Efficient Transformation of Amycolatopsis orientalis (Nocardia orientalis) Protoplasts by Streptomyces Plasmids

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Conditions for efficient transformation of Amycolatopsis orientalis (Nocardia orientalis) protoplasts by Streptomyces plasmid cloning vectors were identified. Three streptomycete plasmid origins of replication function in A. orientalis, as do the apramycin resistance gene from Escherichia coli, the thiostrepton resistance gene from Streptomyces azureus, and the tyrosinase gene from Streptomyces antibioticus. A. orientalis appears to express some restriction and modification, but higher transformation frequencies (10⁶/µg of DNA) were obtained when plasmid pIJ702 was modified by passage in A. orientalis.

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Amycolatopsis orientalis ATCC 19795, recently reclassified as Amycolatopsis orientalis and defined as a type strain for the new genus Amycolatopsis (12), produces the therapeutically important glycopeptide antibiotic vancomycin (6). We were interested in developing gene clonal technology in A. orientalis as a means to study the organization and regulation of vancomycin biosynthesis genes, to improve vancomycin yields through genetic manipulation, and to produce hybrid glycopeptide antibiotics with improved therapeutic and pharmacokinetic properties and minimal toxicity (3). Gene cloning methodologies using transformation of protoplasts have been developed for several Streptomyces species (1, 7, 10, 13, 18), for Streptomyces erythraeus (19), which has been reclassified as Saccharopolyspora erythraea (9), and for Thermomonospora fusca (14). Since Amycolatopsis belongs to the same order (Actinomycetales) as Streptomyces, Saccharopolyspora, and Thermomonospora, we surmised that the development of protoplast transformation with broad-host-range plasmid vectors for Streptomyces spp. might be feasible in A. orientalis.

To test the feasibility of this approach, we first prepared protoplasts of A. orientalis to determine the optimum conditions for regeneration of viable cells from protoplasts. A. orientalis grows well in TS broth (2) up to about 2 x 10⁹ CFU/ml in the stationary phase. The culture contained highly fragmented mycelia, as is characteristic for nocardioforms (12), and treatment of the culture with a 5-s dose of ultrasonic vibration, as described previously (2), resulted in only a twofold increase in the CFU per milliliter. When A. orientalis was grown in TS broth plus 2.5% glycine to the stationary phase, the cells were readily converted to protoplasts by treatment with 5 mg of lysozyme per ml, as described previously (2), and the protoplasts regenerared fairly efficiently (about 5%) on modified R2 agar (2) dehydrated by 8 to 20%; the efficiency of regeneration was about 100-fold lower without dehydration. The need for dehydration of the regeneration agar is common for efficient regeneration of Streptomyces protoplasts (4, 5).

Careful control of temperature during cell growth and during protoplast regeneration was found essential for optimum results. Cells were grown at 29, 34, or 37°C before protoplast formation, and the protoplasts were plated on modified R2 agar and incubated at 29, 34, or 37°C. The most efficient regeneration of cells from protoplasts was observed when cells were grown at 34 or 37°C before lysozyme treatment and when protoplasts were plated at 29°C for the regeneration. The protoplasts prepared at 29, 34, or 37°C regenerated viable cells at 100- to 1,000-fold lower efficiencies at 37°C than at 29°C (data not shown). Since formation and regeneration of protoplasts could be readily demonstrated by the techniques and media used for Streptomyces fradiae protoplast formation, regeneration, fusion, and transformation (2, 4, 13), we attempted to transform A. orientalis with Streptomyces plasmid vectors by methods developed for Streptomyces spp. We first determined if A. orientalis was sufficiently sensitive to any of the antibiotics used to select for transformants in Streptomyces spp.

A. orientalis was resistant to many antibiotics. It grew well on modified R2 agar containing chloramphenicol (10 µg/ml), bacitracin (50 µg/ml), spectinomycin (50 µg/ml), neomycin (5 µg/ml), hygromycin B (250 µg/ml), and viomycin (100 µg/ml). However, it did not grow on modified R2 agar containing 50 µg of apramycin per ml. Therefore, we attempted to transform A. orientalis protoplasts with Streptomyces plasmid vectors containing an apramycin resistance gene from Escherichia coli (15) that is expressed well in Streptomyces spp. (17; R. N. Rao, M. A. Richardson, and S. Kuhstoss, Methods Enzymol., in press). The plasmids used included pOJ148, pOJ160, pOJ192, pKC462a, pKC505, and pKC651 (Table 1). pOJ148, pOJ160, and pOJ185 are related plasmids derived from an SCP2* derivative, pHJL400 (11), and containing a fragment of DNA from pKC309 that contains the apramycin resistance gene (15). pKC462a (17) is a bifunctional cosmid vector that contains the pFJ103 origin for replication in Streptomyces spp. (16). pKC505 is a bifunctional cosmid containing the SCP2* origin for replication in Streptomyces spp. (Rao et al., in press). A. orientalis was partially sensitive to thiostrepton at 25 µg/ml in modified R2 agar and grew at a substantially retarded rate. We therefore also used plasmid pIJ702, a broad-host-range vector that contains the thiostrepton resistance gene (isr) from Streptomyces azureus and the tyrosinase gene (mel) from Streptomyces antibioticus (Table 1). Tyrosinase converts tyrosine to the dark-brown pigment melanin.
TABLE 1. Plasmid or cosmid vectors used

<table>
<thead>
<tr>
<th>Vector</th>
<th>Size</th>
<th>Markers*</th>
<th>Source of replication origin</th>
<th>Other features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIJ702</td>
<td>5.8</td>
<td>Tsr, Mel</td>
<td>pIJ101</td>
<td>High copy number</td>
<td>8</td>
</tr>
<tr>
<td>pKC462a</td>
<td>11.5</td>
<td>Am, Nm</td>
<td>pBR322 3 λ cos sites</td>
<td>17: Rao et al., in press</td>
<td></td>
</tr>
<tr>
<td>pCC505</td>
<td>18.7</td>
<td>Am</td>
<td>pBR322 3 λ cos sites</td>
<td>Rao et al., in press</td>
<td></td>
</tr>
<tr>
<td>pKC561</td>
<td>31.6</td>
<td>Nm, Tsr</td>
<td>SCP2* Adv 1 λ cos site</td>
<td>Rao et al., in press</td>
<td></td>
</tr>
<tr>
<td>pOJ148</td>
<td>7.7</td>
<td>Am, Tsr</td>
<td>pUC19 lacZ*</td>
<td>Stanzak and Schoner, in preparation</td>
<td></td>
</tr>
<tr>
<td>pOJ160</td>
<td>7.0</td>
<td>Am</td>
<td>pUC19 lacZ*</td>
<td>Stanzak and Schoner, in preparation</td>
<td></td>
</tr>
<tr>
<td>pOJ192</td>
<td>10.7</td>
<td>Am, Tsr</td>
<td>pUC19 lacZ*</td>
<td>Stanzak and Schoner, in preparation</td>
<td></td>
</tr>
</tbody>
</table>

* Tsr, Thiostrepton resistance; Mel, melamin production; Am, apramycin resistance; Nm, neomycin resistance; Ap, ampicillin resistance.

We attempted to transform *A. orientalis* with several of the plasmids by using protocols developed for transformation of *Streptomyces* (5, 7, 13, 18) and *Saccharopolyspora* (19) protoplasts. The protocols differ in the media used for growing cells before protoplast formation, for regeneration of protoplasts, and for diluting the protoplasts. They also differ in the agar overlays used for plating protoplasts (for two of the procedures); in the concentration of protoplasts used during transformation; and in the concentration and source of polyethylene glycol, the transformation-inducing agent. None of the transformation protocols gave satisfactory levels of transformants. We then carried out transformations by using many combinations of the important variables and observed the following: *A. orientalis* did not grow in YEME medium (18), so the methods of Bibb et al. (7) and Thompson et al. (18) could not be used directly; protoplasts prepared from cells grown in TS broth plus 2.8% glycine (13) were more transformable than were protoplasts prepared from cells grown in SGGP, a medium used for *S. erythraea* transformation (19); protoplasts were more transformable if they were undiluted or diluted moderately (13), rather than concentrated (7, 18); and transformations were most efficient when protoplasts were prepared in modified P medium (19), were treated with 25% polyethylene glycol 3350 (19), and were plated in R2T20 soft agar overlays on R2T20 agar (19).

The efficiencies of transformation varied over 104-fold, depending on the combinations of variables used, but all of the plasmids yielded transformants. The optimum conditions (see the legend to Fig. 1) gave frequencies of 5.0 × 104 transformants per μg of DNA with plasmid pIJ702 prepared from *Streptomyces lividans* (Fig. 1). pOJ148 gave similar high frequencies of transformants, whereas pKC561, pKC462a, and pKC505 prepared from heterologous hosts transformed at somewhat lower frequencies (102 to 103 transformants per μg of DNA) with this modified procedure. When pIJ702 DNA was used in *A. orientalis,* it transformed *A. orientalis* protoplasts at a frequency of about 105 transformants per μg of DNA. Therefore, it appears that the relatively small pIJ702 is moderately restricted in *A. orientalis* but can be modified by passing in *A. orientalis.*

Since none of the transformation procedures developed for *Streptomyces* or *Saccharopolyspora* spp. was suitable for transformation of *A. orientalis,* a combination of variables from the different protocols yielded a highly efficient procedure, it appears that many of the important variables for successful transformation of actinomycete protoplasts have been identified. Appropriate combinations of these variables might be used to further expand the utility of protoplast transformation in other actinomycetes. Our results also indicate that *A. orientalis* can be efficiently transformed by *Streptomyces* plasmids containing at least three different origins of replication. Transformation of *Saccharopolyspora erythraea* and *T. fusca* by *Streptomyces* plasmids has also been reported (14, 17, 19). The functional expression of broad-host-range *Streptomyces* plasmids in *Amycolatopsis, Saccharopolyspora,* and *Thermomonospora* spp. suggests the possible application of these vectors to an even broader range of actinomycete genera and suggests that such vectors might be used to generate intergeneric recombinants to produce novel antibiotics (3).
We thank D. A. Hopwood, E. Katz, R. N. Rao, B. E. Schoner, R. Stanzak, M. Richardson, and S. Kuhstoss for providing plasmid cloning vectors and B. E. Schoner and E. T. Seno for comments on the manuscript. We also thank Barbara Fogleman for typing the manuscript.

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