolation and phylogenetic characterization of *Mycobacterium* sp. strain C2-3, which is capable of degrading PAHs, such as phenanthrene, pyrene and *n*-alkanes as the sole source of carbon and energy. A phylogenetic (16S rDNA) analysis also differentiated strain C2-3 from other PAH-degrading *Mycobacterium* spp.

Five hundred milligrams of a soil sample, obtained from a petroleum-contaminated area, were suspended in 5 ml of a minimal medium containing 500 ppm of pyrene as a sole carbon and energy source, and incubated for 2 weeks at 28°C with shaking at 130 rpm. The minimal medium contained 30 mg of $(NH_4)_2SO_4$, 2 g of NaCl, 0.5 g of K_2HPO_4, 50 mg of $MgSO_4$-$7H_2O$, 20 mg of $CaCl_2$-H_2O, 10 mg of KNO_3, and 1 ml of a trace metal solution in 1 L of deionized water. One hundred microliters of the culture were transferred to a fresh medium and incubated for 2 weeks. This procedure was repeated 3 times. The enriched culture was plated on a minimal agar plate, 0.5% pyrene in acetone was sprayed uniformly onto the plate, and the plate was incubated at 28°C for 1 week with regular observations (8). Colonies forming clear zones were transferred to a tryptic soy agar plate (Difco) and isolated colonies were obtained.

Each isolated colony was grown in the minimal medium

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supplemented with 300 ppm of pyrene for 10 days and was analyzed by HPLC.

A DNA sequencing analysis of 16S rDNA was performed by the method of Giovannoni *et al.* (1990) with some modifications (3). The primers used for the amplification of 16S rRNA gene were 27F and 1492R which bind specifically to the 16S rRNA gene of members of the domain bacteria. The PCR products were isolated on an agarose gel and sequenced directly using DNA sequencing system (ABI 377). The DNA sequence was compared with the database in GeneBank and EMBL, and used to generate a phylogenetic tree by the neighbor-joining method [14]. Neighbor-joining trees were constructed with the program TreeconW, version 1.3b (16), based on the evolutionary distances estimated by using the Juckes-Cantor correction. Tree reliability was assessed by the bootstrap method (2) with 100 pseudoreplicates. We considered a value of 95% to be statistically significant but values above 50% are reported, since bootstrap may be a conservative estimate for the reliability of a clade.

The isolated strain was grown in a tryptic soy broth (TSB) for 72 hrs, washed 3 times with the minimal medium and then resuspended in the minimal medium for use as an inoculum. Minimal medium containing phenanthrene or pyrene was inoculated to an O.D. value of 0.15 at 440 nm and incubated at 28°C with shaking at 130 rpm. The culture was extracted with 3 volumes of ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated in a vacuum dryer. The extracted pyrene was dissolved in 2 ml of acetonitrile and analyzed by HPLC equipped with an LC-PAH column (Supelco, USA) combined with a UV detector. Isocratic analyses were performed. Twenty microliters of sample were injected, the flow rate was 1 ml of acetonitrile/min, and the pyrene peak was observed at 254 nm (13).

The pyrene degrading strain C2-3 was obtained from petroleum contaminated soil around an oil reservoir using a standard enrichment culture technique. When colonies of the isolate were grown on the pyrene-coated agar plate, clear zones indicating pyrene degradation appeared after 1 week.

Strain C2-3 was found to be a gram positive, non-motile, oxidase-negative, and catalase-positive rod with yellow pigmentation. The organism showed optimal growth on tryptic soy broth at 27°C but did not grow at or above 37°C. Incubation at 27°C yielded visible colonies on TSA after 3 days. Accordingly, the strain was fast-growing mycobacteria as colonies appeared in fewer than 7 days (6).

Table 1. Utilization of aromatic compounds by strain C2-3

Compounds	Degradation
Naphthalene	-
Fluorene	+
Phenanthrene	+
Fluoranthene	+
Pyrene	+
n-Alkanes	+

Strain C2-3 was able to use fluorene, phenanthrene, fluoranthene, pyrene, and n-alkanes such as hexadecane and heptadecane, but was unable to use naphthalene (Table 1). Govindaswami *et al.* (4) reported that *Mycobacterium* sp. PYR-1 can mineralize naphthalene whereas strain RJGII-135 cannot use this compound (4). In addition, strain C2-3 exhibited a significant capacity for degrading aliphatic hydrocarbons. The current results support previous suggestions that the occurrence of both aromatic and aliphatic hydrocarbon degradative capacities within an individual strain is more common than previously thought (1).

The phenanthrene and pyrene degrading abilities of strain C2-3 were measured with various concentrations of these compounds in liquid media. At 50, 100, and 250 ppm of phenanthrene, strain C2-3 degraded more than 95% of the compound within 7 days. Also, it degraded 45% of 500 ppm of phenanthrene within 7 days, and 70%

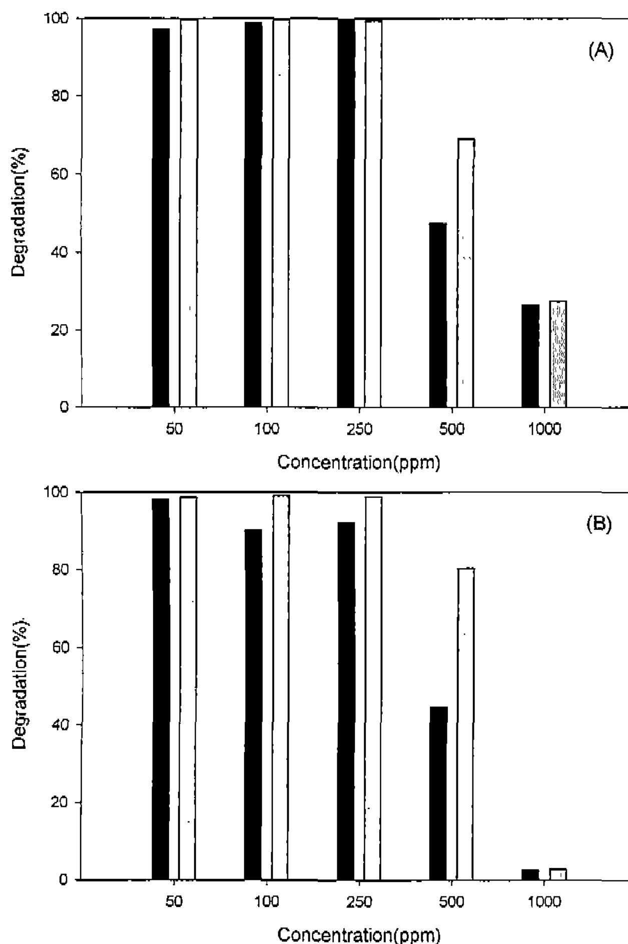

Fig. 1. Degradation of phenanthrene (A: ■ 7 days, ▒ 14 days) and pyrene (B: ■ 14 days, ▒ 28 days) in various concentrations.

within 14 days (Fig. 1A).

In the case of pyrene, strain C2-3 degraded more than 90% within 14 days at concentrations of 50, 100, and 250 ppm. Although strain C2-3 showed a 45% and 80% degradation of pyrene at 500 ppm within 14 and 28 days respectively, it did not degrade pyrene when added at 1,000 ppm (Fig. 1B).

Strain C2-3, which was able to utilize PAHs for growth in the absence of surfactants or cofactors, was different from both strains RJGII-135 and BB1, which require additional cofactors for growth on pyrene (4). However, a recent isolate *Mycobacterium* sp. CH1, was also found to degrade PAHs in the absence of surfactants or cofactors (1). As such, strain CH1 would appear to be very similar to strain C2-3 in that they are both capable of degrading pyrene and n-alkanes, yet incapable of degrading naphthalene. However, the 16S rRNA sequence similarity of C2-3 with strain CH1 in 1,331 bp is relatively low (97.2%). Strain C2-3 was found to be 98.2% (1,452 bp) identical to strain RJGII135 in 1,478 bp and 99.5% (1,421 bp) identical to strain BB1 in 1,428 bp.

The nucleotide sequence of the 1,474 bp DNA segment of the 16S rRNA gene of strain C2-3 was compared with other 16S rRNA gene sequences available in the GenBank data base. From this initial screening, the optimal linear alignment results showed that strain C2-3 was most similar to *Mycobacterium gilvum* with only a 7 bp mismatch in the 1,428 overlapping region.

The phylogenetic tree for the 16S rRNA gene sequences showed that strain C2-3 is a member of the subgroup that contains fast growing mycobacteria (Fig. 2). Strain C2-3 was separated from *M. gilvum* as indicated by the bootstrap value of 100 at the node. The phylogenetic analysis data indicated that strain C2-3 is positioned in the fast growing clade. It is also different from other *Mycobacterium* spp. due to the inclusion of degradative activities in its physiological and phylogenetic characteristics.

Signature structures, based on length and sequence variations of two specific 16S rRNA regions, have been proposed as identifiers for subsets of mycobacteria (15). Using this model, the helix flanked by positions 451 and 482 in *E. coli* 16S rRNA differentiates the fast-growing strains from the slow-growing strains (10, 12, 15). Fig. 3A shows that fast-growing strain C2-3 shares a signature that is identical to that of strains RJGII-135 and PYR-1, and similar to that of *Mycobacterium chelonae*, which are representative fast-growing strains. When compared to slow-growing mycobacteria, many of which are human and animal pathogens, most fast-growing mycobacteria are common saprophytes in natural habitats (6), which have been isolated from a diverse array of habitats, and are able to survive and biotransform a variety of xenobiotic compounds and pollutants (11,17). Such mycobacteria have been demonstrated to be fast growing strains using the model suggested by Stahl and Urbance (1990).

A second signature, corresponding to positions 184 to 193 in *E. coli* 16S rRNA, differentiates thermosensitive strains from thermotolerant ones within fast growers based on the presence of a bulged U (10, 12, 15). Strain C2-3 exhibited a similar structure to strain RJGII-135, which is a known thermosensitive strain (Fig. 3B). The physiological data also indicated that strain C2-3 is thermosensitive (data not shown).

Accordingly, the current data suggest that strain C2-3 is fast-growing, thermosensitive, and able to degrade a

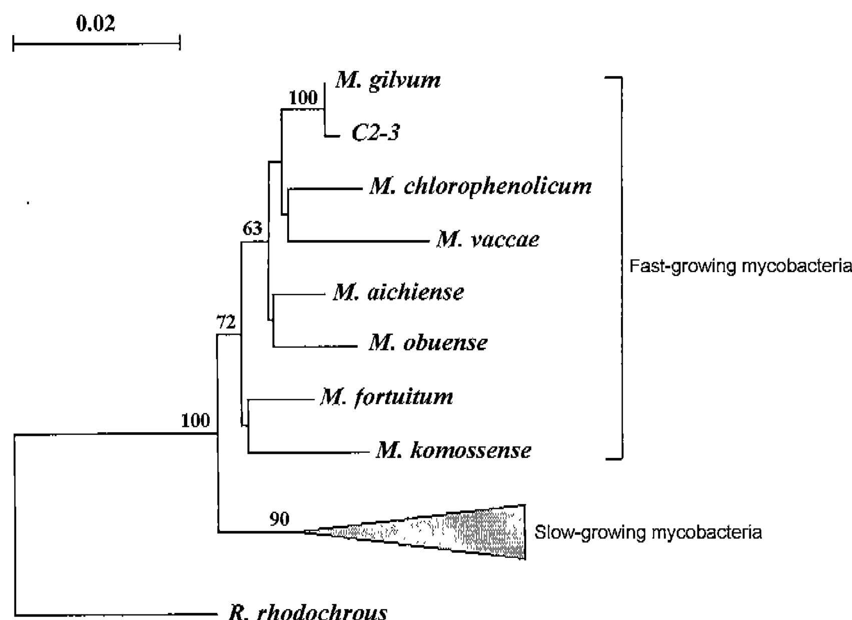

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence analyses.

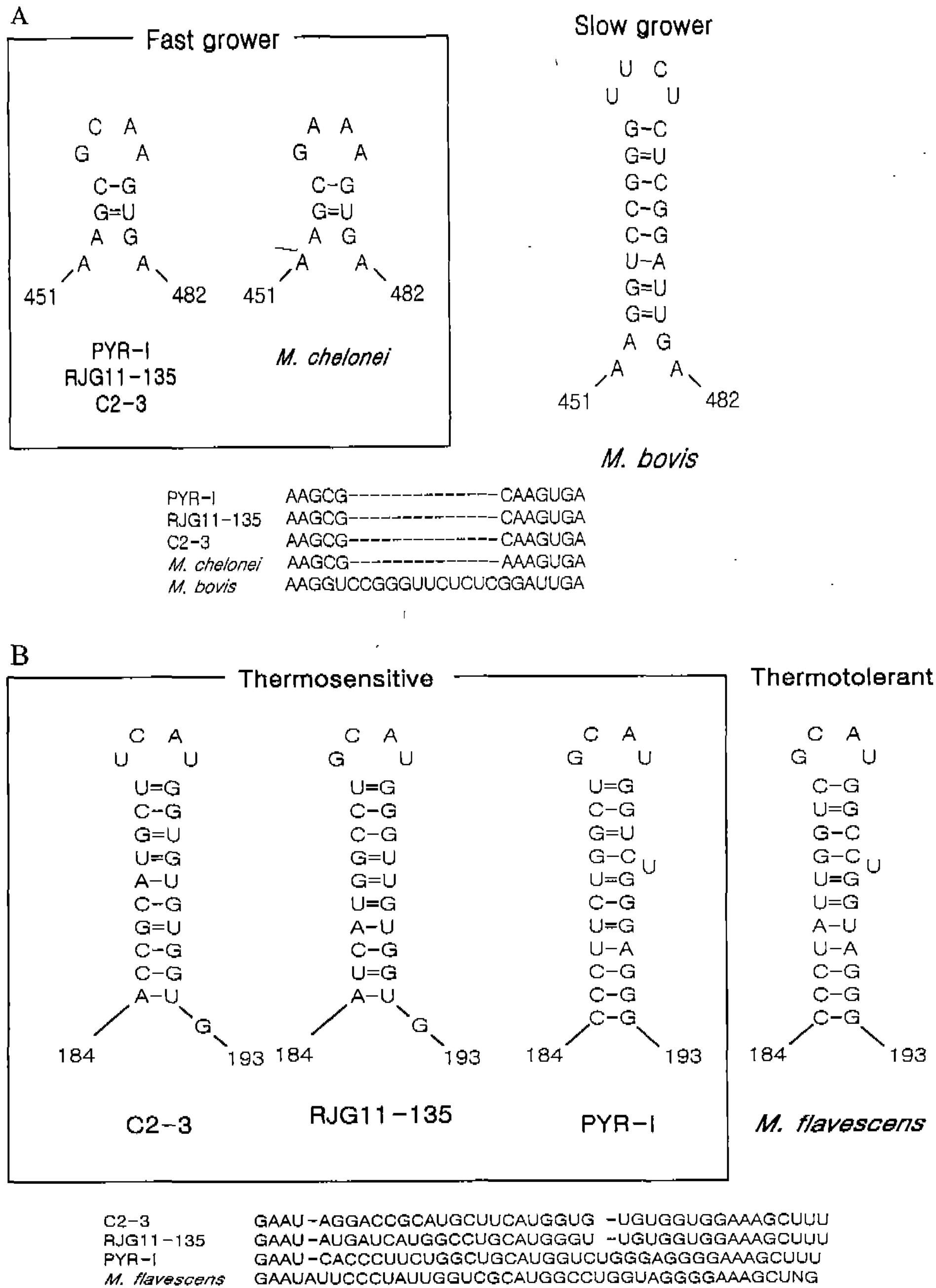

Fig. 3. Signature features for mycobacteria. (A) Secondary structure of 16S rRNA between positions 451 and 482 corresponding to C2-3, RJGII-135, PYR-1, representative fast-grower (*M. chelonei*), and a representative slow-grower (*M. bovis*). (B) Secondary structure of 16S rRNA between positions 184 and 193 corresponding to C2-3, representative thermosensitive (RJGII-135), PYR-1, and representative theromotolerant fast-grower (*M. flavescens*).

broad range of aromatic and aliphatic substrates. Therefore, strain C2-3 would seem to have potential for use in the development of bioremediation techniques for PAH-contaminated areas.

Acknowledgment

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