Use of Ureidopenicillins for Selection of Plasmid Vector Transformants in *Pseudomonas aeruginosa* and *Pseudomonas putida*

DEBRA-LYNN DAY, DEBRA YASINOW, JENNIFER MCDONOUGH, AND C. W. SHUSTER*

Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received 10 September 1983/Accepted 2 December 1983

Broad-host-range plasmids coding for β-lactamase were successfully selected after transformation of *Pseudomonas* strains. Transformants of both *Pseudomonas aeruginosa* and *Pseudomonas putida* containing plasmid pRO1614 were isolated in media containing low concentrations of piperacillin. These strains were also susceptible to other ureidopenicillins. Similar selections of transformants with carbenicillin, ampicillin, or ticarcillin required high concentrations of antibiotics and yielded backgrounds of spontaneous resistant mutants.

Molecular cloning of chromosomal DNA in *Pseudomonas aeruginosa* has required the development of special broad-host-range vectors. Bagdasarian et al. (1), Wood et al. (17), and Olsen et al. (11) have reported the construction of plasmid cloning vectors which overcome the limited host range of pBR322 but take advantage of the utility of special cloning sites within this plasmid and its selective antibiotic resistance features. In practice, tetracycline resistance is an appropriate selective marker in *Pseudomonas* spp. because transformants can be selected reliably, even at low frequency. However, if the *BamHI* site of pBR322 derivatives is used for the insertion of a piece of hetero-DNA, then the tetracycline resistance gene is inactivated, and transformants must be selected by the TEM-type β-lactamase determinant (3, 9) or by direct selection for an acquired trait.

*P. aeruginosa* is unusually resistant to a variety of antibiotics and chemotherapeutic agents, including the penicillins. The broad-spectrum penicillins carbenicillin (7), ticarcillin (16), and ampicillin (12) have been used with limited success in vivo in the treatment of severe *Pseudomonas* infection and in vitro to select for transformants in *P. aeruginosa* (17). One drawback in particular to the use of carbenicillin in a host-vector system for *P. aeruginosa* is the high concentration (0.5 to 0.75 mg/ml) required for selection of recombinant plasmids. Transformation experiments in this laboratory with some strains of *P. aeruginosa* resulted in large numbers of resistant colonies that were not transformants. A satellite phenomenon was also observed on carbenicillin plates, suggesting that β-lactamase was being exported and was permitting the growth of partially resistant mutants. With laboratory strains of *Pseudomonas putida* as recipients, carbenicillin proved to be totally ineffective at any concentration. This communication reports the use of ureidopenicillins (12), a family of broad-spectrum acylaminopenicillin derivatives, for the selection of transformants in *P. aeruginosa* and *P. putida* cloning systems.

The bacterial host strains and plasmids used in this study are listed in Table 1. Piperacillin was a gift of Leder Laboratories, Pearl River, N.Y. Azlocillin and mezlocillin were obtained from Miles Laboratories, West Haven, Conn. Ampicillin and carbenicillin were from the United States Biochemical Corp., Cleveland, Ohio, and ticarcillin was from Beecham Laboratories, Bristol, Tenn. MICs were determined by microdilution susceptibility testing. Overnight cultures of each isolate were diluted in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.), and turbidity was adjusted to a 0.5 McFarland standard (ca. 10⁶ CFU/ml). Serial twofold dilutions of antibiotics in Mueller-Hinton broth and broth control were dispensed into microtiter plates and inoculated with culture suspensions by using a Dynatech multitip plastic inoculator. The plates were covered and incubated at 30°C for 18 to 22 h. The MIC was defined as the lowest concentration of antibiotic which prevented visible growth.

The in vitro activities of the ureidopenicillins, piperacillin, azlocillin, and mezlocillin, were compared with the activities of other beta-lactam antibiotics against *P. aeruginosa* and *P. putida* (Table 2). Piperacillin at a concentration of 8 μg/ml effectively inhibited two strains of *P. aeruginosa*, both of which were resistant to >256 μg of ampicillin per ml. Piperacillin activity was also superior to that of other β-

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Prototroph</td>
<td>K. Klinger</td>
</tr>
<tr>
<td>PAO1/ct(pRO1614)</td>
<td>Cb' Tc'</td>
<td>12</td>
</tr>
<tr>
<td>PAKS-17</td>
<td>Protease negative</td>
<td>B. Wretlind</td>
</tr>
<tr>
<td>PAKS-17(pRO1614)</td>
<td>Cb' Tc', protease negative</td>
<td>This study</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PgG1900</td>
<td>trpA</td>
<td>A. Chakrabarty</td>
</tr>
<tr>
<td>PgG1900(pRO1614)</td>
<td>Cb' Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>12633</td>
<td>No known markers</td>
<td>ATCC 12633</td>
</tr>
<tr>
<td>PgG786</td>
<td>No known markers</td>
<td>ATCC 29607</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JS600(derivative)</td>
<td>Nal' derivative of C600</td>
<td>13</td>
</tr>
<tr>
<td>JS600(pBR322)</td>
<td>Ap' Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>JS600(pMW79)</td>
<td>Ap' Cb' Tc'</td>
<td>This study</td>
</tr>
<tr>
<td><em>Plasmids</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRO1614</td>
<td>Cb' Tc'</td>
<td>11</td>
</tr>
<tr>
<td>pMW79</td>
<td>Ap' Cb' Tc', Sm'</td>
<td>17</td>
</tr>
<tr>
<td>PBRR322</td>
<td>Ap' Tc'</td>
<td>2</td>
</tr>
</tbody>
</table>

* Marker abbreviations: Cb', carbenicillin resistance; Ap', ampicillin resistance; Tc', tetracycline resistance; Sm', streptomycin resistance; Nal', nalidixic acid resistance; trpA, tryptophan requirement.

* Corresponding author.
lactam antibiotics in three strains of *P. putida*. MICs were substantially lower for all ureidopenicillins against each of the strains tested than for any of the other penicillins. Pseudomonads successfully transformed with plasmid vector pRO1614 acquired resistance to >256 μg of any penicillin derivative per ml. Antibiotic susceptibility measurements with *Escherichia coli* strains are included for comparison. A Sau3A-digested gene library of *P. aeruginosa* chromosomal DNA was created by cloning into the unique BamHI site of plasmid vector pRO1614. Initial attempts at isolating transformants in *P. putida* were unsuccessful because of backgrounds approximating 15 to 20% of the total number of transformants, even in the presence of 750 μg of carbicillin per ml. Our inability to isolate reasonable numbers of recombinants in the PpG1900-pRO1614 host-vector system originally prompted our search for a more efficient selective agent.

Plasmid DNA was purified by a modification of the combined methods of Guerry et al. (6) and Olsen et al. (11). Chromosomal DNA was extracted by the procedure of R. Maurer (private communication). Restriction endonuclease digestions and ligation were performed as described by the supplier (Bethesda Research Laboratories, Gaithersburg, Md.). PAO1 genomic DNA was partially digested with Sau3A to maximize the yield of fragments between 1.5 and 6.0 megadaltons, which were inserted into the plasmid BamHI site in a 2.5-fold excess of fragments to vector. Sau3A cleavage of genomic DNA generates a nearly random population of high-molecular-weight, overlapping linear fragments with the recognition sequence GATC (8). Because complementary sequences are also produced by BamHI, plasmid vector and insert fragments could be annealed directly. Recombinant plasmids were introduced into competent host cells by a modification of the method of Mercer and Loutit (10). Transformation reactions were incubated at 30°C for 30 min. Piperacillin was subsequently added to a final concentration of 25 μg/ml, and cultures were grown for an additional 1.5 h. S. faecalis was plated on selective media (Luria agar plus 25 μg of piperacillin per ml). Transformants were screened for the presence of plasmid DNA and insert by agarose gel electrophoresis after alkaline lysis and digestion with *EcoRI* and *SalI*. BamHI sites are not necessarily conserved during Sau3A-BamHI ligation.

As shown in Table 3, 25 μg of piperacillin per ml permitted the unambiguous isolation on the average of over 7,600 vector-containing transformants per μg of plasmid DNA. Despite a decrease in the cloning efficiency of plasmid pRO1614 when ligated to hetero-PAO1 DNA (Table 3), transformants could still be selected expediently. Azlocillin and mezlocillin should be as effective as piperacillin for selection, although these were not tested directly in transformation experiments. Successful introduction of hetero-DNA inactivates the tetracycline resistance region of the vector, resulting in tetracycline susceptibility. An average of 51% of the piperacillin-resistant transformants isolated contained chromosomal inserts at their BamHI sites.

The ureidopenicillins, namely piperacillin, azlocillin, and mezlocillin, are semisynthetic molecules containing substituted side chains in place of the alpha-amino group of AMP (12, 14). Although vulnerable to hydrolysis by different β-lactamases, these aminoacyl derivatives exhibit enhanced activity against a broad range of gram-positive cocci, gram-negative bacilli, and a humber of anaerobic species (4, 14). Piperacillin, a piperazine aminobenzyl derivative, is particularly effective against *P. aeruginosa* (4, 5, 14, 15).

In summary, piperacillin and other ureidopenicillins at low concentrations permit efficient selection of *Pseudomonas* transformants containing vector plasmids coding for TEM-type β-lactamase.

This research was supported by Public Health Service grant AI-17960 from the National Institute of Health.

We thank Jeffrey Klinger, Department of Pediatrics, Rainbow Babies and Childrens Hospital, for aid in the determination of MICs and for supplying samples of antibiotics.

---

**TABLE 2. MICs of penicillin derivatives**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Piperacillin</th>
<th>Azlocillin</th>
<th>Mezlocillin</th>
<th>Ampicillin</th>
<th>Carbencillin</th>
<th>Ticarcillin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>8</td>
<td>4</td>
<td>32</td>
<td>&gt;256</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>PAO1(pRO1614)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>PAKS-17</td>
<td>8</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>128</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>PAKS-17(pRO1614)</td>
<td>64</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PpG1900</td>
<td>8</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td>256</td>
<td>64</td>
</tr>
<tr>
<td>PpG1900(pRO1614)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>12633</td>
<td>16</td>
<td>64</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>PpG786</td>
<td>4</td>
<td>16</td>
<td>128</td>
<td>32</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JS600</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JS600(pBR322)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>JS600(pMW79)</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

**TABLE 3. Selection of transformants with piperacillin**

<table>
<thead>
<tr>
<th>Transformant*</th>
<th>Piperacillin concn (μg/ml)</th>
<th>Background colonies (avg no.)</th>
<th>Transformants per μg of plasmid DNA (avg no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20</td>
<td>160</td>
<td>8,440</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
<td>7,722</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>10</td>
<td>5,280</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0</td>
<td>3,440</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>0</td>
<td>390</td>
</tr>
</tbody>
</table>

* A. Strain PpG1900 transformed with purified pRO1614 plasmid; B. PpG1900 transformed with purified pRO1614 plasmid ligated to PAO1 fragments.
LITERATURE CITED


