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Physiological and Genetic Comparison of Two Aromatic Hydrocarbon-degrading *Sphingomonas* Strains

Kay L. Shuttleworth†, Junghee Sung[1], Eungbin Kim[1,*], and Carl E. Cerniglia*

Division of Microbiology, National Center for Toxicological Research U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA;

[1] Department of Biology and Institute of Life Science, Yonsei University, Seoul 120-749, Korea.

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Sphingomonas yanoikuyae **strain B1 is able to degrade a wider range of aromatic hydrocarbons than *S. paucimobilis* strain TNE12 can degrade. Various culture techniques were used to corroborate that B1 used *m*-xylene, biphenyl, toluene, naphthalene, and phenanthrene as sole carbon and energy sources. In contrast, TNE12 could not mineralize *m*-xylene, biphenyl, toluene, or naphthalene. However, fluoranthene served as carbon and energy source for TNE12 but not B1. Southern blots were performed using the cloned genomic region (approximately 23 kb) containing the degradative genes for the upstream pathways for biphenyl and *m*-xylene and a TOL plasmid-type *meta* operon from B1 as a probe against the *Kpn*I restriction-digested total DNA of TNE12. This 23 kb probe hybridized to three *Kpn*I-digested fragments of TNE12 DNA; thus significant homology existed between the aromatic hydrocarbon-degrading genes of B1 and TNE12. Further work with smaller probes revealed, however, that TNE12 DNA fragments did not hybridize with the probe containing the genes encoding for xylene monooxygenase and part of an aromatic dioxygenase. A recombinant plasmid, which contains only the genes for xylene monooxygenase, is able to complement TNE12 on *m*-xylene. These genes are, therefore, probably missing from TNE12. Hence, TNE12 cannot use monocyclic aromatics whereas B1 can. Pulsed field gel electrophoresis coupled with Southern blotting revealed that the aromatic degradative genes were on an approximately 240 kb plasmid of TNE12; the same genes in B1 are known to be chromosomal.**

Keywords: Aromatic Hydrocarbon; Biodegradation; *Sphingomonas*.

Introduction

Bacteria that can mineralize or otherwise transform organic pollutants to nontoxic products may be used to remediate contaminated sites. Members of the genus *Sphingomonas* are known to degrade various compounds of interest including, but not limited to, dibenzo-*p*-dioxin (Wittich *et al*., 1992), polyethylene glycol (Kawai and Takeuchi, 1996), pentachlorophenol (Ederer *et al*., 1997), carbofuran (Feng *et al*., 1997), and mono- and polycyclic aromatic hydrocarbons (Fredrickson *et al*., 1995; Gibson *et al*., 1973; Khan *et al*., 1996; Mueller *et al*, 1997; Shuttleworth and Cerniglia, 1996a; Ye *et al*., 1996). Indeed, many studies have focused on the isolation and characterization of bacteria, including sphingomonads, capable of degrading polycyclic aromatic hydrocarbons (PAHs), because the PAHs are on the U.S. EPA list of priority pollutants.

Sphingomonas yanoikuyae strain B1 (formerly *Beijerinckia* sp. strain B1; Gibson *et al*., 1973; Khan *et al*., 1996) and *S. paucimobilis* strain TNE12 (Shuttleworth and Cerniglia, 1996a) are two of the *Sphingomonas* spp. that have the capacity to degrade some PAHs. Although both

†Present address: Biotechnical Services, Inc., 4610 W. Commercial Drive, North Little Rock, AR 72116, USA.

* To whom correspondence should be addressed.
Carl E. Cerniglia
Tel: 1-870-543-7567; Fax: 1-870-543-7307
E-mail: ccerniglia@nctr.fda.gov
or
Eungbin Kim
Tel: 82-2-361-2651; Fax: 82-2-312-5657
E-mail: ebkim@biology.yonsei.ac.kr

Abbreviations: PAH, polycyclic aromatic hydrocarbons.

strains grow well on phenanthrene as sole carbon and energy source, previous studies and preliminary work provided indications that B1 might be metabolically more versatile for aromatic compounds than TNE12. Strain B1 can grow on some monocyclic aromatics as well as certain PAHs as sole carbon and energy sources (Kim and Zylstra, 1995). Strain TNE12 has, so far, only been shown to utilize phenanthrene and fluoranthene as sole carbon and energy sources (Shuttleworth and Cerniglia, 1996a). This study was undertaken to directly compare the ability of the two bacteria to metabolize mono- and polycyclic aromatic hydrocarbons and to determine genetic differences that might explain any observed metabolic differences. A better understanding of the physiological and genetic differences between strains of pollutant-degrading microorganisms should aid in the construction of new strain(s) that are metabolically more versatile than the parent strain(s).

Materials and Methods

Bacteria *Sphingomonas paucimobilis* strain TNE12 (Shuttleworth and Cerniglia, 1996a) and *S. yanoikuyae* strain B1 (Gibson *et al*., 1973; Khan *et al*., 1996) were from the authors' culture collection. Stock cultures of strain TNE12 were maintained on a basal mineral salts solution (Shuttleworth and Cerniglia, 1996a) containing 100 mg/L phenanthrene as the sole carbon and energy source. Phenanthrene was dissolved in ethyl acetate, filter-sterilized, and aseptically added to the culture tubes containing mineral salts. The ethyl acetate was evaporated by incubating tubes at 50°C. Strain TNE12 was transferred every 3 to 4 months and was stored at 4°C. Strain B1 was cultured directly from frozen (20% glycerol in Luria broth) stocks.

Carbon source utilization The ability of strain TNE12 and strain B1 to use the volatile carbon sources including naphthalene, biphenyl, *m*-xylene, and toluene was determined by providing the source in the vapor phase for cells that were inoculated on mineral salts agar. Plates were placed in screw-capped jars and were incubated at 30°C. Naphthalene vapor was provided by placing crystals in an uninoculated plate; biphenyl crystals were placed directly in the lids of inoculated plates. Cotton-stoppered glass tubes containing either *m*-xylene or toluene were placed in the lids of uninoculated plates. The potential for phenanthrene, fluorene, anthracene, fluoranthene, and pyrene to serve as sole carbon sources for strains TNE12 and B1 was verified in liquid cultures. The PAH in question was dissolved in methylene chloride, filter-sterilized (0.2 µm Acrodisc CR PTFE, Gelman Science), and added to sterile dry test tubes. After the methylene chloride evaporated, sterile mineral salts were added; the final concentration of the test PAH was 100 mg/L. Controls consisted of uninoculated tubes with PAHs and inoculated tubes containing only mineral salts solution. Cultures were incubated with agitation at room temperature. The PAH was considered to be a sole carbon and energy source if visible turbidity developed and PAH crystals disappeared after two transfers. The ability of strain B1 to cometabolize PAHs was determined by looking for zones of clearing on agar plates containing mineral salts and 20 mM succinate (Shuttleworth and Cerniglia, 1996b). Plates were inoculated and then lightly sprayed with ether containing 0.5% of the PAH in question. Benzoate and *m*-toluate were provided at the concentration of 5 mM.

DNA manipulation Total genomic DNA was prepared by the method of Olsen *et al*. (1982). Plasmid DNA was purified by the QIAprep spin column procedure (Qiagen Inc.). DNA was digested with restriction enzymes as recommended by the supplier (Bethesda Research Laboratories, Inc.). Agarose gel electrophoresis was performed in 40 mM Tris, 20 mM acetate, 2 mM EDTA (TAE) buffer. Transfer of DNA from agarose gels to positively charged nylon membranes (Boehringer Mannheim) was carried out using an LKB vacugene apparatus as recommended by the supplier (Pharmacia-LKB Instruments). DNA restriction fragments to be used as probes in Southern blotting experiments were separated by gel electrophoresis and eluted from gel fragments by the procedure of Vogelstein and Gillespie (1979). DNA fragments were labeled with the DIG kit (Boehringer Mannheim), which is based on the random priming method of Feinberg and Vogelstein (1983). Southern hybridizations were performed as recommended by the supplier of the DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim). The recombinant plasmid to complement strain TNE12 on *m*-xylene was constructed in a broad host range vector pRK415 (Keen *et al*., 1988). The ability of the plasmid to complement the strain was determined by transferring the hybrid plasmid into TNE12 by triparental mating using the helper plasmid pRK2013 (Figurski and Helinski, 1979). One loopful of cells of recipient, donor, and helper strains grown overnight were resuspended in 0.5 ml sterile distilled water. Samples of each suspension were mixed in a 2:1:1 ratio of recipient : donor : helper. An appropriate amount (20 µl) of the mixed suspension was spotted on the surface of an L agar plate, and further incubated for 8 to 10 h at 30°C. The cells grown on the surface were scraped, resuspended in 5 ml of sterile distilled water, and 100 µl of the suspension was plated out on mineral salts plates containing 20 mM succinate and 15 µg/ml tetracycline.

Pulsed field gel electrophoresis (PFGE) Agarose plugs containing genomic DNA were prepared as described by Finney (1994). PFGE was performed using a Bio-Rad Laboratories CHEF Mapper system. Gels (1.0% agarose in 0.5× TBE buffer) were run at 200 volts, 15°C. The pulse duration increased from 15 to 60 s during a 20-h run.

Results and Discussion

Growth of *S. paucimobilis* strain TNE12 and *S. yanoikuyae* strain B1 on aromatics as sole carbon and energy sources In previous work, the only PAHs that served as sole carbon and energy sources for strain TNE12 were phenanthrene and fluoranthene, but strain TNE12 could cometabolize radio-labeled anthracene and fluorene (Shuttleworth and Cerniglia, 1996a). However, previous test substrates did not include monocyclic aromatics and the ability of TNE12 to grow on vapor phase naphthalene was not examined. In this study, it was found that the

aromatic substrate range for *S. paucimobilis* strain TNE12 is indeed limited because it did not grow on toluene, *m*-xylene, biphenyl, or vapor-phase naphthalene (Table 1).

Strain B1 was originally reported to use biphenyl, naphthalene, anthracene, and phenanthrene as sole carbon and energy sources (Gibson *et al.*, 1973); strain B1 has also been reported to transform, but not necessarily mineralize, various PAHs (for a review see Zylstra and Kim, 1997). In order to compare the substrate utilization patterns of strains TNE12 and B1 equitably, reassessment of the metabolic potential of strain B1 was performed under the same conditions. The ability of B1 to use biphenyl, phenanthrene, and naphthalene was confirmed; it did not, however, produce visible growth in liquid cultures containing anthracene as sole carbon and energy source (Table 1). Also, it did not grow on fluorene, fluoranthene, or pyrene. Thus, B1 utilized a wider range of aromatic substrates than strain TNE12, although strain TNE12 grew on fluoranthene and B1 did not. Although both strains utilized phenanthrene as sole carbon and energy source and both cometabolically transformed anthracene and fluorene, there were important metabolic differences between strains TNE12 and B1. The observed variations could be a result of genetic differences or of different substrate specificities of the respective degradative enzymes. Molecular methods were used to address this question.

Southern blots with a probe containing degradative genes for monocyclic and polycyclic aromatic hydrocarbons The previously isolated cosmid clone pGJZ1510 contains many of the genes needed for the degradation of both monocyclic and polycyclic aromatic hydrocarbons by strain B1 (Kim and Zylstra, 1995; Zylstra and Kim, 1997). An ~23 kb *Hin*dIII fragment from pGJZ1510, which has at least 20 of the degradative genes (Fig. 1), was used as a probe in Southern blots to determine if there was homology between the cloned region of strain B1 and the genomic DNA of strain TNE12. Total genomic DNA from strains TNE12 and B1 was completely digested with *Kpn*I and utilized as the target in Southern blot experiments. The results (Fig. 1A) show that strain TNE12 DNA hybridized to the probe, although the hybridizing band pattern was different from that of strain B1. Strains B1 and TNE12 had four (3.0, 6.9, 9.5, and 10.0 kb) and three (9.5, 12, and 20 kb) hybridizing fragments, respectively. The fact that the ~23 kb *Hin*dIII probe from strain B1 hybridizes to multiple genomic *Kpn*I restriction fragments from strain TNE12 suggests that a significant

Table 1. Monocyclic and polycyclic aromatic hydrocarbons used by strains B1 and TNE12.

Compound	*S. yanoikuyae* strain B1	*S. paucimobilis* strain TNE12
Benzoate	Sole carbon and energy source	Sole carbon and energy source
m-Toluate	Sole carbon and energy source	Sole carbon and energy source
Toluene	Sole carbon and energy source	No growth[a]
m-Xylene	Sole carbon and energy source	No growth
Biphenyl	Sole carbon and energy source	No growth. Accumulation of yellow product in plates with biphenyl only is indicative of ring cleavage without further degradation.
Naphthalene	Sole carbon and energy source	No growth
Anthracene	No growth[b]. Cometabolizes anthracene to CO_2 and dead-end metabolites in the presence of succinate.[c]	No growth. Cometabolizes anthracene to CO_2 and dead-end metabolites in the presence of glucose.[d]
Fluorene	No growth. Cometabolism is indicated by the production of large zones of clearing and intense yellow color on fluorene-sprayed succinate plates	No growth. In the presence of glucose, the organism cometabolically produces aqueous and ethyl acetate-extractable dead-end metabolites but not CO_2.[d]
Phenanthrene	Sole carbon and energy source	Sole carbon and energy source
Fluoranthene	No growth. Pale red/orange color in tubes with fluoranthene only indicates possible production of dead-end ring fission metabolites.	Sole carbon and energy source
Pyrene	No growth. Brownish color in tubes with pyrene only indicates production of dead-end ring fission metabolites.	No growth

[a] No growth denotes the compound could not serve as sole carbon and energy source. Growth in liquid culture was determined by turbidity (A_{600} = 1.0 within 48 h) and growth on agar plates exposed to a vapor-phase carbon source was determined by colony formation (1.0 mm in diameter within 48 h).
[b] Strain B1 was originally reported to use anthracene as sole carbon and energy source (Gibson *et al.*, 1973); however, there was no mention of the method used to determine utilization and the strain has been in laboratory culture for more than 25 years.
[c] Data on cometabolism in liquid culture from Kim *et al.* (1997).
[d] Data on cometabolism in liquid culture from Shuttleworth and Cerniglia (1996a).

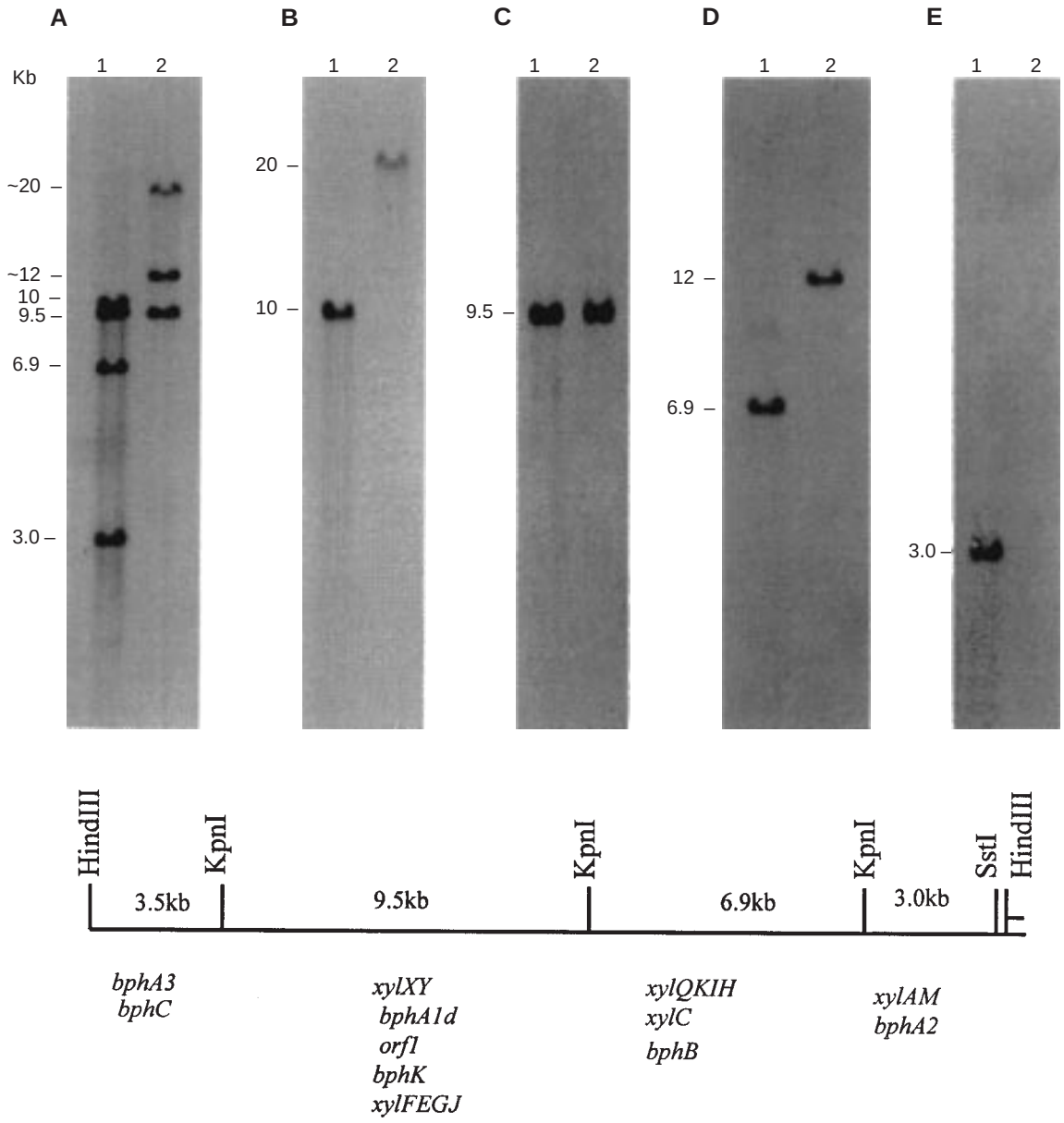

Fig. 1. Southern blots of *Kpn*I-digested DNA from *Sphingomonas yanoikuyae* strain B1 (lane 1) and *S. paucimobilis* strain TNE12 (lane 2). The probes were (**A**) the ~23 kb *Hin*dIII fragment, (**B**) the 3.5 kb *Hin*dIII-*Kpn*I fragment, (**C**) the 9.5 kb *Kpn*I fragment, (**D**) the 6.9 kb *Kpn*I fragment, and (**E**) the 3.0 kb *Kpn*I fragment. The line drawing shows the relative locations of the specific genes in the DNA fragments used as probes for the Southern blots.

amount of homology exists between the regions coding for aromatic hydrocarbon degradation even though the two strains have different substrate ranges.

Southern blots with different *Kpn*I restriction fragment probes Although DNA from strain TNE12 hybridized with the ~23 kb probe, there may have been sections of the probe that were not homologous with strain TNE12 DNA. Thus the *Kpn*I restriction sites on the 23 kb *Hin*dIII DNA fragment were utilized to generate four smaller probes (Fig. 1) for additional Southern hybridization experiments with *Kpn*I-digested total genomic DNA of strain TNE12. This made it possible to determine if homology existed uniformly over the entire 23 kb fragment or if only particular genes were homologous to strain TNE12 DNA.

First, the 3.5 kb *Hin*dIII-*Kpn*I fragment, which contained *bphA3*, *bphC*, and a part of *xylX*, was used as a probe against the *Kpn*I-digested total genomic DNA of each strain. The *bphA3* and *bphC* genes encode for a ferredoxin component for the polycyclic aromatic dioxygenase and a

2,3-dihydroxybiphenyl 1,2-dioxygenase, respectively, which are key enzymes in the upper pathway for PAH degradation by B1. The *xylX* gene encodes for a large subunit of toluate dioxygenase and BphA3 is also required for toluate dioxygenase activity in B1 (Zylstra and Kim, 1997). Strains B1 and TNE12 possessed 10 and 20 kb genomic DNA fragments, respectively, that hybridized to this 3.5 kb *Hin*dIII-*Kpn*I fragment probe (Fig. 1B).

The two adjacent 9.5 and 6.9 kb *Kpn*I fragments (Fig. 1) that were used as Southern probes contain an operon that is similar to the *meta*-cleavage operon found in the TOL plasmid (Zylstra and Kim, 1997). The 9.5 kb fragment contains most of the genes for the degradation of (methyl)benzoates. The remaining genes for (methyl)benzoate degradation and the genes for benzaldehyde dehydrogenase (*xylC*) and *cis*-dihydrodiol dehydrogenase (*bphB*) are on the 6.9 kb *Kpn*I fragment. The Southern blot with the 9.5 kb *Kpn*I probe (Fig. 1C) showed that strains TNE12 and B1 share a common 9.5 kb hybridizing band. These data suggest that the two strains have very similar genes in their *meta*-cleavage operons for (methyl)benzoate degradation. The Southern blot with the 6.9 kb *Kpn*I probe (Fig. 1D) showed that strains B1 and TNE12 have 6.9 and 12 kb hybridizing *Kpn*I fragments, respectively.

The 3.0 kb *Kpn*I probe contained *xylAM* (xylene monooxygenase) and a part of *bphA2* (an iron sulfur protein small subunit of an aromatic dioxygenase). As expected, the Southern blot with the 3.0 kb probe and *Kpn*I-digested B1 DNA revealed a 3.0 kb hybridizing band (Fig. 1E). There was, however, no hybridization between the 3.0 kb probe and the digested DNA of strain TNE12. This observation suggests that the genes *xylAM* and *bphA2* are missing in TNE12. The fact that strain TNE12 cannot grow on toluene or *m*-xylene supports the hypothesis that these genes are missing because xylene monooxygenase, which is encoded by *xylAM*, is required for the initial oxidation of those alkylbenzenes.

Complementation of *S. paucimobilis* strain TNE12 with *xylAM* on *m*-xylene Since the strain TNE 12 did not show hybridization to the 3.0 kb *Kpn*I probe, a complementation experiment was performed by providing TNE12 with a hybrid plasmid containing the 3.0 kb *Kpn*I DNA fragment. The resulting recombinant strain was found to acquire the ability to grow slowly (approximately at half the growth rate of B1) on *m*-xylene as the sole carbon and energy source, and a light yellow color developed, suggesting the accumulation of some *m*-xylene metabolites. The culture supernatant of the strain with the hybrid plasmid grown on *m*-xylene had a UV absorption spectrum (λ_{max} at 388 nm) that was identical to the spectrum of the *meta*-ring cleavage product of 3-methylcatechol (Bayly *et al.*, 1966). These results indicate that the recombinant strain metabolizes *m*-xylene through the TOL plasmid type *meta*-cleavage pathway. The fact that the addition of only the *xylAM* genes confers the ability to grow on *m*-xylene upon *S. paucimobilis* TNE12 suggests that the strain possesses the rest of genes necessary for *m*-xylene degradation. This hypothesis is also supported by the fact that TNE12 can grow on *m*-toluate (Table 1) and by the Southern hybridization data that both *S. paucimobilis* TNE12 and *S. yanoikuyae* B1 show a common 9.5 kb hybridizing band, which contains most of the genes for the *meta*-cleavage operon.

Location of the genes in the genome Kim *et al.* (1996) found that the cross-hybridizing genes for aromatic hydrocarbon degradation in all of the aromatic hydrocarbon-degrading *Sphingomonas* strains isolated from a deep subsurface site are present on large plasmids. The genes were found to be chromosomal, however, in the two surface *Sphingomonas* strains tested, *S. yanoikuyae* B1 and *S. paucimobilis* Q1, even though both strains B1 and Q1 also possess large (>100 kb) plasmids (Kim *et al.*, 1996). More recently, Dagher *et al.* (1997) reported that *Sphingomonas* sp. strain 107, which was isolated from a sand pit and is thus a surface bacterium, contained PAH degradative genes on a 55 kb plasmid. It is therefore evident that the genes for aromatic degradation in *Sphingomonas* spp. may be encoded either on plasmid(s) or the chromosome. In this study, a combination of PFGE and Southern blotting was used to demonstrate that the aromatic degradative genes of strain TNE12 were on an approximately 240 kb plasmid (Fig. 2). Strain TNE12 also had at least one more large plasmid (~680 kb) that did not hybridize to the 23 kb *Hin*dIII probe (Fig. 2). Strain B1 is known to harbor two plasmids, pKG1 (232 kb) and pKG2 (33 kb; Kim *et al.*, 1996; Kiyohara *et al.*, 1983). The smaller plasmid of strain B1, pKG2, was not evident in this study, however, because it ran off the bottom of the gel under the conditions used.

Many members of the genus *Sphingomonas* have been shown to degrade monocyclic and/or polycyclic aromatic compounds (Dagher *et al.*, 1997; Fredrickson, 1995; Mueller *et al.*, 1990; Zylstra and Kim, 1997). The aromatic hydrocarbon-degrading strains *S. yanoikuyae* strain B1 (Zylstra and Kim, 1997), *S. paucimobilis* strain Q1 (Kuhm *et al.*, 1991), and the deep subsurface *Sphingomonas* strains (Fredrickson *et al.*, 1995) all have the ability to grow on both monocyclic and polycyclic aromatic hydrocarbons. *Sphingomonas* sp. strain 107 (Dagher *et al.*, 1997) appears to be able to transform or mineralize a relatively wide range of PAHs, including naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene. In contrast, strain TNE12 has a very narrow aromatic substrate range; it grows only on phenanthrene and fluoranthene and cometabolizes anthracene and fluorene (Table 1). *S. paucimobilis* strain EPA505 also apparently has a broader substrate range than strain

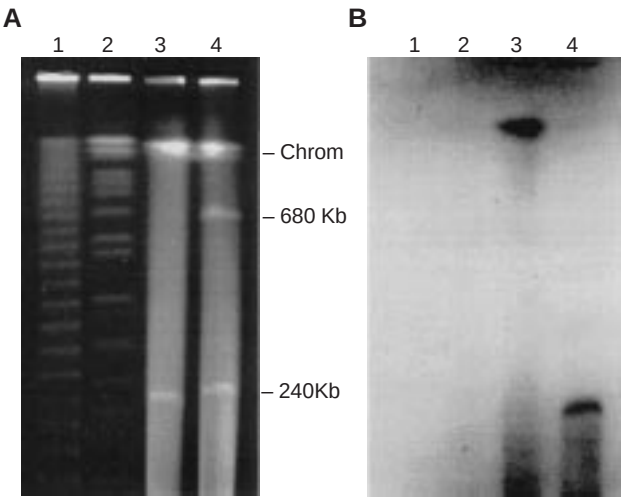

Fig. 2. Localization of the aromatic hydrocarbon degradative genes in *S. yanoikuyae* strain B1 and *S. paucimobilis* strain TNE12. **A.** Pulsed field gel electrophoresis of the total DNA from strains B1 and TNE12. **B.** Southern hybridization of the PFGE-separated DNA from strains B1 and TNE12. The probe was the ~23 kb *Hin*dIII fragment from the pGJZ1510 clone. For both (**A**) and (**B**) the lanes are: (1) CHEF DNA size standard, lambda ladder, (2) CHEF DNA size marker, *Saccharomyces cerevisiae*, (3) *Sphingomonas yanoikuyae* strain B1, and (4) *Sphingomonas paucimobilis* strain TNE12. The CHEF DNA size standards were obtained from Bio-Rad Laboratories.

TNE12. Strain EPA505 reportedly grows best on phenanthrene and fluoranthene, as does strain TNE12, but strain EPA505 also grows to a limited extent with vapor phase naphthalene (Mueller *et al*., 1990). Both strains apparently can cleave biphenyl as determined by the production of colored products, but neither strain can use it as a sole carbon and energy source. Strain EPA505 apparently can also transform and even mineralize higher molecular weight PAHs, such as chrysene and benzo[a]pyrene, under the proper conditions (Ye *et al*., 1996). The ability of strain EPA505 to utilize monocyclic aromatics has apparently not been examined, however. In comparison with other aromatic-degrading *Sphingomonas* spp., strain TNE12 apparently does have a limited ability to mineralize or transform this class of compounds. The limited degradative capability of strain TNE12 might be due to the narrow specificity of the degradative enzymes and/or lack of some key enzymes. The present work shows that some structural genes present in strain B1, including *xylAM*, are missing in strain TNE12; the lack of *xylAM* probably explains the inability of strain TNE12 to grow on monocyclic compounds such as *m*-xylene and toluene. It is unclear if *S. paucimobilis* TNE12 has not had a chance to acquire some peripheral genes such as *xylAM* or if some deletions have occurred.

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