Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics

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The plasmids from 16 sphingomonads which degrade various xenobiotics and polycyclic aromatic compounds were compared with the previously sequenced plasmid pNL1 from Sphingomonas aromaticivorans F199. The replicate genes repAaAb from plasmid pNL1 were amplified by PCR and used as a gene probe for the identification of plasmids belonging to the same incompatibility group as plasmid pNL1. Plasmids were prepared from various sphingomonads and hybridized with the repA gene probe. Positive hybridization signals were obtained with plasmids of approximately 160–195 kb from Sphingomonas subteranea and S. aromaticivorans B0695, which had been isolated from the same subsurface location as S. aromaticivorans F199. The repA probe also hybridized with plasmids from Sphingomonas xenophaga BN6, Sphingomonas sp. HH69 and Sphingomonas macrogoltabidus, which had been isolated from different continents and which utilize different organic compounds than S. aromaticivorans F199 and the other subsurface strains. The results of the hybridization experiments were confirmed by PCR experiments using primers deduced from the repAaAb region of plasmid pNL1. Nucleotide sequence comparisons suggested that three gene clusters were conserved between plasmid pNL1 and plasmid pBN6 from the naphthalenesulfonate-degrading strain S. xenophaga BN6. From these sequence comparisons, PCR primers were derived in order to detect the respective gene clusters in the other strains and to deduce their position relative to each other. These experiments demonstrated that all analysed subsurface strains harboured the same three gene clusters, but that the position and distance from each other of the clusters varied considerably among the different strains.

INTRODUCTION

The genus Sphingomonas consists of Gram-negative, chemoheterotrophic, strictly aerobic, usually yellow-pigmented bacteria that contain glycosphingolipids in their outer membrane and belong to the α-4 subgroup of the Proteobacteria (Takeuchi et al., 2001). Many Sphingomonas strains are able to degrade a wide range of natural and xenobiotic compounds, such as biphenyl, (substituted) naphthalene(s), fluorene, (substituted) phenanthrene(s), pyrene, (chlorinated) diphenylether(s), (chlorinated) furan(s), (chlorinated) dibenzo-p-dioxin(s), carbazole, oestadiol, polyethyleneglycols, chlorinated phenols and different herbicides and pesticides (Cai & Xun, 2002; Feng et al., 1997; Fujii et al., 2003; Harms et al., 1995; Hong et al., 2002; Kilbane et al., 2002; Kumari et al., 2002; Nagata et al., 1997; Nörtemann et al., 1986; Sabaté et al., 2003; Schmidt et al., 1992; Sorensen et al., 2001; Stolz et al., 2000; Story et al., 2001; Tiirola et al., 2002a; Wattiau et al., 2001). The isolation of various Sphingomonas strains which harbour different metabolic pathways for the degradation of a wide range of xenobiotic compounds suggests that the members of this genus have the ability to adapt quickly and efficiently to the degradation of new compounds in the environment.

Previous reports demonstrated that in the naphthalene- and biphenyl-degrading strain Sphingomonas aromaticivorans F199 and in some naphthalenesulfonate-, dibenzo-furan-, dibenzo-p-dioxin- or carbazole-degrading sphingomonads, the relevant degradative pathways are localized on rather large plasmids (Basta et al., 2004; Feng et al., 1997; Ogram et al., 2000; Romine et al., 1999).

Currently, plasmid pNL1 from S. aromaticivorans F199 is the only plasmid from a Sphingomonas strain that has been sequenced, and it has been found that about one-third of the identified ORFs of this plasmid are associated with the catabolism or transport of aromatic compounds (Romine et al., 1999). S. aromaticivorans F199 has been isolated from a deep-subsurface location in the USA from a depth of about 400 m. This is a rather extraordinary niche for a

Abbreviations: LR-PCR, long-range PCR; TAE, Tris/acetate/EDTA.

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micro-organism degrading organic compounds because these sediments are extremely poor in nutrients and thus the organisms have to survive under oligotrophic conditions (Fredrickson et al., 1999).

In our recent study, we demonstrated that large plasmids are ubiquitous in xenobiotic-degrading siphingomonads and that they are important for the dissemination of degradative abilities among these organisms (Basta et al., 2004). We therefore decided to compare these plasmids, with regard to incompatibility grouping and organization of the degradative genes, with plasmid pNL1, in order to gain more information about the diversity and characteristics of *Sphingomonas* plasmids.

**METHODS**

**Bacterial strains and media.** The siphingomonads studied, and some characteristic compounds which are degraded by the strains, are shown in Table 1. Takeuchi et al. (2001) suggested reclassifying the former genus *Sphingomonas (sensu lato)* into the newly defined genera *Sphingomonas (sensu stricto), Sphingobium, Novosphingobium* and *Sphingopyxis*. The members of the former genus *Sphingomonas sensu lato* were therefore also classified in Table 1 according to this suggestion. For those strains listed in Table 1 which are set in quotation marks, no valid new description has been performed, but in the taxonomic system proposed by Takeuchi et al. (2001), most of them probably belong to the genera *Sphingobium or Novosphingobium* (according to their 16S rRNA) and do not belong to the newly defined genus *Sphingomonas sensu stricto*. More recently, the suggestion of Takeuchi et al. (2001) to divide the genus *Sphingomonas* into several genera was rejected by another group and it was suggested that the genus *Sphingomonas sensu lato* be kept (Yabuuchi et al., 2002). Therefore, in the present study, all the strains are referred to as *Sphingomonas*.

The *Sphingomonas* strains were routinely subcultured on nutrient broth (NB) or R2A medium (Atlas, 1995).

**Molecular techniques for the manipulation of DNA.** Genomic DNA from *Sphingomonas* strains was prepared after SDS lysis and phenol extraction, as described elsewhere (Eulberg et al., 1997). The digestion of DNA with restriction endonucleases (New England Biolabs) and agarose gel electrophoresis were performed according to standard procedures (Sambrook et al., 1989). The elution and purification of DNA from agarose gels was performed using the Easy Pure Kit as recommended by the manufacturer (Biozym).

**Preparation of genomic DNA from siphingomonads for the detection of megaplasmids.** Plasmids were isolated and separated from chromosomal DNA using PFGE by the method of Barton et al. (1995), as described previously (Basta et al., 2004).

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**Table 1. Sphingomonas strains analysed in the present study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Compounds degraded</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sphingomonas</em> (Sphingobium) yanoikuyae B1 DSM 6900</td>
<td>Toluene, biphenyl, naphthalene, anthracene, phenanthrene</td>
<td>Yabuuchi et al. (1990)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Sphingobium) herbicidovorans DSM 11019</td>
<td>2-(2,4-Dichlorophenoxy)propionate (dichlor-prop), 2,4-dichlorophenoxyacetate</td>
<td>Zipper et al. (1996)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Sphingobium) chlorophenolica ATCC 33790</td>
<td>Pentachlorophenol, 2,4,6-trichlorophenol</td>
<td>Nohynek et al. (1995)</td>
</tr>
<tr>
<td>'Sphingomonas paucimobilis' Q1</td>
<td>Toluene, xylene, naphthalene, biphenyl, anthracene (Chlorinated) dibenzo-p-dioxin(s), dibenzofuran(s)</td>
<td>Furukawa et al. (1989)</td>
</tr>
<tr>
<td>'Sphingomonas' wittichii RW1 DSM 6014</td>
<td>Fluoranthene, (substituted) naphthalene(s), phenanthrene, anthracene</td>
<td>Wittich et al. (1992); Yabuuchi et al. (2001)</td>
</tr>
<tr>
<td>'Sphingomonas' sp. HH69 DSM 7135</td>
<td>Fluoranthene, (substituted) naphthalene(s), phenanthrene, anthracene</td>
<td>Mueller et al. (1997)</td>
</tr>
<tr>
<td>'Sphingomonas' sp. S33 DSM 6432</td>
<td>Benzene, 1,4-dichlorobenzene</td>
<td>Nohynek et al. (1996)</td>
</tr>
<tr>
<td>'Sphingomonas' sp. EPA505 DSM 7526</td>
<td>(Substituted) naphthalene-2-sulfonate(s)</td>
<td>Nörtemann et al. (1986); Stolz et al. (2000)</td>
</tr>
<tr>
<td>'Sphingomonas' sp. A175 DSM 13477</td>
<td>Polyethyleneglycol 4000</td>
<td>Takeuchi et al. (2001, 1993)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Sphingopyxis) macrogoltabidus DSM 8826</td>
<td>Naphthalene, toluene, biphenyl, dibenzothiophene, fluorene</td>
<td>Balkwill et al. (1997); Takeuchi et al. (2001)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Novosphingobium) subterranea DSM 12447</td>
<td>Naphthalene, toluene, biphenyl, dibenzothiophene, fluorene</td>
<td>Balkwill et al. (1997); Takeuchi et al. (2001)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Novosphingobium) aromaticivorans F199 DSM 12444</td>
<td>2-Methylnaphthalene,acenaphthene,anthracene,fluoranthene,phenanthrene</td>
<td>Shi et al. (2001)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Novosphingobium) aromaticivorans B0695 DSM 10700</td>
<td>2,3,4,6-Tetrachlorophenol, 2,4,6-trichlorophenol</td>
<td>Nohynek et al. (1996); Takeuchi et al. (2001)</td>
</tr>
<tr>
<td><em>Sphingomonas</em> (Novosphingobium) stygia DSM 12445</td>
<td>Toluene, biphenyl, dibenzothiophene, fluorene</td>
<td>Balkwill et al. (1997); Takeuchi et al. (2001)</td>
</tr>
<tr>
<td><em>Sphingomonas paucimobilis</em> DSM 1098</td>
<td>Cannot degrade aromatic hydrocarbons</td>
<td>Yabuuchi et al. (1990)</td>
</tr>
</tbody>
</table>
**Table 2.** Oligonucleotide primers used to amplify various DNA fragments

<table>
<thead>
<tr>
<th>Target/direction*</th>
<th>Primer</th>
<th>Sequence 5′→3′†</th>
<th>Location in the relevant sequence‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicase repAa</td>
<td>Rep-Aa</td>
<td>CCTCTTCTTCTATTTCGCCGG</td>
<td>27778–27798</td>
</tr>
<tr>
<td>Replicase repAb</td>
<td>Rep-Ab</td>
<td>CCGATGAGGGTGATTTGCTGA</td>
<td>31143–31163</td>
</tr>
<tr>
<td>Replicase repAaAb</td>
<td>RepAHST</td>
<td>CATTITGAGCTGCTTCTCCAAGG</td>
<td>28190–28932</td>
</tr>
<tr>
<td>Intergenic region repAa–repAb</td>
<td>RepAori</td>
<td>CGAAAGCCAGGGCATTGGCC</td>
<td>29672–29690</td>
</tr>
<tr>
<td>Replicase repAb</td>
<td>RepAb-Nterm</td>
<td>GTCACTAGGGCAAGTCTGCCG</td>
<td>29831–29850</td>
</tr>
<tr>
<td>Replicase repAb</td>
<td>RepAb-Cterm</td>
<td>GAATCTGCTGTCCTCGTCGGC</td>
<td>30865–30885</td>
</tr>
<tr>
<td>Cluster 1</td>
<td>Block 1-B</td>
<td>tatcgccggcgattggtatg</td>
<td>146901–146884</td>
</tr>
<tr>
<td>Cluster 1</td>
<td>Block 1-C</td>
<td>ccattgcgggacgcctggtctgcag</td>
<td>145368–145384</td>
</tr>
<tr>
<td>Part of cluster 2</td>
<td>Block 2-A</td>
<td>Cgaaggccagcctcctcggtctgcag</td>
<td>161104–161121</td>
</tr>
<tr>
<td>Part of cluster 2</td>
<td>Block 2-C</td>
<td>ctgcagcggaactacccgcctgcag</td>
<td>161336–161357</td>
</tr>
<tr>
<td>Part of cluster 2</td>
<td>Block 2-D</td>
<td>gctgtgctgtaacctgactcgactgcctgcag</td>
<td>162632–162614</td>
</tr>
<tr>
<td>Part of cluster 2</td>
<td>Block 2-E</td>
<td>tcactctccggaaaaatggctgcag</td>
<td>162158–162181</td>
</tr>
<tr>
<td>Part of cluster 2</td>
<td>Block 2-F</td>
<td>cgctgtgctgattacgactcagagtcg</td>
<td>163579–163602</td>
</tr>
<tr>
<td>Cluster 3</td>
<td>Block 3-A</td>
<td>gctgccctccatactgcagatctc</td>
<td>131456–131486</td>
</tr>
<tr>
<td>Cluster 3</td>
<td>Block 3-B</td>
<td>atgtacacgctctgtcagagtcg</td>
<td>132717–132738</td>
</tr>
<tr>
<td>Upstream of cluster 3</td>
<td>Block 3-UP</td>
<td>tcggaaattctctctccttg</td>
<td>131491–131467</td>
</tr>
<tr>
<td>Downstream of cluster 3</td>
<td>Block 3-D-LOW</td>
<td>gctgcgttgtcctgcagagtcg</td>
<td>132717+132728</td>
</tr>
<tr>
<td>Upstream of cluster 1</td>
<td>Block 1-D-UP</td>
<td>acgcggcggagccttcgtcagtcg</td>
<td>146453–146433</td>
</tr>
<tr>
<td>Downstream of cluster 1</td>
<td>Block 1-B-LOW</td>
<td>atcacttcggccgctgataatgtg</td>
<td>146884–146906</td>
</tr>
<tr>
<td>Upstream of cluster 2</td>
<td>Block 2-A-UP</td>
<td>ccaacattggagcgcctgcag</td>
<td>159910–159930</td>
</tr>
<tr>
<td>Downstream of cluster 2</td>
<td>Block 2-E-LOW</td>
<td>ttctactgttattcctgcgcgttca</td>
<td>163679–163706</td>
</tr>
</tbody>
</table>

*Bold letters indicate mismatch nucleotides in the corresponding nucleotide sequence regions of S. xenophaga BN6.  
†The numbers refer to the location of the relevant nucleotides in the sequence of plasmid pNL1 (NCBI accession number NC_002033).

**PCR experiments.** All PCR experiments were performed using a Genius thermal cycler (Techne) in thin-walled 200 μl reaction tubes. The PCR reaction mixtures contained, in a volume of 30 μl, 200 ng DNA, 0.3 μM of each forward and reverse primer (Eurogentec), 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1·5 mM magnesium acetate, 0·2 mM dNTPs (Eppendorf) and 0·5–1·U Taq DNA polymerase (Eppendorf) under the conditions indicated below.

The primers for the detection of replicons with replicative functions similar to those of plasmid pNL1 were derived from the corresponding nucleotide sequence of plasmid pNL1 deposited at the NCBI database (Table 2). The following PCR programme was used for the amplification of the replicon regions from different sphingomonads: an initial denaturation (3 min, 94 °C) was followed by 30 cycles consisting of annealing at 60 °C (30 s), polymerization at 72 °C (40 s) for the amplification of replicons homologues, 45 s for the amplification of the DNA fragments containing iteron sequences, and 3 min for the amplification of the DNA region containing repAaAb, and denaturation at 94 °C (30 s). Finally, an additional polymerization step was performed at 72 °C for 5 min.

The primers for the detection of conserved catabolic gene clusters in different sphingomonads were derived from the consensus sequences of the respective gene clusters from *Sphingomonas xenophaga* BN6 and *S. aromaticivorans* F199, which are available from the NCBI database (Table 2). The PCR program applied consisted of an initial denaturation (3 min, 94 °C), followed by 30 cycles of annealing at 60 °C (30 s), polymerization at 72 °C (40–60 s), and denaturation at 94 °C (30 s). The last polymerization step was extended to 5 min.

The DNA fragments for the generation of the gene probes used for the detection of cluster 1, cluster 2 and cluster 3 were amplified by PCR using primer combinations Block1-B and Block1-C, Block2-A and Block2-F, and Block3-A and Block3-D, respectively (Table 2). The PCR conditions applied were the same as described above, except for the amplification of part of cluster 2. This fragment was amplified using the PCR program consisting of an initial denaturation (3 min, 95 °C), followed by 30 cycles of annealing at 62 °C (30 s), polymerization at 72 °C (4 min), and denaturation at 95 °C (30 s). The last polymerization step was extended to 10 min.

The arrangement of the conserved catabolic gene clusters in sphingomonads was determined using long-range PCR (LR-PCR) experiments. For amplification of long PCR products (5–20 kb), the Expand long template PCR system (Roche), containing a mixture of *Taq* polymerase and *Pwo* proofreading DNA polymerase, was used. The reaction mixtures contained 200–500 ng template, and all other components in the concentrations suggested by the manufacturer. In some cases, 1 M betaine or 6 % (v/v) DMSO was added. The primers used in the LR-PCR experiments were derived from the published nucleotide sequences of catabolic gene clusters of *S. xenophaga* BN6 and *S. aromaticivorans* F199 (Table 2).

The PCR program used consisted of an initial denaturation step at 96 °C (6 min) followed by 10 cycles of denaturation at 94 °C (15 s), primer annealing at 64–68 °C (30 s), polymerization at 68 °C (12–15 min) and 20 cycles of denaturation at 94 °C (15 s), primer annealing at 64–68 °C (30 s), polymerization at 68 °C (12–15 min + 20 s per cycle). The final polymerization step was prolonged to 8 min.

**Hybridization procedures.** A DIG DNA labelling and detection kit was used according to the instructions of the supplier (Roche).
The hybridization temperature in the experiments with the repA probes was set to 56 °C and, with the probes for the detection of the three conserved gene clusters, to 65 °C.

**Sequence comparisons.** Standard sequence analysis, database searches and comparisons were done with the BLAST search facilities at the NCBI. The gene clusters from strains BN6 and F199 were aligned using the MEGALIGN module of the Lasergene program (DNASTAR Inc.).

**RESULTS**

**Hybridization experiments with a repA gene probe from plasmid pNL1**

We attempted to identify plasmids belonging to the same incompatibility group as plasmid pNL1 in our collection of aromatic compound- and xenobiotic-degrading *Sphingomonas* strains (see Table 1). Plasmids of the same incompatibility group have similar replication control mechanisms, which usually involve replication initiation proteins (Rep). Therefore, plasmids can be classified according to nucleotide sequence similarities of rep genes (del Solar et al., 1998). Two genes, repAa and repAb, encoding Rep proteins have been associated with the initiation of replication of plasmid pNL1 (Romine et al., 1999). The repAa and repAb genes were amplified by PCR (see Methods) using genomic DNA of *S. aromaticivorans* F199 as template and the primers Rep-Aa and Rep-Ab specified in Table 2. This resulted in the amplification of a 3–5 kb DNA fragment containing the complete repAa and repAb genes and the intergenic space between them. The 3–5 kb PCR product was purified, labelled with digoxigenin, and hybridized against plasmid DNA from different siphingomonads, which was prepared by PFGE as described previously (Basta et al., 2004). Positive hybridization signals were observed with catabolic plasmids from *S. aromaticivorans* B0695, *Sphingomonas subterranea*, *Sphingomonas* sp. HH69 and *Sphingomonas* sp. EPA505 (Fig. 1). These strains degrade similar aromatic compounds as *S. aromaticivorans* F199 (Table 1). Furthermore, a 260 kb plasmid from *S. xenophaga* BN6, and a 450 kb plasmid from *Sphingomonas macrogoltabidus* also hybridized with the repA gene probe (Table 3).

**PCR identification of plasmids with replication regions similar to that of plasmid pNL1**

The repAa and repAb genes are separated by 495 bp on plasmid pNL1. This intergenic space contains five 17 bp direct repeats termed iterons. It was previously shown for other plasmids that iteron sequences were specific for certain groups of plasmids and that they are major incompatibility determinants (Couturier et al., 1988; del Solar et al., 1998; Giraldo et al., 1998). We therefore attempted to determine the distances between repAa and repAb by PCR for the plasmids which gave a positive hybridization signal with the repA gene probe from plasmid pNL1. The oligonucleotide primers RepAHTH and RepAori were designed (Table 2), which bound to repAa and to the intergenic region between repAa and repAb close to the iteron repeats. This resulted in the amplification of a DNA fragment of about 780 bp from plasmid pNL1. Single PCR products of similar size, which hybridized with the repA probe, were obtained with most of the strains (Fig. 2). The PCR products obtained from *S. aromaticivorans* B0695 and *S. subterranea* demonstrated about the same intensity as that of the positive control *S. aromaticivorans* F199. In contrast, the PCR products that were obtained from *S. macrogoltabidus*, *S. xenophaga* BN6, *Sphingomonas stygia* and *Sphingomonas* sp. HH69 were of much lower intensity.

![Fig. 1](image.png)

**Fig. 1.** Hybridization of a repA gene probe from plasmid pNL1 with plasmids from different siphingomonads separated by PFGE. The hybridization reactions were performed overnight at 56 °C with 20–38 ng ml⁻¹ of repA probe. (a) Lanes: 1, λ-DNA concatamer standard; 2, *S. stygia*; 3, *S. aromaticivorans* F199; 4, *S. subterranea*; 5, *S. chlorophenolica* ATCC 33790; 6, *Sphingomonas* sp. EPA505. (b) Lanes: 1, *S. xenophaga* BN6; 2, *S. aromaticivorans* F199; 3, *S. subarctica* KF1; 4, *S. subterranea*; 5, λ-DNA concatamer standard.
For two of the strains, differences between the results of the PCR and the hybridization experiments were found. Thus, with *S. stygia*, the formation of a weak PCR product but no hybridization with the repA probe was found and with *Sphingomonas* sp. EPA505, a positive hybridization signal was obtained but no PCR product. In order to further

Table 3. *Sphingomonas* strains and plasmids analysed in hybridization experiments with a repA gene probe obtained from plasmid pNL1 of *S. aromaticivorans* F199

<table>
<thead>
<tr>
<th>Strain</th>
<th>Approximate size(s) of harboured plasmids (kb)</th>
<th>Hybridization with repA probe</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aromaticivorans</em> B0695</td>
<td>195, &lt;50</td>
<td>195 kb plasmid</td>
</tr>
<tr>
<td><em>S. subterranea</em></td>
<td>450, 220, 160</td>
<td>160 kb plasmid</td>
</tr>
<tr>
<td><em>S. stygia</em></td>
<td>290, 120</td>
<td>NH</td>
</tr>
<tr>
<td><em>S. paucimobilis</em> Q1</td>
<td>240, 80, &lt;50</td>
<td>NH</td>
</tr>
<tr>
<td><em>S. yanoikuyae</em> B1</td>
<td>240</td>
<td>NH</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. EPA505</td>
<td>200, 160, 50</td>
<td>160 kb plasmid</td>
</tr>
<tr>
<td><em>S. xenophaga</em> BN6</td>
<td>260, 180, 100, 50</td>
<td>260 kb plasmid</td>
</tr>
<tr>
<td><em>S. chlorophenolica</em> ATCC 33790</td>
<td>200, 160, 50, &lt;50</td>
<td>NH</td>
</tr>
<tr>
<td><em>S. subarctica</em> KF1</td>
<td>300, 220</td>
<td>NH</td>
</tr>
<tr>
<td><em>S. wittichii</em> RW1</td>
<td>340, 240</td>
<td>NH</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. HH69</td>
<td>240, 150, 70, 50, &lt;50</td>
<td>240 kb plasmid</td>
</tr>
<tr>
<td><em>S. herbicidivorans</em></td>
<td>300, 160</td>
<td>NH</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. SS3</td>
<td>340, 230, &lt;50</td>
<td>NH</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp. A175</td>
<td>100, &lt;50</td>
<td>NH</td>
</tr>
<tr>
<td><em>S. macrogoltabidus</em></td>
<td>450, 150</td>
<td>450 kb plasmid</td>
</tr>
<tr>
<td><em>S. paucimobilis</em></td>
<td>340, 150</td>
<td>NH</td>
</tr>
</tbody>
</table>

Fig. 2. PCR amplification of DNA fragments corresponding to the intergenic regions between repAa and repAb (a) and of repAb (b) of plasmid pNL1 in different sphingomonads. Approximately 200 ng of the respective genomic DNAs was used as template for PCR. The reactions were performed with a primer-annealing temperature of 60 °C and a polymerization step at 72 °C for 45 s (amplification of intergenic regions between repAa and repAb) and 40 s (amplification of repAb). The PCR reaction mixtures (25 μl each) were analysed by agarose gel electrophoresis in 1× TAE buffer at 6 V cm⁻¹ for 1 h. Lanes: 1, 1 kb ladder standard; 2, *S. yanoikuyae* B1; 3, *S. herbicidivorans*; 4, *S. paucimobilis* Q1; 5, *Sphingomonas* sp. A175; 6, *Sphingomonas* sp. SS3; 7, *S. wittichii* RW1; 8, *Sphingomonas* sp. HH69; 9, *S. subarctica* KF1; 10, *S. macrogoltabidus*; 11, *S. chlorophenolica* ATCC 33790; 12, *Sphingomonas* sp. EPA505; 13, *S. paucimobilis*; 14, *S. xenophaga* BN6; 15, *S. subterranea*; 16, *S. stygia*; 17, *S. aromaticivorans* B0695; 18, *S. aromaticivorans* F199 (positive control).
analyse these contradictions, another set of PCR experiments was performed with two primers that were designed in order to amplify the repAb gene. Thus, oligonucleotide primers repAb-Nterm and repAb-Cterm (Table 2), which bind to the N-terminal and C-terminal region of repAb from plasmid pNL1, were derived. The PCR reactions resulted in the amplification of DNA fragments of the expected size (approx. 1 kb) with genomic DNA from S. xenophaga BN6, S. subterranea, S. stygia, S. aromaticivorans B0695, Sphingomonas sp. HH69 and S. macrogoltabidus (Fig. 2). This confirmed the reliability of the results obtained in the PCR experiments using primers RepAHTH and RepAori.

**PCR detection of conserved catabolic gene clusters in different naphthalene-, biphenyl- and toluene-degrading sphingomonads**

The experiments described above suggested that *S. subterranea* and *S. aromaticivorans* B0695 harboured plasmids with similar replication functions to those of plasmid pNL1. These strains had been isolated at different depths (180–407 m) from the same subsurface location as *S. aromaticivorans* F199 and also degraded a similar range of aromatic compounds (Fredrickson et al., 1995). Previous hybridization studies had demonstrated that in *S. subterranea* (= strain B0478), *S. stygia* (= strain B0712) and *S. aromaticivorans* B0695 the genes that are involved in the degradation of the aromatic compounds were localized on plasmids with sizes of about 130, 270, and 180 plus 800 kb, respectively (Balkwill et al., 1997; Kim et al., 1996). We therefore tested if the organization of the degradative genes in these strains resembled that of *S. aromaticivorans* F199.

The comparison of the genetic organization of the genes involved in the degradation of naphthalene/biphenyl/toluene and naphthalenesulfonates in strains F199 and BN6, respectively, indicated the conservation of three gene clusters in these strains. Recently, two of these clusters have also been reported for a phenanthrene-degrading sphingomonad ("Sphingobium" sp. strain P2") (Pinyakong et al., 2003) (Fig. 3). These gene clusters shared significant nucleotide sequence identities ranging from 60 to 90 %.

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**Fig. 3.** Organization of three conserved gene clusters involved in the degradation of naphthalenesulfonates, naphthalene/biphenyl/toluene and phenanthrene in *S. xenophaga* BN6, *S. aromaticivorans* F199 and *Sphingomonas* sp. P2. The nucleotide sequences of the relevant genes from *S. xenophaga* BN6, *S. aromaticivorans* F199 and *Sphingomonas* sp. P2 were obtained from the NCBI database (accession nos U65001, NC_002033, and AB091692 and AB091693, respectively). The orientation and sizes of the genes within the individual gene clusters are indicated by arrows and are drawn to scale. The orientation of the individual gene clusters towards each other and the distances between them are not shown in their natural order (compare Fig. 7). The number of black dots between the genes indicates the degree of amino acid sequence identity of the encoded proteins in comparison to those of *S. aromaticivorans* F199: one dot, 60–70 %; two dots, 71–80 %; three dots, 81–90 %. The position and orientation of the oligonucleotide primers used for the PCR experiments are indicated by small black arrows.
Table 4. Primer combinations and sizes of the expected PCR products used to detect the presence of conserved degradative gene clusters in different sphingomonads by PCR

<table>
<thead>
<tr>
<th>Gene cluster detected</th>
<th>Primer combination</th>
<th>Expected size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>Block1-B + Block1-C</td>
<td>1550</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>Block2-A + Block2-B</td>
<td>1170</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>Block2-C + Block2-D</td>
<td>1310</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>Block2-E + Block2-F</td>
<td>1444</td>
</tr>
<tr>
<td>Cluster 3*</td>
<td>Block3-A + Block3-D</td>
<td>1280</td>
</tr>
</tbody>
</table>

*The expected size of the PCR product amplified from the genomic DNA of S. xenophaga BN6 was 1690 bp due to the insertion of a transposase-like ORF in xylL (Keck, 2000).

Specific pairs of oligonucleotide primers were derived for each gene cluster after comparison of the published nucleotide sequences from strains BN6 and F199 (Table 2, Fig. 3). Gene cluster 2 (see Fig. 3) was rather large (5.2 kb). Therefore, three pairs of PCR primers were designed in order to specifically amplify parts of this cluster. The oligonucleotide primers used were positioned in such a manner that the DNA fragments generated by PCR overlapped and enclosed all the genes in the gene cluster (Fig. 3). Thus, according to the sizes of the PCR products obtained, it was possible to deduce if the investigated strains encoded gene clusters similar to those found in strains F199 and BN6. The functionality of the deduced primers was shown in PCR experiments with the genomic DNA of S. aromaticivorans F199 as template. Thus, single PCR products of the expected sizes were obtained (Table 4). These primer sets were then used to investigate the presence of the three conserved gene clusters in the deep-subsurface strains, and PCR products of the expected sizes were detected. This indicated that all three gene clusters were conserved in S. stygia, S. subterranea and S. aromaticivorans B0695 (Fig. 4). In contrast, the conserved gene clusters could not be detected in the surface isolates Sphingomonas yanoikuyae B1, Sphingomonas paucimobilis Q1, and Sphingomonas sp. EPA505, which degrade a similar range of compounds as the subsurface strains. Furthermore, no PCR products were obtained with any of the PCR primers with the DNAs from S. paucimobilis, Sphingomonas chlorophenolica ATCC 33790 and Sphingomonas wittichii RW1.

Indications of the presence of the conserved catabolic gene clusters in Sphingomonas sp. HH69 and S. macrogoltabidus

The hybridization experiments with the repA gene probe suggested that plasmids similar to pNL1 were also present in the surface strains Sphingomonas sp. HH69 and S. macrogoltabidus. Therefore, we also attempted to detect the conserved gene clusters in these strains. PCR products with the same sizes as those of naphthalene/biphenyl/toluene-degrading strains were obtained with the primers Block3-A and Block3-D (designed for the detection of cluster 3), and Block2-A and Block2-B and Block2-C and Block2-D (designed for the detection of parts of cluster 2) with strains Sphingomonas sp. HH69 and S. macrogoltabidus, although only with low intensities (Fig. 5). Initially, no amplification signal was observed with the primer combination Block2-E and Block2-F (Fig. 5, lanes 2d and 3d). Therefore, we attempted to amplify a larger DNA fragment using oligonucleotides Block2-C and Block2-F, which enclosed the region amplified by primers Block2-E and Block2-F. This resulted in a single PCR product of the expected size (2280 bp) for both strains (data not shown). Under standard PCR conditions with the primers designed for the detection of cluster 1, several non-specific bands were obtained (Fig. 5, lanes 2a and 3a). Under more stringent conditions (annealing temperature of 65 °C), it

Fig. 4. Detection of conserved catabolic gene clusters in S. stygia, S. subterranea and S. aromaticivorans B0695 by PCR. Genomic DNAs (about 200 ng) were used as templates for PCR using a primer annealing temperature of 60 °C and polymerization steps at 72 °C for 40–60 s. Aliquots (25 μl each) from the PCR reactions were analysed by agarose gel electrophoresis in TAE buffer at 6 V cm⁻¹ for 1 h. Letters indicate the primer combinations used: a, Block1-B + Block1-C; b, Block2-A + Block2-B; c, Block2-C + Block2-D; d, Block2-E + Block2-F; e, Block3-A + Block3-D. Numbers indicate the analysed strains: 1, S. stygia; 2, S. subterranea; 3, S. aromaticivorans B0695; 4, S. paucimobilis Q1; 5, Sphingomonas sp. EPA505. Std, 1 kb ladder standard.
was possible to obtain major PCR products with primers Block1-B and Block1-C; however, they were not of the expected size.

**Arrangement of the conserved catabolic gene clusters in the deep-subsurface sphingomonads**

The nucleotide sequence data revealed that the three gene clusters were located within a 16 kb DNA fragment on plasmid pBN6, whereas the corresponding genes were found in three more distantly located positions on plasmid pNL1. Furthermore, the arrangement of these gene clusters in strains F199 and BN6 was different. If the gene clusters were designated clusters 1, 2 and 3 in the 5'→3' direction in strain BN6, the corresponding gene clusters on plasmid pNL1 were found in a 3-1-2 organization. These findings suggested that these catabolic gene clusters could be part of mobile genetic elements. Therefore, we attempted to compare the arrangement of the corresponding gene clusters by LR-PCR in the three other deep-subsurface strains (S. subterranea, S. stygia and S. aromaticivorans B0695), which also possessed the conserved gene clusters. The PCR primers for these experiments were derived from the sequences used previously for the detection of the single clusters. However, the primers were longer for the LR-PCR and were used in a different orientation (Table 2). The sizes of the expected PCR products in strain F199 with the primer combinations Block 3-D-LOW + Block1-D-UP and Block 1-B-LOW + Block 2-A-UP were 13 736 bp and 13 024 bp, respectively. The LR-PCR reactions were optimized with the genomic DNA of S. aromaticivorans F199 so that major products of the expected sizes were obtained. The LR-PCR was then performed with the other deep-subsurface strains, and the size of the amplified DNA fragments was determined by gel electrophoresis and the arrangement of the conserved gene clusters deduced. The results suggested that the order and the distance between the individual clusters in the deep subsurface strains varied considerably (Fig. 6, Table 5). This was confirmed in a set of subsequent experiments, in which the genomic DNAs of the four subsurface strains were digested with several restriction enzymes (Bsu36I, PmlI, NdeI, BstZ17I) that cut between the conserved gene clusters from plasmid pNL1. The digests were separated by gel electrophoresis and the DNA fragments hybridized with labelled probes of the conserved gene clusters (see Methods). In addition, in these experiments, clear differences in the fragment patterns were observed among the strains investigated. Thus, these experiments strongly indicated that DNA rearrangements had occurred on the plasmids of these strains.

**DISCUSSION**

It was previously demonstrated that large plasmids are ubiquitously present in xenobiotic-degrading Sphingomonas...
strains. These plasmids are in many cases responsible for the degradative capabilities of Sphingomonas strains (Basta et al., 2004; Feng et al., 1997; Kim et al., 1996; Ogram et al., 2000; Romine et al., 1999). Our previous study demonstrated that plasmid pNL1 can be transferred from S. aromaticivorans F199 to a wide range of Sphingomonas strains. In contrast, it appeared that plasmid pBN6, which is the other Sphingomonas plasmid for which a complete degradative pathway has been localized on a single plasmid, had a much more restricted host range (Basta et al., 2004). We therefore analysed in the present study how often plasmids with replication functions similar to those of plasmid pNL1 (which presumably all belong to the same incompatibility group) were present in our collection of aromatic compound- and xenobiotic-degrading sphingomonads. From the hybridization experiments with the repA gene probe originating from plasmid pNL1 and from the PCR experiments it can be deduced that plasmids with a similar replication system are present at quite a high frequency among other Sphingomonas strains with similar degradative abilities that have been isolated from the same deep-subsurface location as S. aromaticivorans F199 (Balkwill et al., 1997; Fredrickson et al., 1995; Shi et al., 2001). This suggests that this group of plasmids was conjugatively disseminated among these subsurface strains and allowed them to degrade simple mono- and bicyclic aromatic compounds. A similar enrichment of a specific allele of a gene involved in the degradation of xenobiotic compounds in one location has been described for pentachlorophenol-degrading Sphingomonas strains. In that study, it was shown that all sphingomonads isolated from a contaminated groundwater in Finland shared almost completely identical pcpB gene homologues (encoding pentachlorophenol-4-monoxygenase), whereas homologous genes were not present in non-sphingomonads from the same site (Tiirola et al., 2002b). These examples might give some indication of the effective dissemination of degradative genes among Sphingomonas strains.

Naphthalene- and biphenyl-degrading Sphingomonas strains have also been isolated from surface locations, for example, S. paucimobilis Q1, S. yanoikuyae B1, and Sphingomonas sp. EPA505 (Furukawa et al., 1989; Mueller et al., 1997; Yabuuchi et al., 1990). It has been suggested previously that the slow-growing deep-subsurface strains may be representatives of common ancestors of surface strains such as S. paucimobilis Q1 and S. yanoikuyae B1. Surprisingly, it has been demonstrated for S. paucimobilis Q1 and S. yanoikuyae B1 that the genes encoding the degradation of biphenyl and naphthalene are located on the chromosomal DNA of these strains (Kim et al., 1996). In accordance with this it was shown in the present study that the plasmids from these strains do not hybridize with the repA probe. This may indicate that in the surface strains the relevant plasmids are lost during or after the transfer of the degradative genes to the bacterial chromosome. However, the results obtained for Sphingomonas sp. EPA505 in the course of the present study, together with those of a previous preliminary communication (Bergeron et al., 1998), suggest that in surface strains the relevant degradative genes can also be encoded on plasmids.

The hybridization and PCR experiments suggested that plasmids belonging to the same incompatibility group as pNL1 are also present in some strains that have not been described as naphthalene- or biphenyl-degrading organisms. Thus, it was shown that a 240 kb plasmid from the dibenzofuran-degrading strain Sphingomonas sp. HH69 possessed a replication system similar to that of plasmid pNL1. Previous hybridization experiments had detected a gene (dnaA) on the same plasmid that encodes the large subunit of a putative dibenzofuran/dibenzop-dioxin dioxygenase (Basta et al., 2004). These results agree with our previous observations following the transfer of a variant of plasmid pNL1 (labelled with a kanamycin-resistance gene) to strain Sphingomonas sp. HH69 and selection for kanamycin resistance in the Sphingomonas sp. HH69 background. In these experiments, substantial rearrangements in the plasmid pattern of strain HH69 were observed. Thus, in all recovered transconjugants, the 240 kb plasmid

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Table 5. Length of DNA fragments (kb) amplified by LR-PCR to determine the arrangement of catabolic gene clusters in several sphingomonads

For S. subterranea it was not possible to amplify the DNA region between cluster 1 and 2 (NA, no amplification; –, not done). This could be due to limitations of LR-PCR, because with this technique, only DNA fragments up to 25 kb DNA can be amplified. In addition, different possible arrangements of the gene clusters (e.g. 2-3-1 and 3-2-1) were tested using appropriate primer combinations, but these did not yield any major PCR products.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Primer combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aromaticivorans F199</td>
<td>~14</td>
</tr>
<tr>
<td>S. aromaticivorans B0695</td>
<td>~23</td>
</tr>
<tr>
<td>S. stygia</td>
<td>–</td>
</tr>
<tr>
<td>S. subterranea</td>
<td>~9</td>
</tr>
<tr>
<td>S. xenophaga BN6</td>
<td>–</td>
</tr>
</tbody>
</table>

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was missing and the gene hybridizing with the \textit{dxnA} gene probe was found either on a different plasmid or on the chromosome of the transconjugants (Basta et al., 2004). This indicated that plasmid pNL1 and the 240 kb plasmid from \textit{Sphingomonas} sp. HH69 have a similar replication system and therefore cannot coexist in the same cell. This observation also suggested that our ‘hybridization typing’ gave biologically useful information and, in addition, demonstrated that plasmids similar to pNL1 are not restricted to deep-subsurface locations in the USA, because strain HH69 has been isolated from a soil sample in Germany (Fortnagel et al., 1990).

The experiments discussed above suggested that plasmids belonging to the same incompatibility group as plasmid pNL1 are also found in \textit{Sphingomonas} sp. EPA505 and \textit{Sphingomonas} sp. HH69, but that similar plasmids are only rarely present in other xenobiotic-degrading \textit{Sphingomonas} strains. Furthermore, this suggests that most of the xenobiotic-degrading strains harboured plasmids that presumably belong to other incompatibility groups. Thus, it was found that the plasmids participating in the degradation of naphthalenesulfonates and dibenzo-p-dioxin in \textit{S. xenophaga} BN6 and \textit{S. wittichii} RW1 do not belong to the same replication group as plasmid pNL1. The assumption that plasmids pNL1 and pBN6 belong to different replication groups was also supported by the previous observation that, among the sphingomonads, plasmid pNL1 shows a different host range to that of plasmid pBN6 (Basta et al., 2004). This indicated that plasmid pNL1 is just one (though not necessarily representative) example of a degradative plasmid from a \textit{Sphingomonas} strain. Thus, it will be necessary to acquire more sequence data about the replication regions in order to obtain a more comprehensive picture about the diversity of \textit{Sphingomonas} plasmids.

The genes for catabolic pathways are often localized in \textit{Sphingomonas} strains separately from each other, or, at least, are not organized in coordinately regulated operons. This has been described for the genes involved in the degradation of naphthalene, biphenyl and toluene by \textit{S. yanoikuyae} B1 and \textit{S. aromaticivorans} F199 (Cho & Kim, 2001; Romine et al., 1999; Zylstra & Kim, 1997), naphthalenesulfonates by \textit{S. xenophaga} BN6 (Keck, 2000), dibenzo-p-dioxin by \textit{S. wittichii} RW1 (Armengaud et al., 1998), \(\gamma\)-hexachlorocyclohexane (lindane) by \textit{S. paoctinobialis} UT26 (Miyauchi et al., 1998; Nagata et al., 1994), pentachlorophenol by \textit{S. chlorophenolica} (Cai & Xun, 2002) and protocatechuate by \textit{S. paoctinobialis} SYK-6 (Masai et al., 1999) (for a current review see Pinyakong et al., 2003). This ‘flexible’ gene organization (e.g. different combinations of conserved gene clusters) could be one of the mechanisms that allow sphingomonads to adapt quickly and efficiently to novel compounds in the environment. From previous work and from the PCR experiments performed in the course of the present study, it is now becoming clear that, at least in strains that degrade biphenyl, naphthalene and compounds that are converted to intermediates common to the naphthalene and biphenyl pathways (such as dibenzo-furan and naphthalenesulfonates), certain conserved gene clusters exist that seem to be part of mobile genetic elements able to change their localization in the genomes of these strains (Fig. 7). Surprisingly, there is no evident biochemical function of the individual gene clusters. A

![Fig. 7. Proposed arrangement of catabolic gene clusters participating in the degradation of aromatic compounds by \textit{S. aromaticivorans} F199 and B0695, \textit{S. stygia}, \textit{S. subterranea} and \textit{S. xenophaga} BN6. The distances between particular gene clusters in different strains were drawn in a 1 : 3 scale. The position of cluster 2 in \textit{S. subterranea} was not determined.](image-url)
good example of this is gene cluster 2, which has been sequenced from plasmids pNL1 and pBN6, and is probably also present in S. subterranea, S. stygia, S. aromaticivorans B0695, Sphingomonas sp. HH69 and S. macroglabellus. This gene cluster probably consists of two transcriptional units, which are transcribed in opposite directions. The annotation of the gene functions of the larger transcriptional unit suggested that the genes code for the large and small subunit of a ring-hydroxylating dioxygenase, a ferredoxin that presumably participates in electron transfer to a ring-hydroxylating dioxygenase, an extradiol ring-fission dioxygenase, and a 2-hydroxychromene-2-carboxylate isomerase which is necessary for the degradation of polycyclic aromatic hydrocarbons such as naphthalene and anthracene (Kuhm et al., 1993; Kim et al., 1997). The enzymes encoded by this transcriptional unit would not be able to synthesize a functional ring-hydroxylating dioxygenase (because of the lack of a ferredoxin reductase), nor convert a ‘standard’ aromatic compound to a diol (because of the lack of a cis-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase), nor convert 1,2-dihydroxynaphthalene (or any other dihydroxylated intermediate of a degradative pathway for an aromatic compound) to a utilizable intermediate of the glycolytic pathway or the citric acid cycle (because of the lack of a 2′-hydroxybenzalpyruvate aldolase or a different enzyme releasing an aliphatic intermediate from a monocyclic ring-fission product). There is also no evident functional biochemical reaction sequence. Thus, there is currently an obvious contradiction between the genetic evidence, which suggests a strong selective pressure for the existence of the conservative mechanisms. A possible solution for this problem might be an incorrect annotation of the function of the proteins that are encoded by the genes of the conserved gene clusters. Therefore, it will be necessary to functionally express the encoded genes and to experimentally determine the encoded enzymic functions.

REFERENCES


