We isolated and characterized two small cryptic indigenous plasmids, pYAN-1 (4,896 bp) and pYAN-2 (4,687 bp), from Sphingobium yanoikuyae, and developed a versatile system that permitted genetic manipulation of the genus Sphingomonas. Nucleotide sequencing of both plasmids revealed that they contained mobA, mobs, and repA genes, which are predicted to encode proteins associated with mobilization and replication, in common. Transformation with each plasmid harboring the antibiotic resistance gene by electroporation was fully successful, using Novosphingobium capsulatum as a host.

Key words: plasmid; electroporation; Sphingobium; Novosphingobium

Recently, the genus Sphingomonas has received increasing attention because it includes various xenobiotic-degrading bacteria. Members of this genus are able to degrade compounds such as polycyclic aromatic hydrocarbons, chlorinated and sulfonated aromatics, herbicides, aromatic ethers, and polyethylene glycol.1) There have been several reports indicating that giant plasmids may be important in the degradation of xenobiotic compounds in sphingomonads isolated from the same location, genes encoding the pathways for degrading biphenyl, naphthalene, m-xylene, and p-cresol have been detected on megaplasmids.2,3) Moreover, strains of genus Sphingomonas have a unique characteristic: they contain glycosphingolipids, which are ubiquitous in eukaryotic cell membranes.4) When Sphingomonas sp. A1 assimilates a macromolecule (alginate), a mouth-like pit (0.02–0.1 μm) is formed on the cell surface through reorganization and/or the fluidity of the pleats, causing extracellular alginate to be concentrated in the pit.5) The pit-dependent system of importing macromolecules was reported for the first time in a prokaryote. This system appears to be the origin of endocytosis and phagocytosis in eukaryotes.

Note

Characterization of Two Compatible Small Plasmids from Sphingobium yanoikuyae

We isolated and characterized two small cryptic indigenous plasmids, pYAN-1 (4,896 bp) and pYAN-2 (4,687 bp), from Sphingobium yanoikuyae, and developed a versatile system that permitted genetic manipulation of the genus Sphingomonas. Nucleotide sequencing of both plasmids revealed that they contained mobA, mobs, and repA genes, which are predicted to encode proteins associated with mobilization and replication, in common. Transformation with each plasmid harboring the antibiotic resistance gene by electroporation was fully successful, using Novosphingobium capsulatum as a host.

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Genetic manipulation of the genus Sphingomonas is necessary to improve the ability of these bacteria to degrade xenobiotic compounds and to determine the unique mechanisms involved in degradation, but no plasmid suitable for genetic manipulation of Sphingomonas has yet been identified. Some vector systems in sphingomonads with a broad-host-range plasmid or a cryptic giant plasmid have been reported by the conjugation method.1,6) Here we report the isolation and characterization of two cryptic indigenous small plasmids, pYAN-1 and pYAN-2, from Sphingobium yanoikuyae, and we describe the construction of shuttle vectors based on these plasmids and the development of an electroporation-transformation system for Novosphingobium capsulatum as a host strain.

The members of the genus Sphingomonas sensu lato were classified according to the advice of Takeuchi et al.7) as members of the newly created genera Sphingomonas, Sphingobium, Novosphingobium, and Sphingopyxis. First we tried to screen the small plasmid from various sphingomonads, Sphingobium amiense JCM11777,8) Sphingomonas cloacae JCM10874,9) Sphingobium yanoikuyae JCM7371,10) Sphingobium chlorophenolicum JCM10275,11) Novosphingobium capsulatum JCM7452,7) and Sphingopyxis alaskensis DSM13593,12) as follows: Plasmid DNA was prepared from these strains by the alkaline lysis procedure.13) Approximately 10⁹ cells were harvested by centrifugation, washed twice with 1 ml of G buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 10% vol/vol glycerol) and resuspended in 100 μl of solution I (25 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 100 mM NaCl, 15% wt/vol sucrose, and 0.8% wt/vol lysozyme) and incubated at 37 °C for 30 min. The cell suspensions were gently mixed with 200 μl of solution II (0.2 mM NaOH and 1.0% wt/vol SDS) and incubated at room temperature until the cells were lysed. Subsequently, the cell lysates were mixed with 150 μl of solution III (3 mM potassium acetate and 11.5% vol/vol glacial acetic acid) and placed on ice for 10 min. Finally, the plasmid DNA was prepared by phenol/chloroform extraction and precip...
Sphingo-Plasmid

![Genetic Organization of Plasmids pYAN-1 and pYAN-2.](image)

**Table 1.** Amino Acids Homologies of Five ORFs in the Two Plasmids

<table>
<thead>
<tr>
<th>Gene</th>
<th>Functional description of closest relative</th>
<th>% Identity</th>
<th>Source</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pYAN-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mobA</td>
<td>Putative MobA/MobL family protein</td>
<td>56</td>
<td>Sulfitobacter sp. NAS-14.1 (genome)</td>
<td>EAP78611</td>
</tr>
<tr>
<td>mobS</td>
<td>Mobilization protein</td>
<td>95</td>
<td>Sphingomonas xenophaga</td>
<td>AAW24019</td>
</tr>
<tr>
<td>orf1</td>
<td>Hypothetical protein</td>
<td>73</td>
<td>Pseudomonas putida KT2440 (genome)</td>
<td>NP_7744647</td>
</tr>
<tr>
<td>orf2</td>
<td>Hypothetical protein</td>
<td>55</td>
<td>Pseudomonas putida KT2440 (genome)</td>
<td>NP_7744648</td>
</tr>
<tr>
<td>repA</td>
<td>Replication protein</td>
<td>64</td>
<td>Sphingomonas xenophaga</td>
<td>AAW24018</td>
</tr>
<tr>
<td><strong>pYAN-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rve</td>
<td>Transposase</td>
<td>96</td>
<td>Sphingopyxis alaskensis RB2256 (genome)</td>
<td>YP_617491</td>
</tr>
<tr>
<td>repA</td>
<td>Replication protein</td>
<td>67</td>
<td>Rodobacter blasticus</td>
<td>NP_775696</td>
</tr>
<tr>
<td>mobS</td>
<td>Mobilization protein</td>
<td>64</td>
<td>Sphingomonas xenophaga</td>
<td>AAW24019</td>
</tr>
<tr>
<td>mobA</td>
<td>Putative MobA/MobL family protein</td>
<td>55</td>
<td>Oceanicola butensis HTCC2597 (genome)</td>
<td>ZP_01001657</td>
</tr>
<tr>
<td>fer</td>
<td>2Fe-2S Ferredoxin</td>
<td>53</td>
<td>Magnetospirillum magnetotacticum MS-1 (genome)</td>
<td>ZP_00049531</td>
</tr>
</tbody>
</table>

The nucleotide sequence data of pYAN-1 and pYAN-2 appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession nos. NC_008246 and AB265741 respectively. The deduced amino acid sequences of all the genes showed in Table 1. MobA and MobS were predicted to function in DNA mobilization and to be contained in both plasmids. The predicted amino acid sequence of repA from pYAN-1 is 64% identical to a putative Rep protein localized on pSx-Qyy of Sphingomonas xenophaga. On the other hand, that of RepA from pYAN-2 is 67% identical to a Rep protein of plasmid pMG106 from Rodobacter blasticus and has no homology to that of RepA from pYAN-1. These results indicate that the replication systems of both plasmids, pYAN-1 and pYAN-2, are quite different and are of different origins. Hence, we concluded that they are compatible in the same cells.

To construct shuttle vectors between *Escherichia coli* and *Sphingomonas*, the regions surrounding the repA genes from pYAN-1 and pYAN-2 were amplified by PCR using KOD-plus polymerase (Toyobo, Tokyo) and the following primers: 5'-CTGAAATTCAATGGCCTTGTGTGTTG-3' (forward, EcoRI site underlined) and 5'-ACGATGTGCCTCCTGTTGTCG-3' (reverse, PstI site underlined) for pYAN-1 (the fragment
from 3,300 bp to 4,890 bp in Fig. 1); and 5'-GTCTGCACTCGTAATCTACG-3' (forward, Psi I site underlined) and 5'-TTTGTGCCGATTGGGGTGCTCTG-3' (reverse, Sau I sites underlined) for pYAN-2 (the fragment from 3,187 bp to 4,687 bp in Fig. 1). The PCR products of pYAN-1 as template DNA were digested with Psi I and Eco RI, and then inserted into Psi I/Eco RI-digested E. coli plasmid vector pHS398 (Takara Shuzo, Tokyo), which harbors a chloramphenicol (Cm) resistance gene, and were designated pYAN-1C. The other plasmid, designated pYAN-2K, was constructed using the E. coli plasmid vector pHS298 (Takara Shuzo), which harbors a kanamycin (Km) resistance gene. Next we chose Novosphingobium capsulatum JCM7452 as a host strain, because no plasmid was detected in the cell in the above results (data not shown). The above plasmids were transformed into N. capsulatum JCM7452 by electroporation, as follows: Cells from 50-ml cultures of N. capsulatum (absorbance at 660 nm = 0.7 to 0.8) were collected by centrifugation and washed twice with 10 ml of chilled 10% glycerol. The cells were resuspended in the same buffer to a final volume of 100 μl and mixed with plasmid DNA (1 μg). Electroporation was performed using a Gene Pulser (Bio-Rad, Hercules, CA) with a buffer to a final volume of 100 μl and mixed with plasmid DNA (1 μg). Electroporation was performed using a Gene Pulser (Bio-Rad, Hercules, CA) with a single pulse at 25 μF at 2.5 kV. The cells were allowed to grow in LB for 2 h, and then were spread on selection plates, and selected by resistance to antibiotic resistance to 15 μg/ml of Cm or 30 μg/ml of Km. Km- and Cm-resistant transformants of pYAN-1C and pYAN-2K respectively were obtained (10^3–10^4 μg DNA). DNA from the transformants was subjected to Southern blot analysis using pHS298 or pHS398 as a probe. Bands corresponding to pYAN-1C and pYAN-2K were observed in all the transformants (data not shown). Hence, these vectors were useful for N. capsulatum as host strain, and we concluded that the replication functions of both plasmids are located within a 1,590-bp fragment (from 3,300 bp to 4,890 bp in Fig. 1) of plasmid pYAN-1, and a 1,500-bp fragment (from 3,187 bp to 4,687 bp in Fig. 1) of plasmid pYAN-2. Moreover, we examined the compatibility of the two plasmids in the same cells. N. capsulatum cells containing plasmid pYAN-1C were successfully transformed by plasmid pYAN-2K by the above method. This result suggests that they are compatible in the cell.

Next we evaluated the stability of the plasmid in the host strain, as follows: Cells harboring plasmids were inoculated in LB supplemented with selective antibiotics and grown at 30 °C to the stationary phase. At this point, cultures were diluted 10^3-fold in fresh LB without antibiotics and grown for 15 generations. Samples of each culture were taken at the beginning and the end of growth, diluted, spread on LB agar plates without antibiotics, and grown to 100–300 colonies per plate. The phenotypes of 100 colonies from each plate were examined by transferring them with toothpicks to selection plates containing antibiotics. We found that plasmids pYAN-1C and pYAN-2K were stably maintained in N. capsulatum cells for at least 15 generations in the absence of selection (data not shown).

In this study, we isolated two small plasmids from S. yanoikuyae JCM7371 and constructed an efficient host-vector system for the genus Sphingomonas. Although these plasmids showed relatively low copy numbers (data not shown), we believe that this should facilitate the molecular design of highly stable cloning vectors for the expression of foreign genes, and prove useful for cis-trans complementation analysis of genes in Sphingomonas.

References

10) Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T., and Yamamoto, H., Proposals of Sphingomonas paucimobilis gen. nov. and comb. nov., Sphingomonas parapauvicnobilis sp. nov., Sphingomonas yanoikuyae sp. nov., Sphingomonas adhaesiva sp. nov.,


