Classification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Mycobacterium* sp. strain PYR-1, as *Mycobacterium vanbaalenii* sp. nov.

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A polycyclic aromatic hydrocarbon (PAH)-utilizing *Mycobacterium* strain, PYR-1^T, was isolated from petroleum-contaminated estuarine sediments and has been shown by 16S rRNA gene sequencing to be closely related to *Mycobacterium aurum* ATCC 23366^T and *Mycobacterium vaccae* ATCC 15438^T. In this investigation, the 16S rDNA, fatty acid methyl esters, DNA–DNA hybridization, PFGE analysis of restriction-digested total genomic DNA and biochemical tests were used to determine the taxonomic relationship of strain PYR-1^T to other closely related *Mycobacterium* species. The sequence of the 16S rRNA gene of strain PYR-1^T was similar to that of *Mycobacterium austroafricanum* ATCC 33464^T, except for one gap at position 43. Fatty acid methyl ester analysis also showed similarity to *M. austroafricanum* ATCC 33464^T; however, the Euclidean distance was greater than 4.0, indicating that these strains were not identical. Dot-blot DNA–DNA hybridization of strain PYR-1^T with *M. austroafricanum* indicated less than 40% relatedness. When the total chromosomal DNA of *M. aurum* ATCC 23366^T, *M. austroafricanum* ATCC 33464^T and strain PYR-1^T was digested with restriction enzyme *Xba*I and analysed by PFGE, all three organisms gave different restriction patterns. Previous studies from our laboratory have shown that the reverse-phase HPLC elution profiles of mycolic acids of strain PYR-1^T and *M. austroafricanum* ATCC 33464^T have different patterns. Based on phylogenetic analysis using 16S rRNA gene sequences, fatty acid analysis, DNA–DNA hybridization and PFGE analysis and physiological and chemotaxonomic characteristics, it is concluded that strain PYR-1^T (= DSM 7251^T = NRRL B-24157^T) represents a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium vanbaalenii* sp. nov. is proposed.

Keywords: *Mycobacterium vanbaalenii*, rapidly growing bacteria, polycyclic aromatic hydrocarbon metabolizing bacteria, 16S rDNA, fatty acid analysis

INTRODUCTION

Members of the genus *Mycobacterium* have been reported to degrade a wide variety of environmentally

hazardous chemicals, including high-molecular-mass polycyclic aromatic hydrocarbons (PAH) (Boldrin *et al.*, 1993; Cerniglia, 1981; Churchill *et al.*, 1999; Dean-Ross & Cerniglia, 1996; Fritzsche, 1994; Gros-ser *et al.*, 1991, 1995; Guerin & Jones, 1988; Jimenez & Bartha, 1996; Kästner *et al.*, 1994; Kelley *et al.*, 1990; Kleespies *et al.*, 1996; Lloyd-Jones & Hunter, 1997; Molina *et al.*, 1999; Rehmann *et al.*, 1996, 1998; Schneider *et al.*, 1996; Tiehm & Fritzsche, 1995; Tongpim & Pickard, 1999). A rapidly growing, acid–alcohol-fast, scotochromogenic, PAH-degrading *Mycobacterium* strain, PYR-1^T, was isolated from

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A table and a figure showing the peak areas of esters of mycolic acids analysed by reversed-phase HPLC are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Abbreviations: FAME, fatty acid methyl ester; PAH, polycyclic aromatic hydrocarbon.

oil-contaminated sediment (Heitkamp *et al.*, 1988). It degrades chrysene, anthracene, fluoranthene, 1-nitropyrene, phenanthrene, biphenyl and benzo[a]pyrene (Heitkamp *et al.*, 1988; Heitkamp & Cerniglia, 1989; Kelley & Cerniglia, 1995; Kelley *et al.*, 1991; Moody *et al.*, 2001, 2002; Rafii *et al.*, 1994). *Mycobacterium* sp. strain PYR-1^T has both mono- and dioxygenases to catalyse the initial attack on PAHs (Kelley & Cerniglia, 1995; Moody *et al.*, 2001). Recently, we cloned and sequenced genes for the degradation of PAHs from *Mycobacterium* sp. strain PYR-1^T and found a unique genetic organization (Khan *et al.*, 2001; Wang *et al.*, 2000). Molecular techniques indicate that it survives and performs well in the microbial populations of mixed sediments (Wang *et al.*, 1996).

Since strain PYR-1^T has potential use in the bioremediation of PAH-contaminated environmental sites (Wang *et al.*, 1996), we characterized the strain with biochemical tests, 16S rDNA phylogenetic analysis, fatty acid methyl ester (FAME) analysis, dot-blot DNA–DNA hybridization, analysis of the total genomic restriction pattern by PFGE and mycolic acid analysis. On the basis of the results, a novel *Mycobacterium* species, *Mycobacterium vanbaalenii* sp. nov., is described.

METHODS

Strains and media. Strain PYR-1^T was isolated from sediments in an oil-contaminated site in Redfish Bay, TX, USA (Heitkamp *et al.*, 1988). The bacterium was isolated from a 500-ml microcosm. After incubation of the microcosm for 25 days under aerobic conditions, the sediment samples were serially diluted and screened for the presence of pyrene-degrading micro-organisms. One colony that was able to degrade pyrene was designated strain PYR-1^T and was further identified as a *Mycobacterium* species on the basis of morphological, biochemical, chemotaxonomic and 16S rRNA properties (Heitkamp *et al.*, 1988; Rafii *et al.*, 1992; Wang *et al.*, 1995). The type strains of *Mycobacterium aurum*, ATCC 23366^T, and *Mycobacterium austroafricanum*, ATCC 33464^T, used in this study were obtained from the Centers for Disease Control, Atlanta, GA, USA. Strain PYR-1^T was maintained in a mineral salts medium containing 250 µg peptone l⁻¹, 250 µg yeast extract l⁻¹ and 0.5 µg pyrene ml⁻¹ dissolved in dimethylformamide (Heitkamp *et al.*, 1988). The strain also grows on tryptic soy, brain/heart infusion and minimal basal salts (MBS) media.

Chemical and biochemical characterization. The organism was characterized biochemically by standard methods (Kent & Kubica, 1985). The G+C content was determined by thermal denaturation (Heitkamp *et al.*, 1988). Bacterial cultures were harvested from Middlebrook 7H10 agar medium (Remel) for analysis of total cellular FAMES. Fatty acids were extracted by following the instructions of the Microbial Identification System (MIDI; Microbial ID Inc.). A dendrogram based on the fatty acid compositions of strain PYR-1^T and other closely related *Mycobacterium* strains was created by cluster analysis using the Sherlock Library Generation System version 4.0 software (Khan *et al.*, 1996).

Dot-blot DNA–DNA hybridization. DNA from *M. austroafricanum* ATCC 33464^T and strain PYR-1^T was isolated by using the Qiagen DNeasy tissue kit. DNA was quantified

spectrophotometrically at 260 nm. DNA from each strain was denatured in 0.4 M NaOH/10 mM EDTA and 10, 1 and 0.1 ng portions were transferred onto a nylon membrane (GeneScreen Plus; Dupont) as described previously (Khan *et al.*, 1996). The filter was air-dried, wrapped in plastic and exposed to UV radiation using a UV Stratalinker (Stratagene) to fix the DNA to the filter matrix. Total chromosomal DNA of strain PYR-1^T was labelled non-isotopically by using a random-primed labelling kit (DIG DNA labelling kit, Roche Molecular Biochemicals) according to the instructions supplied by manufacturer. Dot-blot DNA–DNA hybridization was performed as described by Jalava *et al.* (1998).

PFGE. Bacterial cultures were grown on tryptic soy agar and incubated at 37 °C. After 72 h, a suspension of each culture with a turbidity adjusted to 0.69–0.73 % in 100 mM Tris/100 mM EDTA was made using a turbidity meter (Dade Behring). Agarose plugs were prepared by mixing equal volumes of the bacterial suspension with 1 % agarose and 1 % SDS in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) plus 0.2 mg proteinase K (Gibco-BRL) and casting into disposable moulds (Bio-Rad Laboratories). Bacterial cells in the agarose plugs were lysed by treatment with lysis solution containing 0.1 mg proteinase K ml⁻¹ in a solution containing 50 mM Tris, 50 mM EDTA and 1 % *N*-lauryl sarcosine for 2 h at 54 °C. Agarose plugs were washed twice in sterile distilled water and four times in TE at 50 °C for a minimum of 20 min for each wash. A 5 × 5 × 1.5 mm section of each plug was equilibrated for 10 min with enzyme buffer supplied with the restriction enzyme and was then placed into 200 µl fresh buffer containing 50 U of the restriction enzyme *Xba*I for 6 h at 27 °C. High-molecular-mass restriction fragments were resolved in a 1 % agarose gel in 0.5 × TBE buffer using a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DR II, Bio-Rad Laboratories). Electrophoresis was performed at 6 V cm⁻¹ with a 2.2–63.8 s linear ramp time for 18 h. Gels were cooled at 14 °C throughout the run. Gels were stained with ethidium bromide and destained with distilled water. Banding patterns were visualized and photographed with a UV transilluminator.

Phylogenetic analysis. The 16S rDNA sequence of strain PYR-1^T was aligned with those of representatives of the genus *Mycobacterium* using CLUSTAL X version 1.8 (Thompson *et al.*, 1997). Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987) and Fitch–Margoliash (Fitch & Margoliash, 1967) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant tree topology was evaluated in bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. The resulting tree was depicted by using TreeView version 1.6.6 (Page, 1996).

RESULTS AND DISCUSSION

Phenotypic and biochemical characteristics

Cells of strain PYR-1^T were Gram-positive, acid-fast rods (1.4 × 0.7 µm), smooth and saffron yellow on Middlebrook medium. It grew well on brain/heart infusion, trypticase soy, tryptone yeast extract or minimal balanced salt media. The 15 biochemical tests, a G+C content of 66 mol %, characterization of mycolic acids with a carbon chain length of C₅₈–C₆₄

Table 1. Cellular fatty acid compositions of strain PYR-1^T and related strains

Values are percentages of total fatty acids. tr, Less than 1 %.

Fatty acid	PYR-1 ^T	<i>M. austroafricanum</i> ATCC 33464 ^T	<i>M. aurum</i> ATCC 23366 ^T
C14:0	2.81	3.16	5.93
C16:1 ω 9c	1.88	1.53	2.19
C16:1 ω 6c	13.17	11.34	5.58
C16:0	22.36	22.89	30.12
Summed feature 2*	16.70	19.11	10.01
C18:1 ω 9c	11.57	12.15	27.35
C18:0	1.41	tr	2.66
C18:0 10-methyl	19.56	17.72	9.09
Summed feature 3	10.57	12.08	3.74

* Summed features consist of one or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 2: C17:1 ω 7c and/or C17:1 ω 6c. Summed feature 3: C20:0 alcohol, C19:0 cyclopropane ω 10c and/or an unidentified fatty acid with an equivalent chain length of 18.838.

and fatty acid analysis were consistent with the assignment of this organism to the genus *Mycobacterium* (Heitkamp *et al.*, 1988; Rafii *et al.*, 1992). The bacterium grew well at 24 and 37 °C with minimal or no growth at 42 °C. This organism is classified as a rapidly growing *Mycobacterium* strain with a generation time of 2.7–4.0 h in MBS medium and is strictly aerobic. It has arylsulfatase and utilizes naphthalene, biphenyl, phenanthrene, anthracene, fluoranthene, pyrene, 1-nitropyrene, 3-methylcholanthrene, 6-nitrochrysene and benzo[*a*]pyrene (Heitkamp *et al.*, 1988; Kelley & Cerniglia, 1995; Kelley *et al.*, 1991; Rafii *et al.*, 1994; Moody *et al.*, 2001, 2002). Strain PYR-1^T metabolizes salicylic acid, hydrolyses Tween 80, reduces tellurite, is urease-positive and catalase-positive and reduces nitrate to nitrite. It does not produce niacin and does not utilize glucose, xylose, lactose, Simmons' citrate, sucrose, maltose, adonitol, arabinose, cellobiose, dulcitol, galactose, malonate, melizitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, starch, trehalose, gelatin or aesculin (Heitkamp *et al.*, 1988). It does not produce H₂S and there is no reaction for the motility assay, aesculin hydrolysis, lysine decarboxylase, arginine dihydrolase or ornithine decarboxylase (Heitkamp *et al.*, 1988).

FAME and mycolic acid analysis

Total cellular FAMES were determined from strain PYR-1^T, *M. austroafricanum* ATCC 33464^T and *M. aurum* ATCC 23366^T. Table 1 shows the cellular fatty acid compositions of these strains. Strain PYR-1^T can be differentiated from *M. austroafricanum* ATCC 33464^T by the presence of C18:0; the profile did not match those of *M. aurum* ATCC 23366^T, *M. austroafricanum* ATCC 33464^T or any other *Mycobacterium* species. A dendrogram based on the fatty acid compositions of strain PYR-1^T, *M. austroafricanum* ATCC 33464^T and other phylogenetically related *Mycobac-*

Fig. 1. Similarity dendrogram of *Mycobacterium* sp. strain PYR-1^T and related species generated by cluster analysis of FAME profiles. Levels of correlation are expressed as coefficients of similarity generated by Euclidean distances between pairs of bacteria.

terium species from the fatty acid composition library was created by cluster analysis. The relationships of the FAME profile of strain PYR-1^T to other strains based on the coefficients of similarity generated by Euclidean distances between pairs of bacteria are depicted in Fig. 1. The Euclidean distance between strains PYR-1^T and *M. austroafricanum* ATCC 33464^T was greater than 4.0. These results suggest that these two strains are different. Similar dendrograms have been used to differentiate closely related *Mycobacterium* species (Willumsen *et al.*, 2001) and clinical isolates of *Pseudomonas cepacia* (Mukwaya & Welch, 1989).

The mycolic acids from strain PYR-1^T and *M. austroafricanum* ATCC 33464^T were analysed in our laboratory as described previously by Rafii *et al.* (1992); a table and a figure adapted from Rafii *et al.* (1992), showing the peak areas of esters of mycolic acids analysed by reversed-phase HPLC, are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>). Three peaks, 4, 17 and 27, were absent in strain PYR-1^T and four peaks, 5, 6, 8 and 9,

Fig. 2. Dot-blot DNA–DNA hybridization assay with *Mycobacterium* sp. PYR-1^T as a probe. Rows: A; PYR-1^T; B, *M. austroafricanum* ATCC 33464^T. Columns: 1, 10 ng; 2, 1 ng; 3, 0.1 ng.

Fig. 3. Neighbour-joining tree based on 16S rDNA sequences showing relationships between strain PYR-1^T and members of the genus *Mycobacterium*. Numbers at nodes indicate levels of bootstrap support based on neighbour-joining analyses of 1000 resample datasets. Bar, 0.01 nucleotide substitutions per nucleotide position.

were absent in *M. austroafricanum*. These data indicate that these two strains have different mycolic acids.

Dot-blot DNA–DNA hybridization

The dot-blot DNA–DNA hybridization experiment revealed that chromosomal DNA of strain PYR-1^T did not hybridize intensively with the chromosomal DNA of strain *M. austroafricanum* ATCC 33464^T. The hybridization was less than 40 % (Fig. 2). These results suggest that strain PYR-1^T is distinct from *M. austroafricanum* ATCC 33464^T.

16S rRNA gene phylogenetic analysis

The sequence of the 16S rRNA gene of strain PYR-1^T was determined previously (Wang *et al.*, 1995). Phylogenetic analysis was conducted by comparing the 16S rDNA sequence for this species with other *Mycobacterium* species obtained from GenBank (Fig. 3). Wang *et al.* (1995) previously compared 41 other mycobacterial species and found that strain PYR-1^T is different

from all other known mycobacterial species in the group tested. The most notable variation was at the V2 region of the 16S rRNA gene, positions 168–200 (in the *Escherichia coli* numbering system; Brosius *et al.*, 1978). Wang *et al.* (1995) also reported that the 16S rDNA sequence of strain PYR-1^T is closely related to that of *M. aurum* ATCC 23366^T; however, at that time, the 16S rDNA sequence of *M. austroafricanum* ATCC 33464^T had not been determined. The 16S rDNA sequence of *M. austroafricanum* ATCC 33464^T has now been determined (accession number AF190800). When we aligned the 16S rDNA sequences of strain PYR-1^T and *M. austroafricanum* ATCC 33464^T, we found one gap, at sequence position 43. The 16S rDNA sequence of strain PYR-1^T also exhibited a high level of similarity to those of other fast-growing *Mycobacterium* species. For example, the similarity values for the 16S rDNA sequence of strain PYR-1^T was 99.8 % to *M. austroafricanum* ATCC 33464^T, 99 % to *M. aurum* ATCC 23366^T, 98 % to *Mycobacterium vaccae* ATCC 15438^T and 94–97 % to other *Mycobacterium* species. These high sequence similarity values confirm that strain PYR-1^T is closely related to *M. austroafricanum* ATCC 33464^T (Kleespies *et al.*, 1996). Recently, Brown *et al.* (1999) have reported two novel species of fast-growing mycobacterium, from which the 16S rDNA of strain PYR-1^T differed by only 3 bases.

A phylogenetic tree based on the neighbour-joining method is given in Fig. 3. Strain PYR-1^T was recovered in a monophyletic clade containing the type strains *M. austroafricanum* ATCC 33464^T, *M. aurum* ATCC 23366^T and *M. vaccae* ATCC 15438^T. The relationship was strongly supported by bootstrap values and by the Fitch–Margoliash treeing algorithms.

PFGE analysis

In order to characterize strain PYR-1^T further and to confirm it as a novel species of *Mycobacterium*, we conducted a PFGE analysis. Agarose plugs were prepared containing DNA from *Mycobacterium* sp. strain PYR-1^T, *M. austroafricanum* ATCC 33464^T and *M. aurum* ATCC 23366^T and digested with *Xba*I restriction endonuclease to compare the total genomic DNA digestion patterns. After restriction digestion, the digested plugs were subjected to PFGE. Fig. 4 shows the *Xba*I digestion patterns of chromosomal DNA from strain PYR-1^T, *M. austroafricanum* ATCC 33464^T and *M. aurum* ATCC 23366^T; all three bacteria gave unique restriction patterns. Previously, we reported that strain PYR-1^T and *M. austroafricanum* ATCC 33464^T have different DNA restriction patterns, which was confirmed by Southern hybridization (Rafii *et al.*, 1992).

Conclusion

The phylogenetic and morphological properties, analysis of 16S rDNA, FAMES and mycolic acids (Table 1 and Fig. 1; Supplementary material), dot-blot

Fig. 4. *Xba*I-digested PFGE patterns of *Mycobacterium* species. Lanes: 1, *M. aurum* ATCC 23366^T; 2, *M. austroafricanum* ATCC 33464^T; 3, *Mycobacterium* sp. PYR-1^T; 4, low-range PFGE marker. Molecular mass marker band sizes are in kb.

DNA–DNA hybridization and PFGE restriction analysis (Figs 2 and 4) clearly demonstrate that strain PYR-1^T is a member of the genus *Mycobacterium*. It can be clearly distinguished from *M. austroafricanum* ATCC 33464^T and *M. aurum* ATCC 23366^T by FAME and PFGE analyses. Therefore, we propose that this organism be classified in the genus *Mycobacterium* as *Mycobacterium vanbaalenii* sp. nov.

Description of *Mycobacterium vanbaalenii* sp. nov.

Mycobacterium vanbaalenii (van.baa'len.i.i. N.L. gen. n. *vanbaalenii* of Van Baalen, in memory of Dr Chase Van Baalen, late Professor at The University of Texas Marine Science Institute, Port Aransas Marine Laboratory, Port Aransas, TX, USA).

Cells are aerobic, Gram-positive, acid-fast rods (1.4 µm long and 0.7 µm wide). Colonies are smooth and saffron yellow. Catalase- and urease-positive, reduces nitrate to nitrite, metabolizes salicylic acid, hydrolyses Tween 80, reduces tellurite and utilizes pyrene, anthracene, fluoranthene, naphthalene, phenanthrene, 1-nitropyrene, 6-nitrochrysene, 3-methylcholanthrene and benzo[a]pyrene. Can grow well at 24 and 37 °C, with minimal or no growth at 42 °C. Classified as a rapidly growing *Mycobacterium* species with a generation time of 2.7–4.0 h in MBS medium. Strictly aerobic. Does not produce

niacin and does not utilize glucose, xylose, lactose, Simmons' citrate, sucrose, maltose, adonitol, arabinose, cellobiose, dulcitol, galactose, malonate, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, starch, trehalose, gelatin or aesculin. Does not produce H₂S and there is no reaction for the motility assay, aesculin hydrolysis, lysine decarboxylase, arginine dihydrolase or ornithine decarboxylase.

The type strain is strain PYR-1^T (= DSM 7251^T = NRRL B-24157^T).

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REFERENCES

- Boldrin, B., Tiehm, A. & Fritzsche, C. (1993). Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. *Appl Environ Microbiol* **59**, 1927–1930.
- Brosius, J., Palmer, J. L., Kennedy, J. P. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.
- Brown, B. A., Springer, B., Steingrube, V. A. & 10 other authors (1999). *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* **49**, 1493–1511.
- Cerniglia, C. E. (1981). Aromatic hydrocarbons: metabolism by bacteria, fungi and algae. *Rev Biochem Toxicol* **3**, 321–361.
- Churchill, S. A., Harper, J. P. & Churchill, P. F. (1999). Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. *Appl Environ Microbiol* **65**, 549–552.
- Dean-Ross, D. & Cerniglia, C. E. (1996). Degradation of pyrene by *Mycobacterium flavescens*. *Appl Microbiol Biotechnol* **46**, 307–312.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, USA.
- Fitch, W. M. & Margoliash, E. (1967). Construction of phylogenetic trees. *Science* **155**, 279–284.
- Fritzsche, C. (1994). Degradation of pyrene at low defined oxygen concentrations by a *Mycobacterium* sp. *Appl Environ Microbiol* **60**, 1687–1689.
- Grosser, R. J., Warshawsky, D. & Vestal, J. R. (1991). Indigenous and enhanced mineralization of pyrene, benzo[a]pyrene, and carbazole in soils. *Appl Environ Microbiol* **57**, 3462–3469.
- Grosser, R. J., Warshawsky, D. & Vestal, J. R. (1995). Mineralization of polycyclic and *N*-heteropolycyclic aromatic compounds in hydrocarbon-contaminated soils. *Environ Toxicol Chem* **14**, 375–382.
- Guerin, W. F. & Jones, G. E. (1988). Mineralization of phenanthrene by a *Mycobacterium* sp. *Appl Environ Microbiol* **54**, 937–944.
- Heitkamp, M. A. & Cerniglia, C. E. (1989). Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. *Appl Environ Microbiol* **55**, 1968–1973.

- Heitkamp, M. A., Franklin, W. & Cerniglia, C. E. (1988). Microbial metabolism of polycyclic aromatic hydrocarbons: isolation and characterization of a pyrene-degrading bacterium. *Appl Environ Microbiol* **54**, 2549–2555.
- Jalava, K., On, S. L. W., Vandamme, P. A. R., Happonen, I., Sukura, A. & Hanninen, M.-L. (1998). Isolation and identification of *Helicobacter* spp. from canine and feline gastric mucosa. *Appl Environ Microbiol* **64**, 3998–4006.
- Jimenez, I. Y. & Bartha, R. (1996). Solvent-augmented mineralization of pyrene by a *Mycobacterium* sp. *Appl Environ Microbiol* **62**, 2311–2316.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kästner, M., Breuer-Jammali, M. & Mahro, B. (1994). Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). *Appl Microbiol Biotechnol* **41**, 267–273.
- Kelley, I. & Cerniglia, C. E. (1995). Degradation of a mixture of high-molecular-weight polycyclic aromatic hydrocarbons by a *Mycobacterium* strain PYR-1. *J Soil Contam* **4**, 77–91.
- Kelley, I., Freeman, J. P. & Cerniglia, C. E. (1990). Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp. *Biodegradation* **1**, 283–290.
- Kelley, I., Freeman, J. P., Evans, F. E. & Cerniglia, C. E. (1991). Identification of a carboxylic acid metabolite from the catabolism of fluoranthene by a *Mycobacterium* sp. *Appl Environ Microbiol* **57**, 636–641.
- Kent, P. T. & Kubica, G. P. (1985). Public health mycobacteriology. *A Guide for the Level III Laboratory*, pp. 71–157. Atlanta, GA: US Department of Health and Human Services, Public Health Service, Centers for Disease Control.
- Khan, A. A., Wang, R.-F., Cao, W.-W., Franklin, W. & Cerniglia, C. E. (1996). Reclassification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Beijerinckia* sp. strain B1, as *Sphingomonas yanoikuyae* by fatty acid analysis, protein pattern analysis, DNA-DNA hybridization, and 16S ribosomal DNA sequencing. *Int J Syst Bacteriol* **46**, 466–469.
- Khan, A. A., Wang, R., Cao, W.-W., Doerge, D. R., Wennerstrom, D. & Cerniglia, C. E. (2001). Molecular cloning, nucleotide sequence, and expression of genes encoding a polycyclic aromatic ring dioxygenase from *Mycobacterium* sp. strain PYR-1. *Appl Environ Microbiol* **67**, 3577–3585.
- Kleespies, M., Kroppenstedt, R. M., Rainey, F. A., Webb, L. E. & Stackebrandt, E. (1996). *Mycobacterium hodleri* sp. nov., a new member of the fast-growing mycobacteria capable of degrading polycyclic aromatic hydrocarbons. *Int J Syst Bacteriol* **46**, 683–687.
- Lloyd-Jones, G. & Hunter, D. W. F. (1997). Characterization of fluoranthene- and pyrene-degrading *Mycobacterium*-like strains by RAPD and SSU sequencing. *FEMS Microbiol Lett* **153**, 51–56.
- Molina, M., Araujo, R. & Hodson, R. E. (1999). Cross-induction of pyrene and phenanthrene in a *Mycobacterium* sp. isolated from polycyclic aromatic hydrocarbon contaminated river sediments. *Can J Microbiol* **45**, 520–529.
- Moody, J. D., Freeman, J. P., Doerge, D. R. & Cerniglia, C. E. (2001). Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. *Appl Environ Microbiol* **67**, 1476–1483.
- Moody, J. D., Doerge, D. R., Freeman, J. P. & Cerniglia, C. E. (2002). Degradation of biphenyl by *Mycobacterium* sp. strain PYR-1. *Appl Microbiol Biotechnol* **58**, 364–369.
- Mukwaya, G. M. & Welch, D. F. (1989). Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. *J Clin Microbiol* **27**, 2640–2646.
- Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Rafii, F., Butler, W. R. & Cerniglia, C. E. (1992). Differentiation of a rapidly growing, scotochromogenic, polycyclic-aromatic-hydrocarbon-metabolizing strain of *Mycobacterium* sp. from other known *Mycobacterium* species. *Arch Microbiol* **157**, 512–520.
- Rafii, F., Selby, A. L., Newton, R. K. & Cerniglia, C. E. (1994). Reduction and mutagenic activation of nitroaromatic compounds by a *Mycobacterium* sp. *Appl Environ Microbiol* **60**, 4263–4267.
- Rehmann, K., Noll, H. P., Steinberg, C. E. W. & Ketrup, A. A. (1996). Branched metabolic pathway for phenanthrene degradation in a pyrene-degrading bacterium. *Polycycl Aromat Comp* **11**, 125–130.
- Rehmann, K., Noll, H. P., Steinberg, C. E. W. & Ketrup, A. A. (1998). Pyrene degradation by *Mycobacterium* sp. strain KR2. *Chemosphere* **36**, 2977–2992.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W. & Warshawsky, D. (1996). Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. *Appl Environ Microbiol* **62**, 13–19.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Tiehm, A. & Fritzsche, C. (1995). Utilization of solubilized and crystalline mixtures of polycyclic aromatic hydrocarbons by a *Mycobacterium* sp. *Appl Microbiol Biotechnol* **42**, 964–968.
- Tongpim, S. & Pickard, M. A. (1999). Cometabolic oxidation of phenanthrene to phenanthrene *trans*-9,10-dihydrodiol by *Mycobacterium* strain S1 growing on anthracene in the presence of phenanthrene. *Can J Microbiol* **45**, 369–376.
- Wang, R.-F., Cao, W.-W. & Cerniglia, C. E. (1995). Phylogenetic analysis of polycyclic aromatic hydrocarbon degrading mycobacteria by 16S rRNA sequencing. *FEMS Microbiol Lett* **130**, 75–80.
- Wang, R.-F., Luneau, A., Cao, W.-W. & Cerniglia, C. E. (1996). PCR detection of polycyclic aromatic hydrocarbon-degrading mycobacteria. *Environ Sci Technol* **30**, 307–311.
- Wang, R.-F., Wennerstrom, D., Cao, W.-W., Khan, A. A. & Cerniglia, C. E. (2000). Cloning, expression, and characterization of the *katG* gene, encoding catalase-peroxidase, from the polycyclic aromatic hydrocarbon-degrading bacterium *Mycobacterium* sp. strain PYR-1. *Appl Environ Microbiol* **66**, 4300–4304.
- Willumsen, P., Karlson, U., Stackebrandt, E. & Kroppenstedt, R. M. (2001). *Mycobacterium frederiksbergense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species. *Int J Syst Evol Microbiol* **51**, 1715–1722.