The TraA relaxase autoregulates the putative type IV secretion-like system encoded by the broad-host-range *Streptococcus agalactiae* plasmid pIP501

Brigitta Kurenbach, Jolanta Kopeć, Marion Mägedefrau, Kristin Andreas, Walter Keller, Christine Bohn, Mouhammad Y. Abajy and Elisabeth Grohmann

Abstract

The conjugative multiple antibiotic resistance plasmid pIP501 can be transferred and stably maintained in a variety of Gram-positive genera, including multicellular *Streptomyces lividans*, as well as in Gram-negative *Escherichia coli*. The 15 putative pIP501 transfer (*tra*) genes are organized in an operon-like structure terminating in a strong transcriptional terminator. This paper reports co-transcription of the pIP501 *tra* genes in exponentially growing *Enterococcus faecalis* JH2-2 cells, as shown by RT-PCR. The *tra* genes are expressed throughout the life cycle of *Ent. faecalis*, and the expression level is independent of the growth phase. Electrophoretic mobility shift assays indicated that the TraA relaxase, the first gene of the *tra* operon, binds to the *tra* promoter *P*_{tra}, which partially overlaps with the origin of transfer (oriT). DNase I footprinting experiments further delimited the TraA binding region and defined the nucleotides bound by TraA. β-Galactosidase assays with *P*_{tra}–lacZ fusions proved *P*_{tra} promoter activity, which was strongly repressed when TraA was supplied in trans. Thus, it is concluded that the pIP501 *tra* operon is negatively autoregulated at the transcriptional level by the conjugative DNA relaxase TraA.

INTRODUCTION

Plasmid-mediated conjugative transfer of antibiotic resistance genes is one of the major reasons for the tremendous spread of multiple antibiotic resistant pathogenic bacteria. pIP501 is a 30599 bp conjugative Inc18 plasmid, originally isolated from *Streptococcus agalactiae* (Horodniceanu et al., 1976), conferring resistance to the MLS group of antibiotics (erythromycin) and to chloramphenicol. pIP501 exhibits a very broad host range for conjugative transfer, including streptococci, lactobacilli, enterococci, lactococci, staphylococci, bacilli, *Listeria* and clostridia. Recently, Zuniga and co-workers have transferred pIP501 and derivatives thereof to *Oenococcus oeni*, a lactic acid bacterium used as a commercial starter culture, and for which no suitable genetic tools for transfer of exogenous DNA are available (Zuniga et al., 2003). We have demonstrated conjugative transfer of pIP501 to the multicellular Gram-positive (*G + *) organism *Streptomyces lividans* and to the Gram-negative (*G − *) organism *Escherichia coli*. The pIP501 antibiotic resistance, replication and *tra* genes are functional in the heterologous hosts, and the plasmid is stably maintained for at least 50 generations in *E. coli* XL-1 Blue cells (Kurenbach et al., 2003).

The pIP501 *nic* site, at which plasmid DNA transfer initiates by the relaxase-mediated introduction of a site- and strand-specific nick, has been mapped by Wang & Macrina (1995a). They also proved *in vivo* nicking activity of the first gene product encoded by the operon, the TraA relaxase (Wang & Macrina, 1995b). TraA- and TraAN293* (the protein comprising the N-terminal 293 amino acids of TraA) mediated *in vitro* relaxation of supercoiled oriT_{pIP501} DNA has been demonstrated by Kurenbach et al. (2002), and relaxation activity of TraAN246 (the protein comprising the N-terminal 246 amino acids of TraA) by Kopec et al. (2005). The pIP501 relaxase TraA contains a positively charged tail sequence at the C terminus, as found in putative type IV secretion substrates and in relaxases of conjugative plasmids from various *α*-Proteobacteria (Schulein et al., 2005). P_{tra} has been mapped by primer extension analysis. Co-transcription of...
the tra genes, orf1 to orf15, has been shown by RT-PCR on RNA from Enterococcus faecalis cells harbouring pIP501 (Kurenbach et al., 2002).

Three of the pIP501 tra genes exhibit significant similarity to type IV secretion system components (Grohmann et al., 2003; Kurenbach et al., 2003) involved in conjugative transfer in G− bacteria (for a review see Llosa & de la Cruz, 2005) and transport of effector molecules from G− pathogens to eukaryotic host cells (reviewed by Cascales & Christie, 2003; Christie, 2004; Christie et al., 2005; Christie & Cascales, 2005). Possible roles for the type IV homologues in a presumably simplified type IV secretion process through the G+ cell envelope have been discussed in Grohmann et al. (2003) and Grohmann (2005).

Regulation of conjugative transfer has been studied in some detail for G− bacteria, whereas in G+ bacteria knowledge of regulation of conjugative transfer is mainly based on data on the Ent. faecalis sex-pheromone-responding conjugative plasmids pCF10, pAD1 and pPD1 (Bae & Dunny, 2001; Bae et al., 2003) and Grohmann (2005).

In this report, we present data on the regulation of the pIP501 tra operon (Fig. 1). By two different approaches we demonstrate that the regulation is exerted at the transcriptional level: i) Ptra:: lacZ reporter fusions proved repression of the tra operon promoter in the presence of the TraA DNA relaxase; and ii) electrophoretic mobility shift assays (EMSAs) and DNase I protection experiments showed binding of TraA to DNA fragments containing the tra operon promoter and parts thereof.

**METHODS**

**Bacterial strains and growth conditions.** Ent. faecalis JH2-2 (Jacob & Hobbs, 1974) was cultivated in brain heart infusion medium (Oxoid) at 37°C. For the selection of Ent. faecalis JH2-2 (pIP501), 20 µg chloramphenicol ml−1 was added to the medium. E. coli JM109 (Promega) and E. coli HB101 (Promega) were grown in LB medium at 37°C, supplemented with 100 µg ampicillin ml−1 for the selection of pQE30-traAN (Kopeč et al., 2005) and pQF120 (Ronald et al., 1990) and derivatives thereof. LB medium was supplemented with 20 µg chloramphenicol ml−1 and 10 µg tetracycline ml−1 for the selection of pACYC184, and supplemented with 20 µg chloramphenicol ml−1 alone for derivatives thereof. For the β-galactosidase assays, E. coli JM109 cells harbouring the respective plasmids were cultivated in the medium suggested by Miller (1972).

**DNA preparation and transformation.** Extraction and purification of plasmid DNAs from E. coli were performed using the Qiagen kit or the Gen Elute Plasmid Miniprep kit (Sigma). Restriction endonucleases were purchased from Promega and New England Biolabs, T4 DNA ligase and Shrimp alkaline phosphatase from Roche Diagnostics, and Gen Therm DNA polymerase from Rapi- dozym. The enzymes were used as specified by the suppliers. PCR fragments for cloning experiments were purified by Wizard PCR Prep (Promega). Preparation of competent cells and E. coli transformations with plasmid DNA were performed by standard methods (Sambrook et al., 1989).

**Construction of plasmids for the β-galactosidase assay.** The promoter probe plasmid pQF120 containing a promoterless lacZ gene was used to clone Ptra, Pna was amplified as a 179 bp PCR fragment with the oligonucleotide primers Ptra_fw and Ptra_rev with added BglII restriction sites at their 5’ ends and a lysate of Ent. faecalis JH2-2 (pIP501) as template. All oligonucleotides used in this work are listed in Table 1. The 171 bp BglII–BglII fragment was ligated with BglII-cut and dephosphorylated pQF120 (9200 bp). E. coli JM109 cells harbouring recombinant plasmids (pQF120-Ptra)

---

**Fig. 1.** Organization of the pIP501 tra region. The overlapping orf7 and Pna regions are indicated (not to scale). The operon (orf1 to orf15) is terminated by a putative strong rho-independent termination signal (hairpin). Hatched genes encode proteins similar to those identified in G− type IV secretion systems. The ORF5 protein shows the conserved features, a nucleotide binding site motif A (Walker A box 250GLSGGGKT256) and motif B (Walker B box 509DEFHFLLS10), of proteins belonging to the VirB4 family of NTP-binding proteins (COG3451). The ORF10 protein is a member of the pfm02534 family of TraG/TrvB/TraD/VirD4 coupling proteins. It shows the P-loop motif (Walker A box) and a Walker B motif for nucleotide binding. The ORF7 protein (similar to VirB1 of the Agrobacterium T DNA transfer system) contains the SLT domain present in bacterial lytic transglycosylases. This domain catalyses the cleavage of the β-1,4-glycosidic bond between acetyl-muramic acid and N-acetylgalactosamine in the cell wall.
were selected as blue colonies on LB agar plates supplemented with 100 \( \mu \)g ampicillin ml\(^{-1}\) and 40 \( \mu \)g X-Gal ml\(^{-1}\). \( P_{\text{tac}} \) and the glutathione S-transferase gene (\( P_{\text{tac}} \): : GST) were PCR-amplified from pGEX-2T (Amersham Biosciences) with primers containing \( \text{Sal} \)I and \( \text{Sph} \)I restriction sites at the 5’ ends, respectively; the fragment was cut with \( \text{Sal} \)I/\( \text{Sph} \)I and inserted into the respective sites of pACYC184 (Chang & Cohen, 1978), thereby interrupting the tetracycline-resistance gene. The \( \text{traA} \) expression cassette (\( P_{\text{tac}} \): : GST-\( \text{traA} \)) was amplified from pGEX-2T-\( \text{traA} \) (Kurenbach et al., 2002) with the same primers and inserted into the \( \text{Sal} \)I/\( \text{Sph} \)I sites of pACYC184. Transformants resistant to chloramphenicol but sensitive to tetracycline were PCR-verified for the presence of \( P_{\text{tac}} \): : GST and \( P_{\text{tac}} \): : GST-\( \text{traA} \), respectively. The nucleotide sequence of the insertions was verified by dideoxy chain termination sequencing in an automated sequencer (ABI prism 310, Perkin Elmer).

**\( \beta \)-Galactosidase assay.** Samples were taken from exponentially growing cultures (OD\(_{600}\) = 0.3) in a minimal medium. Cells were permeabilized by the addition of chloroform and 0.1% SDS. The expression of \( \text{traA} \) was induced by addition of 1 mM IPTG (time of

---

**Table 1. Oligonucleotides used in this work**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Position</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1 fw</td>
<td>GATCAATCCCAAGAATTGGATA</td>
<td>3185–3206*</td>
<td>Growth phase (orf1–2)</td>
</tr>
<tr>
<td>orf2 rev</td>
<td>CTTGCCAACAAGGATCCACCC</td>
<td>3571–3591*</td>
<td>Growth phase (orf1–2)</td>
</tr>
<tr>
<td>orf3 fw</td>
<td>GGTAGCAGCATTAAATAGGCT</td>
<td>3901–3020*</td>
<td>Growth phase (orf3–4)</td>
</tr>
<tr>
<td>orf4 rev</td>
<td>CGGTTCAATGAAGAGGTTGGAG</td>
<td>4401–4423*</td>
<td>Growth phase (orf3–4)</td>
</tr>
<tr>
<td>orf6 fw</td>
<td>CCAATGACTTGTTAGAAGA</td>
<td>8048–8066*</td>
<td>Growth phase (orf6–7)</td>
</tr>
<tr>
<td>orf7 rev</td>
<td>CCGTCATATGCTACGCTTGT</td>
<td>240–260†</td>
<td>Growth phase (orf6–7)</td>
</tr>
<tr>
<td>pIPSeq10</td>
<td>GAGAGTTTGGCTACCGGGG</td>
<td>4187–4203†</td>
<td>Co-transcription (orf11–12)</td>
</tr>
<tr>
<td>orf3 rev</td>
<td>GTTGGCTCTATGAGG</td>
<td>4915–4929†</td>
<td>Co-transcription (orf11–12)</td>
</tr>
<tr>
<td>Seq13</td>
<td>TCTTTCGCGGTTCGCGCGGCCG</td>
<td>5302–5322†</td>
<td>Co-transcription (orf12–13)</td>
</tr>
<tr>
<td>orf4 rev</td>
<td>AATAGTAGAAGAAGATCATTAGTC</td>
<td>6135–6153†</td>
<td>Co-transcription (orf12–13)</td>
</tr>
<tr>
<td>orf5 rev</td>
<td>AAGTAGATATATCGGTATGAA</td>
<td>6437–6457†</td>
<td>Co-transcription/growth phase (orf13–14)</td>
</tr>
<tr>
<td>Seq16</td>
<td>CAGAAGAAAGAAGAAGGTTAAGGG</td>
<td>6718–6740†</td>
<td>Co-transcription (orf14–15)</td>
</tr>
<tr>
<td>orf6 rev</td>
<td>CTCAACAGTGGGGCTA</td>
<td>7164–7179†</td>
<td>Co-transcription (orf14–15)</td>
</tr>
<tr>
<td>orf6 fw</td>
<td>GACGGAACGTAAACGG</td>
<td>7823–7838†</td>
<td>Co-transcription (orf15–copR)</td>
</tr>
<tr>
<td>copR rev</td>
<td>CTCTAAATAGCAGTACCGAAG</td>
<td>8284–8303†</td>
<td>Co-transcription (orf15–copR)</td>
</tr>
<tr>
<td>GAP-DH fw</td>
<td>GGCCCAATTCTCTGACCC</td>
<td>1484236–1484253</td>
<td>Growth phase (standard)</td>
</tr>
<tr>
<td>GAP-DH rev</td>
<td>GGCCCAACTTGAGGCGGCGGCCGGGGCGG</td>
<td>1484772–1484790</td>
<td>Growth phase (standard)</td>
</tr>
<tr>
<td>prom-10 fw</td>
<td>GACCTTATACGGCAGTAACTTC</td>
<td>1264–1283*</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>prom-10 rev</td>
<td>AATGAGCGAAGATCTACAGGGTA</td>
<td>1264–1283*</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>prom-35 fw</td>
<td>TTATAGAACTCCTAGCGATTATATATAGGATC</td>
<td>1230–1259*</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>prom-35 rev</td>
<td>TTATAGAACTCCTAGCGATTATATATATAGGATC</td>
<td>1230–1259*</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>promoter fw</td>
<td>ATTCCCTAGATTATTTACTAAGGCG-</td>
<td>1238–1274*</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>promoter rev</td>
<td>CGTATAAGTGCGCCCTTAGAATAA-TCTAAGGAT</td>
<td>1238–1274*</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>42control fw</td>
<td>AGAATGTTGGAACGACTAATAGAATTAGC-</td>
<td>2179–2220$§$</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>42control rev</td>
<td>ATTCAACCCGCTAGTCATTTTGTTAAGCTGTAACATTCTCT</td>
<td>2179–2220$§$</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>FP_forward</td>
<td>TTCCATGAAATACGCCCTCT</td>
<td>1097–1116*</td>
<td>DNase I footprint</td>
</tr>
<tr>
<td>FP_reverse</td>
<td>CAAACAGCTTGAGCATAAAAFAGCCACTG</td>
<td>1326–1346*</td>
<td>DNase I footprint</td>
</tr>
<tr>
<td>pGEX-2TSphl fw</td>
<td>GGGCATGACATCGGATTAC</td>
<td>146–1581</td>
<td></td>
</tr>
<tr>
<td>pGEX-2TSall rev</td>
<td>GGGTGCACTGCGCTTGAAAGAAGAAGAAGA</td>
<td>1141–1152</td>
<td></td>
</tr>
<tr>
<td>Pra fw</td>
<td>CTCAAGTCTTAATAGGATTCC</td>
<td>1231–1241*</td>
<td>Cloning of ( \text{tra} )</td>
</tr>
<tr>
<td>Pra rev</td>
<td>CTCAAGTCTTAATAGGATTCC</td>
<td>1382–1392*</td>
<td>Cloning of ( \text{tra} )</td>
</tr>
</tbody>
</table>

Accession nos: *L39769, †AJ505823, §NC_0046688 (locus EF1526), §AJ301605, ‡U13850. Restriction sites are underlined.
induction 4 h) to E. coli JM109 cultures harbouring pACYC184- 
P_tac::GST-traA. Determination of β-galactosidase activity was performed as described by Miller (1972).

Estimation of plasmid copy number. Aliquots taken from cultures at the time of sampling for β-galactosidase assays were lysed, and plasmid DNA was prepared with the Gen Elute Plasmid Mini-prep kit (Sigma) and analysed on 0-7% agarose gels. The gels were stained and the amount of plasmid DNA per unit of culture optical density quantified by the Easy win 32 software (Herolab).

RT-PCR analysis. RNA of exponentially growing Ent. faecalis JH2-2 and Ent. faecalis JH2-2 cells harbouring pIP501 (OD_{600}=0-6) was isolated and purified as described in Kurenbach et al. (2002). RT-PCR was performed with primer pairs designed to amplify two successive ORFs (orf11 to orf15) in the pIP501 tra region. Prior to use in RT-PCR, RNA was treated with DNase I (Promega). A 0-5 μg quantity of RNA and 50 pmol of each primer were used in each RT-PCR performed with the Access-RT-PCR kit (Promega). RNA samples were denatured for 2 min at 70°C and kept on ice prior to addition of polymerases. The orf11 to orf12 fragment was amplified as follows: 48°C for 60 min for reverse transcription, followed by inactivation of avian myeloblastosis virus reverse transcriptase (AMV-RT) and denaturation of the template at 94°C for 90 s. The program 94°C for 30 s/55°C for 90 s/68°C for 4 min was applied (40 cycles) and terminated by a final elongation step of 10 min. For the amplification of orf12 to orf13, orf13 to orf14, orf14 to orf15, and orf15 to copR, the program 94°C for 30 s/66°C for 90 s/68°C for 2 min (30 cycles) was applied and terminated by a final elongation step of 10 min. Control reactions were performed with RNA from the plasmid-free isogenic strain, without template RNA, and with 2 μl100 mM pyrocarbonate-treated water were added to a final volume of 20 μl. TraAN246 was overexpressed and purified as described in Kopec et al. (2005). Ten nanograms of the 250 bp fragment (6-5 nM) were incubated at 37°C for 10 min with increasing TraA concentrations from 200 nM to 2 μM in binding buffer (20 mM Tris/HCl, pH 8-0, 200 mM NaCl, 0-1 mM EDTA) in a total volume of 10 μl. DNase I (0-01 Kunitz units, Fermentas) was added to each sample. Ten microlitres of stop buffer (100 μg yeast tRNA ml^{-1}, 30 mM EDTA, 1% SDS, 200 μM NaCl) (Leblanc & Moss, 1994) were added to each RNA sample. The RNA was then mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and precipitated with ethanol. The pellets were air-dried, dissolved in loading buffer (33 mM NaOH, 32% formamide, 0-1% bromophenol blue, 0-1% xylene cyanol) and run on a 5% denaturing polyacrylamide gel. DNA and RNA were visualized with a Typhoon 9400 imaging system (Amersham Biosciences) and read by using the online help (www.promega.com/biomath) for single-strand DNA calculations. ii) PCR: 1 μg cDNA was applied to PCRs containing 2 mM MgCl₂, 10 pmol dNTPs, 5 μl 1 × PCR buffer, 1-5 U Genthern DNA polymerase and 20 pmol of each primer in a total volume of 50 μl. For amplification of orf1 to orf2, orf6 to orf7, and orf13 to orf14, the cycle program 94°C for 30 s/60°C for 90 s/ 68°C for 2 min (30 cycles) was applied and terminated by a final elongation step of 10 min. As a constitutively expressed control, the glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) gene was reverse transcribed and amplified by the same procedure. For amplification of orf3 to orf4 and the gene encoding GAP-DH, the annealing temperature was decreased to 56°C. The PCR products were analysed in 1-5% agarose gels and quantified by the Easy win 32 software (Herolab).

DNA labelling. Synthetic oligonucleotides were purchased from VBC-GENOMICS and Sigma-Genosys. They were labelled with [γ-^{32}P]ATP (1×10^{14} Bq mmol^{-1}) by T4 polynucleotide kinase (Roche Diagnostics). Unbound [γ-^{32}P]ATP was removed by a Sephadex G-50 (Amersham Biosciences) column. For EMSAs, the 5' end-labelled oligonucleotides were annealed to the complementary unlabelled oligonucleotide to generate double-stranded DNA. A 1:1 mixture of the oligonucleotides was diluted in Tris/EDTA buffer (10:1), completely denatured (5 min at 95°C) and annealed by slowly cooling down to room temperature. The labelled DNA fragments were applied immediately to the EMSAs or stored at 4°C.

EMSA. TraAN246 was overexpressed and purified as described in Kopec et al. (2005). All oligonucleotides used to generate double-stranded DNA fragments are shown in Table 1. Binding mixtures (20 μl) containing 10 fmol radiolabelled DNA fragment and increasing TraAN246 concentrations in 20 mM Tris/HCl, pH 7-5, 0-1 mM EDTA, 200 mM NaCl, were incubated at 37°C. Binding reactions and electrophoresis were performed as described in Kopec et al. (2005). As a negative control, a randomly chosen 42-mer DNA fragment with no sequence identity with the pIP501 promoter region (nucleotides 2179 to 2220 on pIP501, accession no. AJ301605) was incubated with increasing TraAN246 concentrations up to 4 μM. No binding was observed.

DNase I footprint. 5'-Labelled FP_forward and unlabelled FP_reverse primer or 5'-labelled FP_reverse and unlabelled FP_forward primer were used to generate a 250 bp F_{tra} and F_{orf1} DNA fragment (nucleotides 1097 to 1346, accession no. L39769) with labelled coding and non-coding strands. Prior to the DNase I footprint reaction, the fragments were heated to 95°C for 10 min and allowed to cool to 37°C. TraA was purified as described in Kopec et al. (2005). Ten nanograms of the 250 bp fragment (6-5 nM) were incubated at 37°C for 10 min with increasing TraA concentrations from 200 nM to 2 μM in binding buffer (20 mM Tris/HCl, pH 8-0, 200 mM NaCl, 0-1 mM EDTA) in a total volume of 10 μl. DNase I (0-01 Kunitz units, Fermentas) was added to each sample. Ten microlitres of stop buffer (100 μg yeast tRNA ml^{-1}, 30 mM EDTA, 1% SDS, 200 mM NaCl) (Leblanc & Moss, 1994) were added to each sample. The RNA was then mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and precipitated with ethanol. The pellets were air-dried, dissolved in loading buffer (33 mM NaOH, 32% formamide, 0-1% bromophenol blue, 0-1% xylene cyanol) and run on a 5% denaturing polyacrylamide gel with 8 M urea in 1× TBE buffer at constant wattage. Reference sequencing reactions were prepared with the Cycle Sequencing kit (Fermentas) with primers FP_forward and FP_reverse. Only the nucleotide numbers are shown in Fig. 5. The gel was exposed overnight to a Storage Phosphor GP Screen (Amersham Biosciences) and read by using the online help (www.promega.com/biomath) for single-strand DNA calculations.

RESULTS AND DISCUSSION

The pIP501 tra region is transcribed as a single operon in Ent. faecalis

The pIP501 tra region shows an operon-like structure [short distance between translational stop and start codons of neighbouring genes, overlapping stop (orf11) and start codons (orf12)]. In order to test co-transcription of orf1 to orf15, we performed RT-PCR with RNA isolated from Ent. faecalis JH2-2 (pIP501) cells harvested during the mid-exponential growth phase, at OD_{600}=0-6. Primer pairs were selected to amplify two successive genes of the pIP501 tra region, starting with orf11. Co-transcription of orf1 to orf11 has already been demonstrated (Kurenbach et al., 2002).
RT-PCR resulted in products of the expected size for orf11 to orf12, orf12 to orf13, orf13 to orf14, and orf14 to orf15 (Fig. 2, lanes 1, 2, 3 and 4). We tested for the existence of transcription products beyond orf15 using primers which would generate an orf15/copR product of 480 bp. Using RNA as template, the respective product was never observed (Fig. 2, lane 5). Transcription of the pIP501 tra operon appears to be terminated by a strong rho-independent transcriptional terminator [free energy of about −46 kJ mol−1], for the following setting: DNA sequence at 37°C, 1 M NaCl; MFold web server for nucleic acid folding and hybridization prediction: http://www.bioinfo.rpi.edu/applications/mfold (Zuker, 2003)] approximately 10 bp downstream of the orf15 translational stop codon (data not shown).

The expression level of the tra genes is independent of growth phase

To investigate the potential influence of growth phase on the expression level of the tra genes, total RNA from Ent. faecalis JH2-2 (pIP501) was isolated at three different time-points of growth: in the early exponential (OD600 = 0·2), the mid-exponential (OD600 = 0·6), and the stationary growth phase (OD600 = 1·0). First, we tested if the tra genes are expressed in all three growth phases. The selected RT-PCR products from orf3 to orf4, orf6 to orf7, and orf13 to orf14 were obtained with RNA from all three growth phases. For the semi-quantitative RT-PCRs, 2 µl each RNA sample was subjected to reverse transcription reactions. The cDNA obtained was quantified by A260 measurement. The amount of cDNA template for PCR was selected taking two requirements into account: i) the amount of cDNA had to be under the level of saturation of the respective PCRs; and ii) the amount of cDNA should yield good visible and quantifiable PCR products. The use of 1 µg of each cDNA fulfilled both criteria. Amplification of products from orf1 to orf2, and orf13 to orf14, of the pIP501 tra region was chosen to investigate the expression levels of different tra genes under varying physiological conditions. As a control, the constitutively expressed gene for GAP-DH was also amplified by RT-PCR, applying RNA isolated from Ent. faecalis JH2-2 cells harvested at OD600 = 0·2, 0·6 and 1·0 as template. Identical volumes of PCR samples were loaded onto 1·5% agarose gels and subjected to electrophoresis. In Fig. 3, PCR samples of orf1 to orf2, orf13 to orf14, and GAP-DH are shown. The amounts of obtained PCR products were compared for the different tra gene fusions at the three different time-points by quantification of the DNA bands with Easy win 32 software. No significant differences were detected for the analysed PCR products. The constitutively expressed gene for GAP-DH yielded the same amount of PCR product under all the conditions tested.

The only variable parameter in the experiment was the growth phase of the Ent. faecalis JH2-2 (pIP501) cells. We cannot exclude, however, that tra gene expression decreases at a later stage in stationary phase, as we have observed slightly lower transfer frequencies (two- to threefold decrease) for donors and/or recipients grown to high cell densities (OD600 > 1·1; C. Bohn and E. Grohmann, unpublished observations). However, a phenomenon such as ‘F− phenocopies’, in which F+ cells become transfer-deficient in stationary phase (Hayes, 1964), was never observed. The expression of several F-encoded tra genes decreases at the transcriptional level in mid-exponential or stationary phase, coincident with a rapid decline in transfer efficiency in mid-exponential phase (Frost & Manchak, 1998).

We conclude that the pIP501 tra genes are expressed during the whole growth cycle of Ent. faecalis and that their level of expression is independent of growth phase.

The TraA relaxase binds to the Ptra promoter

The compact organization of the pIP501 oriT region (Fig. 4), in the sense that the Ptra −10 and −35 boxes overlap with the left half repeat of inverted repeat structures (IR-1 and IR-2), presumably representing the TraA recognition and binding site (Kopec et al., 2005), makes autoregulation of the tra operon by the TraA relaxase likely. To study relaxase binding to the Ptra promoter, we selected three DNA fragments, the first comprising the whole promoter (−35 and −10 region), the second the −35 region alone, and the
third the −10 region alone. The shortest N-terminal portion of TraA exhibiting relaxase activity, the 246 amino acid TraAN246 protein (Kopeć et al., 2005), was used in the bandshift assays. The oligonucleotide representing the tra coding strand was 5′ labelled and annealed to the complementary unlabelled strand to generate double-stranded substrates for the EMSAs. As an example, the data for the −10 region fragment are shown. Applying increasing TraAN246 concentrations to this fragment, we detected one retarded DNA–protein complex (Fig. 4).
Incubation of the −35 region and the promoter fragment with TraAN246 concentrations of greater than 1·0 μM resulted in a complete shift, which represented large protein–DNA complexes that were excluded and so did not enter the gel. Binding affinity for the −35 region and for the whole promoter region was lower than for the −10 region fragment (data not shown). This could be due to presence of the complete left half repeat of the inverted repeat structure (IR-2) in the −10 region fragment.

The complete left half repeat of IR-2 was present in all single-strand oligonucleotides shown to bind TraAN246 and TraA. An oligonucleotide comparable with the −10 region fragment, but additionally containing the right half repeat, results in similar binding affinity (Kopeč et al., 2005). TraA showed similar binding affinities for the different promoter fragments to those of the N-terminal domain TraAN246 (data not shown).

Incubation of TraAN246 at concentrations up to 4 μM with a 42-mer control fragment (8000-fold excess of protein) resulted in no visible shift (data not shown). We conclude that the TraA DNA relaxase binds to the Ptra promoter region. This is in good agreement with relaxase binding to oligonucleotides composed of i) the complete IR-2 structure, ii) IR-2 and the region up to the nic site, and iii) IR-2, the nic site and seven further 5’ bases (Kopeč et al., 2005).

DNase I footprinting analyses with a 250 bp DNA fragment composed of Ptra and the complete IR-1 and IR-2 structures showed protection of both the Ptra −35 region and the −10 region, with hypersensitive sites on the non-cleaved strand close to the nic site (nucleotide numbers 1253 and 1256), at the nic site (nucleotide number 1262) and two nucleotides downstream from the −10 region. On the cleaved strand, DNase I protection extends eight nucleotides to the nic site, with the nic site itself as hypersensitive site (Fig. 5). The DNase I hypersensitive sites could be generated by a conformational change of the oriT region induced by TraA binding, resulting in greater exposure to DNase I attack.

Our data indicate that the left half repeats of IR-1 and IR-2 are the preferential binding sites for the TraA relaxase. Binding of TraA to its target DNA would be a prerequisite for the recognition and cleavage of DNA at the 5’-GpC-3’ dinucleotide in the nic site, which would remain accessible to the enzymic activity of TraA.

**Fig. 5.** DNase I footprinting analysis of the oriT region of pIP501. One hundred nanograms of the 250 bp oriT fragment were incubated with increasing TraA concentrations: lane 1, no TraA added; lane 2, 200 nM TraA; lane 3, 500 nM TraA; lane 4, 1 μM TraA; lane 5, 2 μM TraA. The nucleotide positions on the pIP501 sequence (accession no. L39769) are shown on the left; the −10 and −35 Ptra regions are marked by filled rectangles. The open and solid arrowheads indicate the inverted repeats IR-1 and IR-2, respectively. The open and solid arrowheads to the right of each panel show the bands decreased and increased in intensity, respectively, with increasing concentrations of TraA. The nic site is marked with a horizontal arrow. In the right-hand panel, the gel image is composed of three parts with different brightness and contrast for better clarity.

**Autoregulation of the tra operon: the TraA relaxase negatively regulates transcription from the Ptra promoter**

To prove that TraA binding to the Ptra promoter region affects promoter activity, we put the promoterless lacZ gene in pQF120 under the control of the Ptra promoter. *E. coli* JM109 cells harbouring this construct, pQF120-Ptra::lacZ, gave blue colonies on LB X-Gal plates and resulted in a β-galactosidase activity of 401 Miller units. The effect of traA expression in trans on Ptra activity was tested by co-transformation of E. coli JM109 cells with pQF120-Ptra::lacZ and pACYC184-Plac::GST-traA expressing traA under the control of the IPTG-inducible tac promoter. When traA expression was induced by the addition of 1 mM IPTG, the β-galactosidase activity dropped to 6 Miller units. As a control, the effect of co-resident pACYC184-Plac::GST on the Ptra activity of
pQF120-Prac::lacZ was tested. No significant change in \( \beta \)-galactosidase activity (407 Miller units) was observed. The copy number of the pACYC184 derivatives (pACYC184-Prac::GST and pACYC184-Prac::GST-traA) is considerably smaller (10–12 copies per cell) than that of pQF120-Prac::lacZ (500–700 copies per cell, pMB1 ori of pUC18). The \( \beta \)-galactosidase activities were corrected for apparent copy-number variations. The data (mean values of three independent measurements) clearly indicate that the \( \text{tra} \) operon is regulated at the level of transcription by the TraA relaxase.

The compact organization of the pIP501 \( \text{oriT} \) region: partial overlapping of relaxase promoter and \( \text{nic} \)-region, resembles that of the rolling-circle-replicating plasmid pMV158 (Farias et al., 1999; Grohmann et al., 1999), which is efficiently mobilized by pIP501 (van der Lelie et al., 1990; Kurenbach et al., 2003). For the pMV158-encoded relaxase MobM, autoregulation is currently under investigation. de Antonio and co-workers have proposed that a Leu-zipper motif between residues 317 and 338 in MobM might be involved in dimerization and autoregulation (de Antonio et al., 2004). In TraA, no putative Leu-zipper motif was found. For Mob, the mobilization protein encoded by the mobilizable broad-host-range plasmid pBBR1, the \( \text{nic} \) site of which is identical with that of pMV158, autoregulation by the binding of Mob to its promoter region overlapping with \( \text{oriT} \) has been demonstrated (Szpirer et al., 2001).

Autoregulation of \( \text{tra} \) gene expression mediated by the transfer initiator protein, the DNA relaxase, seems to be an effective mechanism to shut down the energy-consuming process of plasmid transfer in a controlled way at a very early stage of plasmid spread. It has been demonstrated that this mechanism is not restricted to small non-self-transmissible plasmids, but seems also to control the conjugative transfer of multiple resistance plasmids of \( \text{G}^+ \) origin (pIP501 and pRE25, the transfer region of which is virtually identical to that of pIP501). All these transfer-control systems as well as those from \( \text{G}^- \) bacteria appear to be designed to achieve an optimum balance between the maximum transfer potential and the lowest metabolic burden for the host.

ACKNOWLEDGEMENTS

We are very grateful to E. Zechner and S. Kohlwein (Karlfranz-Universität Graz) for providing access to the radioisotope experimental facility and the Typhoon 9400 imaging system, respectively. This work was supported by the Deutsche Forschungsgemeinschaft, grants GR1792/1-1 and 1-2, the Austrian Science Foundation (FWF projects P15040 and F01805) and by travel grants of the Deutscher Akademischer Austauschdienst (DAAD) and the Dr Heinrich Jörg-Stiftung. The generous support of the Institute of Ecology of the Berlin University of Technology is gratefully acknowledged. J.K. is a recipient of a PhD scholarship of the Berliner Programm zur Förderung der Chancengleichheit für Frauen in Forschung und Lehre. M.Y.A. of a doctoral fellowship of the University of Aleppo, Syria.

REFERENCES

Bae, T. & Dunny, G. M. (2001). Dominant-negative mutants of \( \text{prgX} \) gene expression mediated by the transfer initiator protein, the DNA relaxase, seems to be an effective mechanism to shut down the energy-consuming process of plasmid transfer in a controlled way at a very early stage of plasmid spread. It has been demonstrated that this mechanism is not restricted to small non-self-transmissible plasmids, but seems also to control the conjugative transfer of multiple resistance plasmids of \( \text{G}^+ \) origin (pIP501 and pRE25, the transfer region of which is virtually identical to that of pIP501). All these transfer-control systems as well as those from \( \text{G}^- \) bacteria appear to be designed to achieve an optimum balance between the maximum transfer potential and the lowest metabolic burden for the host.

Acknowledgements

We are very grateful to E. Zechner and S. Kohlwein (Karlfranz-Universität Graz) for providing access to the radioisotope experimental facility and the Typhoon 9400 imaging system, respectively. This work was supported by the Deutsche Forschungsgemeinschaft, grants GR1792/1-1 and 1-2, the Austrian Science Foundation (FWF projects P15040 and F01805) and by travel grants of the Deutscher Akademischer Austauschdienst (DAAD) and the Dr Heinrich Jörg-Stiftung. The generous support of the Institute of Ecology of the Berlin University of Technology is gratefully acknowledged. J.K. is a recipient of a PhD scholarship of the Berliner Programm zur Förderung der Chancengleichheit für Frauen in Forschung und Lehre. M.Y.A. of a doctoral fellowship of the University of Aleppo, Syria.

REFERENCES

Bae, T. & Dunny, G. M. (2001). Dominant-negative mutants of \( \text{prgX} \) gene expression mediated by the transfer initiator protein, the DNA relaxase, seems to be an effective mechanism to shut down the energy-consuming process of plasmid transfer in a controlled way at a very early stage of plasmid spread. It has been demonstrated that this mechanism is not restricted to small non-self-transmissible plasmids, but seems also to control the conjugative transfer of multiple resistance plasmids of \( \text{G}^+ \) origin (pIP501 and pRE25, the transfer region of which is virtually identical to that of pIP501). All these transfer-control systems as well as those from \( \text{G}^- \) bacteria appear to be designed to achieve an optimum balance between the maximum transfer potential and the lowest metabolic burden for the host.

Acknowledgements

We are very grateful to E. Zechner and S. Kohlwein (Karlfranz-Universität Graz) for providing access to the radioisotope experimental facility and the Typhoon 9400 imaging system, respectively. This work was supported by the Deutsche Forschungsgemeinschaft, grants GR1792/1-1 and 1-2, the Austrian Science Foundation (FWF projects P15040 and F01805) and by travel grants of the Deutscher Akademischer Austauschdienst (DAAD) and the Dr Heinrich Jörg-Stiftung. The generous support of the Institute of Ecology of the Berlin University of Technology is gratefully acknowledged. J.K. is a recipient of a PhD scholarship of the Berliner Programm zur Förderung der Chancengleichheit für Frauen in Forschung und Lehre. M.Y.A. of a doctoral fellowship of the University of Aleppo, Syria.

REFERENCES

Bae, T. & Dunny, G. M. (2001). Dominant-negative mutants of \( \text{prgX} \) gene expression mediated by the transfer initiator protein, the DNA relaxase, seems to be an effective mechanism to shut down the energy-consuming process of plasmid transfer in a controlled way at a very early stage of plasmid spread. It has been demonstrated that this mechanism is not restricted to small non-self-transmissible plasmids, but seems also to control the conjugative transfer of multiple resistance plasmids of \( \text{G}^+ \) origin (pIP501 and pRE25, the transfer region of which is virtually identical to that of pIP501). All these transfer-control systems as well as those from \( \text{G}^- \) bacteria appear to be designed to achieve an optimum balance between the maximum transfer potential and the lowest metabolic burden for the host.

Acknowledgements

We are very grateful to E. Zechner and S. Kohlwein (Karlfranz-Universität Graz) for providing access to the radioisotope experimental facility and the Typhoon 9400 imaging system, respectively. This work was supported by the Deutsche Forschungsgemeinschaft, grants GR1792/1-1 and 1-2, the Austrian Science Foundation (FWF projects P15040 and F01805) and by travel grants of the Deutscher Akademischer Austauschdienst (DAAD) and the Dr Heinrich Jörg-Stiftung. The generous support of the Institute of Ecology of the Berlin University of Technology is gratefully acknowledged. J.K. is a recipient of a PhD scholarship of the Berliner Programm zur Förderung der Chancengleichheit für Frauen in Forschung und Lehre. M.Y.A. of a doctoral fellowship of the University of Aleppo, Syria.


