Transformation of *Arthrobacter* and studies on the transcription of the *Arthrobacter ermA* gene in *Streptomyces lividans* and *Escherichia coli*

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We report the development of a plasmid-mediated transformation system for *Arthrobacter* sp. NRRLB3381, using the *Streptomyces* cloning vector pIJ702. Our procedure gives a transformation frequency of $10^8$/µg of plasmid DNA. In addition we have explored the expression of the *Arthrobacter ermA* gene in *Streptomyces lividans* and *Escherichia coli*, and shown that the *ermA* promoter is recognized in *S. lividans* not *E. coli*. The relationship between *Arthrobacter*, *Streptomyces* and *E. coli* promoters is discussed.

**INTRODUCTION**

*Arthrobacter* belongs to the Coryneform group of bacteria, which also includes such genera as *Corynebacterium, Cellulomonas, Brevibacterium, Curtobacterium* and *Microbacterium*. Interest in the genus *Arthrobacter* stems from the fact that its members have an extensive secondary metabolism. Relevant features that have been assigned to at least some *Arthrobacter* species include the capacity to degrade herbicides, pesticides and chitin, steroid transformation, and the ability to produce antibiotics, phytohormones, polysaccharides and riboflavin. In general the group is nutritionally versatile and can use a diverse range of organic substrates (Keddie & Jones, 1981). The particular species of *Arthrobacter* we have studied (NRRLB3381) possesses a cell wall containing 1-diaminopimelic acid and a genome composed of 70.5% G+C, two properties that distinguish the *simplex* group of arthrobacters (DNA G+C content of 70–74% ; Keddie & Jones, 1981) but which are shared by the *Streptomyces*. In addition, *Arthrobacter* sp. NRRLB3381, like *Streptomyces erythreus*, is able to produce the macroclide antibiotic erythromycin (French et al., 1970). However, *Arthrobacter* sp. NRRLB3381 differs from *Streptomyces* in two significant ways: it is asporogenous and it grows as unicellular isolated colonies, not as a mycelium characteristic of *Streptomyces*. These features may facilitate the use of this organism for the expression of cloned genes, and may offer certain advantages over *Streptomyces*. We have developed plasmid-mediated transformation of *Arthrobacter* sp. NRRLB3381 to investigate the utility of *Streptomyces* cloning vectors in *Arthrobacter*. The expression of the *Arthrobacter ermA* gene in *S. lividans* and *E. coli* is also analysed.

**MATERIALS AND METHODS**

**Bacterial strains**

*Arthrobacter* sp. NRRLB3381 (French et al., 1970) and *Streptomyces lividans* 1326 (Schottel et al., 1981) were used for this study. The culture conditions for these two organisms have been described (Roberts et al., 1985; Chater et al., 1982). *Arthrobacter* sp. NRRLB3381 will be simply referred to as *Arthrobacter* throughout the rest of the paper.

**Transformation**

*S. lividans* was transformed by standard methods (Chater et al., 1982). A transformation procedure for *Arthrobacter* was adapted from the *Streptomyces* protocol (Hopwood et al., 1985). Buffer P, R2YE plates and soft nutrient agar were made as described (Chater et al., 1982; Hopwood et al., 1985). An overnight culture of *Arthrobacter* was diluted 1:10 and grown for 4 h at 30°C. The following procedure applies to a 10 ml culture sample. The cells were harvested by centrifugation at 5000 rev./min and 22°C for 7.5 min. The pellet was washed with 7.5 ml of 10.3% sucrose and resuspended in 2 ml of P buffer containing 5 mg of lysozyme/ml. The culture was incubated at 30°C with intermittent shaking. After 1 h, 2.5 ml of P buffer was added and incubation continued for a further 1 h, resulting in the formation of osmotically sensitive protoplasts. These were centrifuged at 5000 rev./min and 22°C for 10 min, washed with 5 ml of P buffer and then resuspended in 0.3 ml of P buffer. Aliquots (50 µl) of this protoplast suspension, dispersed in Minifuge tubes, were used for each transformation. DNA, contained in a volume of 1–10 µl, was added and mixed by gently tapping the tube. This was followed immediately by the addition of 200 µl of P buffer containing 50% poly(ethylene glycol) 1000 (Koch-Light) and pipetting slowly up and down four times in order to mix. The sample was diluted with 750 µl of P buffer and the tube was inverted two or three times. Appropriate dilutions of this mixture were plated out directly on R2YE agar and incubated at 30°C for approx. 24 h, or until faint growth was just visible. Thiostrepton-resistant transformants were selected by overlaying the plates with 2.5 ml of soft nutrient agar containing 600 µg of the antibiotic/ml. Transformants were scored after 5–6 days at 30°C.

**Preparation of DNA and cloning techniques**

General methods used for cloning steps, preparation of DNA and nick-translation have been described

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Table 1. ‘Prime, cut’ probes used for S1 mapping and primer extension experiments

Each probe is named after the number of the M13 clone and the restriction enzyme used for cutting. The size of each probe includes 48 nucleotides of M13 sequence (51 nucleotides for EcoRI-cut probes). Each probe is complementary to the non-coding sequence of the ermA gene described in Roberts et al. (1985).

<table>
<thead>
<tr>
<th>Name</th>
<th>Positions covered in ermA sequence</th>
<th>Size (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.X/Smal</td>
<td>303-4</td>
<td>299*</td>
</tr>
<tr>
<td>03.X/BglI</td>
<td>443-155</td>
<td>336</td>
</tr>
<tr>
<td>03.X/Apal</td>
<td>443-294</td>
<td>197</td>
</tr>
<tr>
<td>10.X/BglI</td>
<td>485-155</td>
<td>378</td>
</tr>
<tr>
<td>41.X/Apal</td>
<td>659-294</td>
<td>413</td>
</tr>
<tr>
<td>22.X/NcoI</td>
<td>695-336</td>
<td>407</td>
</tr>
<tr>
<td>06.S/EcoRI</td>
<td>1410-1191</td>
<td>270</td>
</tr>
<tr>
<td>64.S/NciI</td>
<td>1457-1249</td>
<td>256</td>
</tr>
<tr>
<td>61.S/EcoRI</td>
<td>1644-1429</td>
<td>266</td>
</tr>
<tr>
<td>56.S/PstI</td>
<td>1822-1556</td>
<td>314</td>
</tr>
</tbody>
</table>

* Clone 23.X contains an additional Smal site which was formed fortuitously when insert DNA was blunt-end ligated into the Smal site of mp8. Therefore the size of the probe does not include any M13 sequence since cleavage with Smal totally removes all M13 DNA.

(Roberts et al., 1985). Plasmid DNA from *S. lividans* was prepared by the method of Hopwood et al. (1983). Plasmid DNA was extracted from *Arthrobacter* cells by the Birnboim & Doly (1979) method, after incubation in lysozyme (5 mg/ml) at 30 °C for 1 h. Genomic DNA was prepared as described by Chater et al. (1982)

Subcloning ermA into Streptomyces lividans

A 4 kb BamHI fragment containing the entire ermA gene (previously cloned into pUC8; Roberts et al., 1985) was subcloned into the BglII site of the multicopy plasmid pIJ702 (Katz et al., 1983) and transformed into *S. lividans* 1326 (Schottel et al., 1981). This construct was called pAR4.

Transcript analysis

Transcription of the *ermA* gene, cloned in pIJ702 (pAR4) in *S. lividans* or cloned in pUC8 (pAR2) in *E. coli*, was analysed by S1 mapping and primer extension studies. These experiments were carried out using ‘prime, cut’ probes prepared on M13 templates that hybridized to the appropriate regions of RNA. The method by which RNA was prepared, conditions for S1 mapping and primer extension analysis and formation of ‘prime, cut’ probes were described previously (Roberts et al., 1985). Table 1 lists the probes prepared.

RESULTS AND DISCUSSION

Plasmid-mediated transformation of Arthrobacter

The method used to transform *Arthrobacter*, adapted from the standard Streptomyces protocol (Hopwood et al., 1985), is described in the Materials and methods section. Many changes to this procedure simply reflect physical differences between the unicellular *Arthrobacter* and mycelial *Streptomyces*. Additional significant changes were: (i) a higher lysozyme concentration which improved the yield of osmotically sensitive protoplasts, (ii) an increased incubation time in lysozyme, although as for *Streptomyces* this was not critical; protoplast formation was monitored by phase-contrast microscopy and cells were routinely used after 1.5–2 h, and (iii) in our hands a higher poly(ethylene glycol) concentration improved *Streptomyces* transformation frequency and was therefore adopted for *Arthrobacter*. Using these conditions we could demonstrate that 5–10% of the cells regenerated and of those that regenerated 99% were osmotically fragile. The *Streptomyces* plasmid pIJ702, which confers thiostrpton resistance was used for all transformations (Hopwood et al., 1985). Confirmation that pIJ702 was transforming *Arthrobacter* to thiostrpton resistance was obtained from Southern analysis of total DNA prepared from three putative transfectants. These DNA samples, cut with *SmaI* and *BamHI* gave the identical banding pattern as pure pIJ702 when probed with nick-translated pIJ702 (results not shown). Examination of plasmid DNA prepared from seven thiostrpton-resistant transfectants revealed the presence of a vector the same size (5.8 kb) as pIJ702. This DNA also hybridized with nick-translated pIJ702. The plasmid DNA prepared from *Arthrobacter* transformed *S. lividans* to thiostrpton resistance and melanin secretion characteristic of pIJ702 (Katz et al., 1983). From these experiments it was concluded that pIJ702 can transform *Arthrobacter* to thiostrpton resistance and can replicate autonomously. The plasmid is maintained in its entirety since its total size, the position of restriction sites and ability to transform *S. lividans* are preserved. In the absence of thiostrpton, the plasmid is maintained stably for at least seven generations as judged by the plate cell count in the presence and absence of antibiotic. We obtained a frequency of 10⁵ transformants/µg of pIJ702 plasmid DNA.

Expression of erythromycin resistance from ermA in Streptomyces lividans

We have previously described (Roberts et al., 1985) the cloning and characterization of a gene (*ermA*) coding for a presumed erythromycin resistance determinant isolated from an erythromycin-producing strain of *Arthrobacter* sp. NRRLB3381. The identity of the gene was established by (i) cross-hybridization to the *S. erythreus* erythromycin-resistance gene *ermE*, and (ii) the predicted amino-acid sequence of the protein encoded by *ermA* showed substantial homology to three other erythromycin resistance determinants.

The plasmid pAR4 (described in the Materials and methods section) which contains the *ermA* gene was transformed into *S. lividans* by selecting for thiostrpton resistance. It was found that the thiostrpton-resistant transformants obtained had also acquired erythromycin resistance. All of these colonies were white in colour since cloning into the BglII site of pIJ702 inactivates the melanin-producing tyrosinase (*mel*) gene. Restriction analysis (results not shown) confirmed that the 4 kb *BamHI* fragment had been correctly inserted into pIJ702, producing an erythromycin-resistant derivative of this vector. In each of ten erythromycin-resistant transformants examined, the *ermA* gene was inserted in the same orientation as the *mel* promoter of pIJ702 (Hopwood 1987).
et al., 1985). This confirms the previous assignment of erythromycin resistance to the ermA gene.

Transcription from the Arthrobacter ermA promoter in Streptomyces lividans

It has been demonstrated that Streptomyces, like E. coli (Grossman et al., 1984; Landick et al., 1984), Bacillus (Losick & Per0, 1981) and recently the nitrogen-regulated genes of enteric bacteria (Hirschman et al., 1985) possess multiple sigma (σ) factors (Westpheling et al., 1985; Buttner & Brown, 1985) which are each believed to initiate transcription from a specific class of promoter. In S. coelicolor one of the σ-factors identified, probably the σ80 found by Buttner & Brown (1985) or σ85 of Westpheling et al. (1985), may be analogous to the E. coli σ70 factor that recognizes the consensus promoter of TTGACA (−35 region) and TATAAT (−10 region), and may be partly responsible for the capacity of Streptomyces to recognize and initiate transcription from promoters derived from a diverse source of bacteria including E. coli (Bibb & Cohen, 1982; Jaurin & Cohen, 1984; Westpheling et al., 1985; Herbert et al., 1986).

As an increasing number of Streptomyces genes have been sequenced and S1 mapped and promoter probe vectors become available (Brawner et al., 1985; Ward et al., 1986), it appears that most Streptomyces promoters belong loosely into one of two groups. The first group shows no similarity with the E. coli and vegetative Bacillus consensus promoter and includes such promoters as that from the hygromycin B phosphotransferase (HPH) gene from S. hygroscopicus (Zalacain et al., 1986), the rRNA methylase (tsr) gene from S. azureus, the aminoglycoside phosphotransferase (aph) gene from S. fradiae, the viomycin phosphotransferase (vph) gene from S. vinaceus (Bibb et al., 1985b; Janssen et al., 1985) and the endoglucosidase-H (endoH) gene from S. plicatus (Westpheling et al., 1985). Not surprisingly, those promoters tested for transcriptional activity in E. coli (tsr, aph and vph) do not function (Bibb et al., 1985b).

Shown in Table 2 is the second group of Streptomyces promoter which is characterized by at least a partial similarity to the E. coli canonical promoter sequence. Comparison of these sequences enables a consensus sequence of TtGaoc8a (−35 region) and tAgaaT (−10 region) to be reached, in which capital letters indicate the bases most strongly conserved. It has been proposed that these promoters may be analogous to those read by σ70 of E. coli or σ85 of Bacillus and may represent the Streptomyces ‘vegetative’ promoter (Bibb et al., 1985a). It would be necessary therefore, for the Streptomyces RNA polymerase to have less stringent promoter sequence requirements than E. coli RNA polymerase. Alternatively, Streptomyces may possess many more σ-factors than other bacteria, each of which recognizes a specific subset of promoter. Of those sequences tested for their promoter activity in E. coli, SEP2, SEP3 and SEP6, which closely match the E. coli promoter sequence, are active and the promoter pIJ101A, which differs considerably from the E. coli sequence, functions only weakly.

The Arthrobacter ermA promoter (Table 2) shows partial similarity to the E. coli consensus promoter. The transcription initiation site of the ermA gene in S. lividans was determined by S1 mapping using probe 10X/BglI (see Table 1) which extends over the transcription start site of this gene in Arthrobacter and which previously protected a fragment of 200 nucleotides (Roberts et al., 1985). The lower arrow in Fig. 1a shows that RNA from Arthrobacter (lane 3) and from S. lividans containing plasmid pAR4 (lane 4) protected the same sized fragment (197 nucleotides) from S1 nuclease digestion, strongly suggesting that transcription of ermA in S. lividans begins from the Arthrobacter promoter. This was confirmed by a primer extension experiment (Fig. 1b) which showed that transcription of ermA in S. lividans containing pAR4 (lane 3) proceeds from the same two residues as found in Arthrobacter (lanes 2, 4 and 5). Although there is a slight ‘smile’ in the gel, it is clear, nevertheless, that the S. lividans primer extension corresponds to the same position as that of Arthrobacter. From the equivalent gel of primer extension of the ermA gene in Arthrobacter (Roberts et al., 1985), the transcription start of the ermA gene was assigned to an A at 286 and a G at 289, complementary to the T and C residues shown in Fig. 1b. The upper arrow in Fig. 1a denotes a band corresponding to full length protection of the probe by pAR4 RNA (lane 4) which probably corresponds to transcriptional read-through from the mel promoter of pIJ702 (Hopwood et al., 1985). These

Table 2. Streptomyces promoter sequences

<table>
<thead>
<tr>
<th>Promoter</th>
<th>−35 sequence</th>
<th>Space (bp)</th>
<th>−10 sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli and vegetative Bacillus</td>
<td>TTGACA</td>
<td>17±1</td>
<td>TATAAT</td>
<td>Moran et al. (1982)</td>
</tr>
<tr>
<td>ermE'P1</td>
<td>TTGACA</td>
<td>14</td>
<td>TAGGAT</td>
<td>Bibb et al. (1985a)</td>
</tr>
<tr>
<td>ermE'P2</td>
<td>TTAGGC</td>
<td>18</td>
<td>GAGGAT</td>
<td>Bibb et al. (1985a)</td>
</tr>
<tr>
<td>ORI</td>
<td>CTTGCA</td>
<td>18</td>
<td>TAGCAT</td>
<td>Bibb et al. (1985a)</td>
</tr>
<tr>
<td>gylP1</td>
<td>TTTGAC</td>
<td>17</td>
<td>GAGACT</td>
<td>Janssen et al. (1985)</td>
</tr>
<tr>
<td>gylP2</td>
<td>TTTGAC</td>
<td>19</td>
<td>TAGGAT</td>
<td>Janssen et al. (1985)</td>
</tr>
<tr>
<td>gylR</td>
<td>TTTGAC</td>
<td>19</td>
<td>TAGGT</td>
<td>Janssen et al. (1985)</td>
</tr>
<tr>
<td>SEP2</td>
<td>TTTGAC</td>
<td>18</td>
<td>TAAAT</td>
<td>Jaurin &amp; Cohen (1985)</td>
</tr>
<tr>
<td>SEP3</td>
<td>TTTGAC</td>
<td>16</td>
<td>CATCAT</td>
<td>Jaurin &amp; Cohen (1985)</td>
</tr>
<tr>
<td>SEP6</td>
<td>TTTGAC</td>
<td>17</td>
<td>TTTAT</td>
<td>Jaurin &amp; Cohen (1985)</td>
</tr>
<tr>
<td>pIJ101A</td>
<td>TTTGAC</td>
<td>18</td>
<td>CAGACT</td>
<td>Buttner &amp; Brown (1985)</td>
</tr>
<tr>
<td>Streptomyces 'consensus'</td>
<td>TGGACG</td>
<td>18</td>
<td>TagaT</td>
<td>Roberts et al. (1985)</td>
</tr>
</tbody>
</table>

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results clearly demonstrate that the S. lividans RNA polymerase recognizes the Arthrobacter ermA promoter and initiates transcription at precisely the same position as the RNA polymerase of Arthrobacter. We find no evidence for transcription of the ermA gene proceeding from two start sites, as found for the ermE gene in S. erythreus (Bibb et al., 1985a).

The available evidence indicates that there are few heterospecific barriers affecting transcription between Arthrobacter and Streptomyces: the Streptomyces vector pIJ702 replicates autonomously and the S. azureus tsr gene, which contains a promoter that differs from the E. coli consensus, is expressed in Arthrobacter; the Arthrobacter ermA gene is expressed in S. lividans.

Since tsr and ermA represent the two groups of Streptomyces promoter so far discussed, it is likely that other Streptomyces genes are expressed in Arthrobacter. Thus it may be possible to take advantage of detailed molecular biology that has been carried out in Streptomyces for the development of cloning vectors in Arthrobacter.

**Transcription of the ermA gene in Escherichia coli**

The effectiveness of the ermA promoter in E. coli was assessed by galactokinase assays and S1 mapping. A fragment of DNA (extending from bp 158 to 307 of the sequenced ermA gene; Roberts et al., 1985) containing the ermA promoter was inserted in the appropriate
Transformation of *Arthrobacter*: expression of the *ermA* gene

orientation into the promoter-probe vector pKOS00 (McKenny et al., 1981). No galactokinase activity was detected (results not shown), indicating that the *ermA* promoter does not function in *E. coli*. This was confirmed by S1 mapping RNA derived from *E. coli* containing pAR2. This vector comprises the entire *ermA* gene contained on a 4 kb BamHI fragment cloned into the BamHI site of pUC8 (Roberts et al., 1985). Restriction mapping characterized two isolates: pAR2A in which the *ermA* gene opposes the lacZ promoter of pUC8, and pAR2B in which the *ermA* gene is in the same orientation as lacZ. S1 mapping was carried out using 03/X/BglI (results not shown) which extends over the transcription start point of the *ermA* gene in *Arthrobacter*.

RNA from pAR2A did not protect the probe from S1 nuclease activity, whereas RNA from pAR2B fully protected probe 03/X/BglI and all other probes tested (23.X/SmaI, 41.X/ApaI, 22.X/NcoI, 06.S/EcoRI, 64.S/NcoI, 61.S/EcoRI and 56.S/PvuII; see Table 1) from S1 nuclease digestion (results not shown). These probes mapped regions of RNA between bp 4 and 1822 of the sequenced *ermA* gene. These results indicate that transcription of the *ermA* gene does not proceed from its own promoter in *E. coli* in either pAR2A or pAR2B. In pAR2B, transcription of *ermA* is initiated further upstream, presumably from the lacZ promoter of the vector. Taken together, these results demonstrate that, although the *ermA* promoter is partially homologous to the *E. coli* consensus promoter and has a near optimal spacing of 16 bp between the −10 and −35 region, it is not sufficiently similar to meet the relatively more stringent requirements of the *E. coli* RNA polymerase.

Relationship between the expression of erythromycin resistance genes

Erythromycin resistance genes examined are either expressed constitutively or induced by erythromycin by a translational attenuation mechanism (Dubnau, 1984). Genes that are inducible by erythromycin are characterized by a leader sequence preceding the coding sequence (Gryczan et al., 1980, 1984; Horinouchi & Weisblum, 1980; Horinouchi et al., 1983; Murphy, 1985) which is absent from constitutively expressed genes (Lampson & Parisi, 1986; Monod et al., 1986). Examination of the nucleotide sequence and S1 mapping data of the erythromycin resistance genes *ermA* and *ermE* from two organisms that produce the antibiotic, *Arthrobacter* and *S. erythreus* (Roberts et al., 1985; Bibb et al., 1985a; Uchiyama & Weisblum, 1985), show that a leader sequence is not present. Therefore it seems reasonable to propose that in organisms producing erythromycin, e.g. *Arthrobacter* and *S. erythreus*, the gene responsible for erythromycin resistance is constitutively expressed. If antibiotic resistance genes originated in antibiotic-producing organisms (Benveniste & Davies, 1973; Walker & Walker, 1970) then it would appear that the inducibility of erythromycin resistance in non-antibiotic-producing bacteria must have been acquired later.

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