Phylogenetic Comparison of Two Polycyclic Aromatic Hydrocarbon-Degrading Mycobacteria

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Two mycobacterial strains previously isolated from fossil-fuel-contaminated environments and shown to degrade four- and/or five-ring polycyclic aromatic hydrocarbons were further characterized. The two strains, PYR-I and RJGII-135, had similar growth characteristics, colony morphologies, and scotochromogenic pigments. DNA amplification fingerprints obtained with total genomic DNA indicated some strain similarities but with several distinctly different bands. Moreover, phylogenetic analysis based upon essentially full-length 16S rRNA gene sequences separates the two strains as distinct species within the fast-growing group of mycobacteria. Although both strains are thermosensitive, strain PYR-I has the bulged U between positions 184 and 193 characteristic of thermotolerant mycobacteria. Both strains are of potential use for reintroduction into and bioremediation of polycyclic aromatic hydrocarbon-contaminated soils.

Polycyclic aromatic hydrocarbons (PAHs) include a number of widespread pollutants which are products of the incomplete combustion of fossil fuels and organic compounds. The environmentally refractory four- to six-ring PAHs include known carcinogens and other compounds suspected of altering gene regulation and DNA structure; hence, the presence of these compounds in the environment is of considerable interest. Coal gasification waste sites contain complex mixtures rich in such PAHs. The existence of more than 3,000 of these sites in the United States alone adds to the concern about risks to human health due to PAHs in the environment.

There is increased interest in developing biotechnologies to detoxify PAH-contaminated sites. We are investigating microorganisms indigenous to PAH-contaminated soils, which are capable of degrading these hazardous wastes. An initial goal of this work is to carry out genetic and biochemical analyses of the degradative pathways involved, as well as phylogenetic analyses of the PAH-degrading microorganisms. Molecular probes based upon these results will facilitate ecological studies and will help us in our eventual goal of reintroducing these degradative microorganisms into contaminated environments for the purpose of bioremediation through enhanced biodegradation.

Although the catabolism of low-molecular-weight PAHs is well studied (6), research on the biodegradation of the larger recalcitrant PAHs has been limited by the general lack of microbial isolates or consortia which can degrade these toxicants. In 1988 Heitkamp and Cerniglia (14) reported the isolation of a *Mycobacterium* strain, PYR-I, from a drainage pond chronically exposed to petrogenic chemicals, downstream of an oil tank farm near Port Aransas, Tex. This strain mineralizes pyrene and fluoranthene more rapidly than the two- and three-ring naphthalene and phenanthrene; although some metabolites of benzo[a]pyrene (BaP) were detected, no mineralization of BaP was observed. In 1991 Grosser et al. (12) reported on a

Mycobacterium strain, RJGII-135, abbreviated here as 135, that they isolated from soil obtained from an abandoned coal gasification plant in southern Illinois. In addition to mineralizing pyrene, strain 135 also mineralized approximately 20% of added BaP. More recently, Boldrin et al. (3) reported on another *Mycobacterium* strain, BB1, isolated from a former coal gasification site at Karlsruhe, Germany, which was able to metabolize phenanthrene, pyrene, fluoranthene, and a few other aromatic compounds as sole sources of carbon and energy and degraded fluorene cometabolically; there was no mention of BaP metabolism.

This study describes a comparative analysis of strains PYR-I and 135. Reported here are general growth characteristics, genetic differences shown by DNA amplification fingerprinting (DAF) using one arbitrary GC-rich decanucleotide primer, and phylogenetic analyses based upon 16S rRNA gene (rDNA) sequences. In addition, these DNA sequences will be used to design strain-specific DNA probes for use in monitoring the presence, survival, and growth of these isolates in PAH-contaminated soils.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The two bacterial strains were obtained from their laboratories of origin (12, 14). *Escherichia coli* DH5 α was obtained from Bethesda Research Laboratories.

Yeast extract-peptone-dextrose (YEPD) agar containing 1% yeast extract, 2% peptone, 2% glucose, and 2% agar was used as a multipurpose growth medium. Trypticase soy broth supplemented with 1% glucose and 0.05% Tween 80 (TSB+) was used for some growth experiments. To check for the maintenance of degradative traits, aliquots of fresh cultures were checked routinely by plating them onto quarter-strength YEPD plates, spraying them with a 2% solution of pyrene or other PAHs in acetone (16), and noting clear zones around isolated colonies following incubation.

Nucleic acid extraction. For total genomic DNA preparation both mycobacterial strains were cultured by shaking at 28°C in TSB+. Cells in exponential growth were pelleted by centrifugation at 12,100 $\times g$ for 5 min at 4°C. The cells were washed once, and genomic DNA was isolated as described by R. N. Husson et al. (15). Plasmid DNA was prepared by standard methods (2, 19).

DAF. DAF PCRs were performed with the decanucleotide primer 5'-CCG CAGCCAA3' using the Stoffel fragment DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) in a Perkin-Elmer DNA thermal cycler. Reactions were generally carried out in a total volume of 102 μ l with 10 to 20 pg of template DNA, 1 μ g of primer, and 2.5 U of enzyme, in reaction buffer (10 mM Tris HCl [pH 8.3], 10 mM KCl, and 2.5 mM MgCl₂) containing a 200 μ M concentration of each

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deoxynucleoside triphosphate (Pharmacia). The cycling program was as described by Breen et al. (4). DNA amplification fragments were electrophoretically separated after loading 20 μ l of the reaction mixture on 2% gels of Meta-phor agarose (FMC Bioproducts, Rockland, Maine).

PCR cloning and sequencing. The nearly full-length 16S rDNA of each mycobacterial strain was amplified by PCR with the oligodeoxynucleotide primers and PCR conditions as described previously (11). Following amplification the DNA mixture was phenol-chloroform extracted, chloroform extracted, and ethanol precipitated; the DNA was then suspended in 1 $\times$ Tris-EDTA (19).

The purified PCR product of strain 135 was treated with Klenow DNA polymerase (New England Biolabs) and ligated as a blunt-end fragment into *Sma*I (New England Biolabs)-digested Bluescript KS(–) (Stratagene) by standard protocols (19). A sticky-end ligation was used for strain PYR-I because the blunt-end approach (19) gave ambiguous results with respect to the size of the cloned fragment. PCR primers for this were made with *Not*I linkers and had 10 extra bases to optimize efficiency of digestion (to >90% upon incubation for 20 h). Digestion of the PCR product with *Not*I (New England Biolabs) provided 6 extra bases on either end for ligation into *Not*I-digested pNEB (New England Biolabs) by standard protocols (19). For each strain, four PCR mixtures were pooled for ligation and cloning. The ligation mixture in both cases was transformed into *E. coli* and plated onto Luria-Bertani agar plates containing 100 μ g of ampicillin per ml (13).

Plasmid DNA preparations from recombinant transformants were pooled (seven for strain 135 and nine for strain PYR-I) and used as templates for direct dideoxy sequencing, using Sequenase, version 2, with 32 P according to the protocol recommended by the manufacturer for sequencing double-stranded plasmid DNA (United States Biochemical). The cloned 16S rDNA segments from strains 135 and PYR-I were sequenced on both strands by using primers complementary to conserved regions of the *E. coli* 16S rDNA sequence. Additional primers were designed on the basis of sequenced DNA in order to confirm the sequence of neighboring ambiguous regions. In both cases the entire sequence was determined on both strands except for portions of the constant region complementary to some of the oligonucleotide primers where the sequence was determined from replicate sequencing reactions using one strand.

During the review of this paper we became aware of work submitted by Carl Cerniglia's laboratory (25a) on the same two strains which showed a difference in our respective sequence data for strain 135. Fresh genomic template DNA was isolated, and the 16S rDNA amplification by PCR was repeated. The approximately 1.5-kb PCR product from four pooled PCRs was sequenced with the Applied Biosystems automated sequencer model 3738A by using the fluorescent dye terminator cycle sequencing kit. Both strands were sequenced in their entirety with the previously described forward and reverse primers. A systematic error unique to a segment of our original strain 135 sequencing data was detected and has been corrected.

Phylogenetic analysis. To infer the phylogenetic relationship of strains PYR-I and 135, we conducted maximum likelihood and parsimony analyses of their 16S rDNA sequences together with similar sequences from 14 other strains of *Mycobacterium*. A sequence from *Nocardia asteroides* was used as the out-group. Sequence data for these 15 other strains were downloaded from the Ribosomal Database Project at the University of Illinois (18) and from the National Center for Biotechnology Information (20). Homologous nucleotide positions were aligned on the basis of conserved features of primary and secondary structures. The number of nucleotide positions used in the alignment (the noncoding or RNA-like strand was used) was 1,342; the first nucleotide corresponded to position 38 and the last corresponded to position 1377 of the *E. coli* 16S rRNA.

Phylogenetic trees were inferred by the maximum likelihood method as implemented by PHYLIP, version 3.5c (8). Heuristic parsimony analyses of the same sequences were implemented with the computer program PAUP, version 3.1 (25). Because the heuristic search does not explore all possible topologies to find the shortest tree, we repeated the search 100 times for each parsimony analysis. Each search was initiated with a different randomly constructed starting topology, thus minimizing the possibility that the algorithm will find a local parsimony optimum rather than the universal optimum for a particular data set. Random stepwise addition of taxa was used in both maximum likelihood and parsimony analyses.

Nucleotide sequence accession numbers. The sequence results from this study are available from GenBank (accession numbers U30662 and U30661).

RESULTS

Growth comparison. Strains PYR-I and 135 showed optimal growth on YEPD agar at 28°C; appreciable growth was observed at room temperature (approximately 23°C), while growth was slow at 37°C. Both strains failed to grow at 45°C. Incubations at 28°C yielded visible colonies after 24 to 30 h and heavy growth within 4 to 5 days (10). Colony morphologies for both mycobacterial strains are somewhat similar: medium-size, smooth, shiny colonies with characteristic scotochromogenic pigmentation (10, 22). Colonies of strain PYR-I are bright

FIG. 1. DAF profiles of strains PYR-I and 135. Lane 1, no DNA control; 2, ϕ X174 replicative-form DNA-*Hae*II marker; 3, 135; 4, PYR-I; 5, 123-bp DNA ladder. Arrows indicate products prominent in one strain but absent or present in small quantities in the other.

yellow, while strain 135 produces a deeper yellow-gold pigment which darkens as colonies age.

Growth rates in liquid culture were determined with TSB+ at 28°C and 120 rpm in a rotary water bath shaker. Both strains have a doubling time of approximately 8 h (data not shown). Loss of degradative ability was not observed during routine checks conducted on fresh cultures of either strain.

DAF comparison. DAF is an in vitro method in which genomic DNA template is amplified by PCR using a single short arbitrary GC-rich primer of typically 8 to 10 nucleotides in length (5). Figure 1 shows DAF patterns generated from strains PYR-I and 135 by using the primer described in Materials and Methods. In order to achieve consistency in the banding patterns, it was important to maintain constant concentrations of each reaction component for both strains.

DAF reactions with both strains resulted in complex profiles. Some products observed with one strain are also seen with the other strain. Indicated by arrows in Fig. 1 are a few products which are very prominent in one strain but are either absent or present in very small quantities in the other. Overall, the DAF profiles show high similarity but with distinct differences, indicating that the strains are unique.

16S rDNA analysis. The PCR and sequencing strategy used generated sequence information for a DNA segment comprising approximately 95.7% of the 16S rDNA for each strain. Sequence comparisons with the Genetics Computer Group program demonstrated 97.0% similarity between strains PYR-I and 135 in the 1,486-bp overlap region (Fig. 2). A search performed using the Similarity Rank Program of the Ribosomal Database Project (18) identified these two strains most closely with *Mycobacterium* species. Optimal linear alignment results of 16S rDNA sequences showed that strain PYR-I was most similar to *Mycobacterium aurum* (similarity = 0.952), while strain 135 was most similar to *Mycobacterium aichiense* (similarity = 0.938). *Mycobacterium* strain BB1, the other environmental isolate reported to degrade PAHs, was stated to

FIG. 2. Optimal sequence alignment of the 16S rDNAs of strains PYR-I and 135. Identical sequences are shown in boldface lettering, and dissimilar sequences are underlined. The similarity in the overlap region is 97%.

be closely related to *Mycobacterium gilvum*, but no data were presented (9).

Signature structures, based upon length and sequence variations of two specific 16S rRNA regions, have been proposed as identifiers for subsets of mycobacteria (24). By this model, the helix flanked by positions 451 to 482 in the *E. coli* 16S rRNA numbering differentiates the fast-growing from the slowly growing strains (21, 23, 24). In Fig. 3A we show that the two fast-growing strains PYR-I and 135 share a signature that is also identical to that of *Mycobacterium komossense* and similar to that of *Mycobacterium chelonae*, both representative fast

growers. A second signature, corresponding to positions 184 to 193 in *E. coli* 16S rRNA, differentiates the thermosensitive from the thermotolerant among the fast growers (21, 23, 24). As our physiological data show, strains 135 and PYR-I are both thermosensitive. In Fig. 3B we show that strain 135 has a structure similar to that of *M. komossense*, a typical thermosensitive strain. However, PYR-I shares the structural signature proposed for thermoresistance, shown in Fig. 3B for thermoresistant strain *Mycobacterium flavescens*. The linear alignment in Fig. 3B shows the similarity relationships for these sequences in the overall region from positions 177 to 202.

Phylogenetic analysis. The maximum likelihood tree generated by PHYLIP for the 17 16S rDNA sequences is shown in Fig. 4. As observed by others (21, 23, 24), our analysis also shows two lines of descent among *Mycobacterium* strains, the ancestral fast-growing strains and the derived slowly growing strains. The two new bacterial taxa (PYR-I and 135) fall within the fast-growing *Mycobacterium* clade. Strains 135 and PYR-I are distinct from each other, with strain 135 branching off deeper in the fast-growing clade and most closely related to *M. aichiense*, while strain PYR-I is most closely related to *M. aurum* and *Mycobacterium vaccae*.

Parsimony analysis yielded two trees of 445 steps based on a 2:1 weighting of transversions-transitions (data not shown). Both trees are congruent with the maximum likelihood analysis. The only difference between the two parsimony trees is exact placement of *M. flavescens* within the fast-growing clade.

DISCUSSION

Table 1 summarizes work of others on the mineralization of PAHs by strains PYR-I and 135. From their results it is apparent that the two have some differences in substrate specificity but are both promising isolates for testing in bioremediation systems. The intent of this study was to conduct a comparative analysis of the two strains, including their phylogenetic relatedness based upon 16S rDNA data, as a prelude to the use of these strains in studies of biodegradation.

Our observation that the two strains are not thermotolerant supports the finding for PYR-I reported recently by F. Rafii et al. (22), although they used slightly different temperatures and growth conditions. For PYR-I Heitkamp et al. (14) reported a generation time of 2.7 to 4.0 h in minimal basal salts medium supplemented with nutrients and pyrene. Using Trypticase soy broth medium supplemented with 0.05% Tween 80 to prevent clumping, we obtained a doubling time of approximately 8 h for both strains. Colony formation in fewer than 5 days is a characteristic of fast-growing mycobacteria (26).

DAF is a convenient approach to characterize organisms because it does not require prior DNA sequence information to generate fingerprints from DNA of any origin. It detects changes in DNA sequence at arbitrary sites in the genome which are defined by the primer; these changes are manifest in the number and length of amplified products and are not linked to particular loci (5). DAF has been extensively used in the genetic analysis of single organisms, including humans, plants, pathogenic bacteria, and yeasts (1, 5, 7, 17). It has been observed that bands tend to fall into two categories, those that are phylogenetically conserved and those that are species specific (5). Under the conditions that we used (see Materials and Methods), our data (Fig. 1) indicated that in addition to the formation of similar bands, there are bands present that distinguish between the two strains. Such distinguishing bands from both strains could be cloned and sequenced, and if the

FIG. 4. Maximum likelihood tree for 14 species in the genus *Mycobacterium*, strains 135 and PYR-I, and an out-group microorganism, *N. asteriodes* (N.ast). The scale bar corresponds to 0.01 estimated nucleotide substitution per sequence position. The 14 other mycobacterial taxa used in this study are *M. fortuitum* (M.for), *M. vaccae* (M.vac), *M. aurum* (M.aur), *M. flavescens* (M.flu), *M. obuense* (M.obu), *M. aichiense* (M.aic), *M. xenopi* (M.xen), *M. gadium* (M.gad), *M. avium* (M.avi), *M. chlorophenolicus* (M.chl), *M. komossense* (M.kom), *M. fallax* (M.fal), *M. gilvum* (M.gil), and *M. nonchromogenicum* (M.non).

soil, strain BB1, has been reported to degrade higher multiring PAHs (3). Since BB1 was reported to have high 16S rRNA sequence similarity to *M. gilvum* (9), it also presumably is a fast grower.

Inasmuch as strains PYR-I and 135 are both positioned as discrete taxa in our phylogenetic analysis of 16S rDNA se-

quences, it seems likely that additional PAH-degrading mycobacteria could be found. It may be that a collection of these organisms could provide broad substrate specificity for the degradation of complex PAH mixtures in environmental sites. Strain-specific probes based upon fluorescently labelled antibodies to cell surface antigens, variable segments of 16S rDNA, and possibly products developed in DAF analysis could be used in ecological studies of these strains in degradative microbial communities.

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TABLE 1. PAH mineralization by strains PYR-I and 135

Compound	Mineralization ^a by:	
	PYR-I	135
Naphthalene	+	ND
Phenanthrene	+	+
Anthracene	(+)	-
Fluoranthene	+	ND
Pyrene	+	+
BaP	(+)	+
Benz[a]anthracene	ND	-

^a + or -, mineralization detected or not detected with ^{14}C -labelled substrates; ND, not tested; (+), result from Carl Cerniglia (5a).

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