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

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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 Ptra, 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 Ptra–lacZ fusions proved Ptra 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 conjunctive transfer of antibiotic resistance genes is one of the major reasons for the tremendous spread of multiple antibiotic resistant pathogenic bacteria. pIP501 is a 30 599 bp conjunctive 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 conjunctive 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 conjunctive 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 plasmid 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 Kopeć 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 conjunctive plasmids from various α-Proteobacteria (Schulein et al., 2005). Ptra has been mapped by primer extension analysis. Co-transcription of

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Abbreviations: EMSA, electrophoretic mobility shift assay; GAP-DH, glyceraldehyde-3-phosphate dehydrogenase; G−, Gram negative; G+, Gram positive; GST, glutathione S-transferase.
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 of the *Ent. faecalis* sex-pheromone-responding conjugative plasmids pCF10, pAD1 and pPD1 (Bae & Dunny, 2001; Bae et al., 2002; Bae et al., 2003) and Grohmann (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).

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) *P*$_{tra}$::*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-*traA$_{JH2}$* (Köpeć 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 pACC184, 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 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 *P*$_{tra}$ in *P*$_{ra}$ was amplified as a 179 bp PCR fragment with the oligonucleotide primers *Ptra*$_{fw}$ and *Ptra*$_{rev}$ with added Bgl II 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–BglIII fragment was ligated with BglII–cut and dephosphorylated pQF120 (9200 bp). *E. coli* JM109 cells harbouring recombinant plasmids (pQF120-*Ptra*) were grown in LB medium at 37°C, supplemented with 100 µg ampicillin ml$^{-1}$ and 10 µg tetracycline ml$^{-1}$ for the selection of pACC184, 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).

**Fig. 1.** Organization of the pIP501 *tra* region. The overlapping *orf7* and *Ptra* 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 250 GLSGGGKT 256) and motif B (Walker B box 509 DEFHFLL 515), of proteins belonging to the VirB4 family of NTP-binding proteins (COG3451). The ORF10 protein is a member of the pfam02534 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-acetylmuraminic acid in the cell wall.
were selected as blue colonies on LB agar plates supplemented with 100 μg ampicillin ml⁻¹ and 40 μg X-Gal ml⁻¹. P_tac and the glutathione S-transferase gene (P_tac::GST) were PCR-amplified from pGEX-2T (Amersham Biosciences) with primers containing SalI and SphI restriction sites at the 5’ ends, respectively; the fragment was cut with SalI/SphI and inserted into the respective sites of pACYC184 (Chang & Cohen, 1978), thereby interrupting the tetracycline-resistance gene. The traA expression cassette (P_tac::GST-traA) was amplified from pGEX-2T-traA (Kurenbach et al., 2002) with the same primers and inserted into the Sall/SphI sites of pACYC184. Transformants resistant to chloramphenicol but sensitive to tetracycline were PCR-verified for the presence of P_tac::GST and P_tac::GST-traA, respectively. The nucleotide sequence of the insertions was verified by dideoxy chain termination sequencing in an automated sequencer (ABI prism 310, Perkin Elmer).

β-Galactosidase assay. Samples were taken from exponentially growing cultures (OD600=0.3) in a minimal medium. Cells were permeabilized by the addition of chloroform and 0.1% SDS. The expression of traA was induced by addition of 1 mM IPTG (time of

Table 1. Oligonucleotides used in this work

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<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Position</th>
<th>Experiment</th>
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<tbody>
<tr>
<td>orf1 fw</td>
<td>GATCAATCCCAAGAATTGGATA</td>
<td>3185–3206*</td>
<td>Growth phase (orf1–2)</td>
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<td>3571–3591*</td>
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<td>3901–3020*</td>
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<td>4401–4423*</td>
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<td>orf6 fw</td>
<td>CCAATGACTTGGTAAAGA</td>
<td>8048–8066*</td>
<td>Growth phase (orf6–7)</td>
</tr>
<tr>
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<td>240–260†</td>
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<tr>
<td>pitPSeq10</td>
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<td>4187–4203†</td>
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</tr>
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<td>4915–4929†</td>
<td>Co-transcription (orf11–12)</td>
</tr>
<tr>
<td>Seq13</td>
<td>TTTTGGGCTTTTCCATTGCC</td>
<td>5302–5322‡</td>
<td>Co-transcription (orf12–13)</td>
</tr>
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<td>6135–6153‡</td>
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<td>Bandshift assay</td>
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</tr>
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<td>2179–2220§</td>
<td>Bandshift assay</td>
</tr>
<tr>
<td>42control rev</td>
<td>ATTCAACCGCAGATCTATTTTGTAGT-AGTCGTTGAA</td>
<td>2179–2220§</td>
<td>Bandshift assay</td>
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<td>1097–1116*</td>
<td>DNase I footprint</td>
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<td>1326–1346*</td>
<td>DNase I footprint</td>
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<td>146–158Ⅱ</td>
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<td>CTCAAGTCTTATTAGATTGCGT</td>
<td>1382–1392*</td>
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</table>

Accession nos: *L39769, †AJ505823, §NC_004668 (locus EF1526), §AJ301605, ||U13850. Restriction sites are underlined.
induction 4 h) to E. coli JM109 cultures harbouring pACYC184-P$_{cat}$::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 *Escherichia coli* strain JH2-2 and *Enterococcus 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 copyR, 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 template RNA but omitting the reverse transcription step (data not shown). RT-PCR products were analysed on 2% agarose gels.

**RT-PCR of tra mRNA isolated under different growth conditions.** Total RNA was isolated from *Escherichia coli* strain JH2-2 (pIP501) cells harvested at different stages of bacterial growth: in the early exponential phase, OD$_{600}$=0.2; in the mid-exponential phase, OD$_{600}$=0.6; and during the stationary growth phase, OD$_{600}$=1.0. The semi-quantitative RT-PCR was performed in two steps. i) Reverse transcription (cDNA synthesis): 2 μl DNase-treated RNA was incubated with 0.5 μg random hexamer primer (Promega) in a volume of 5 μl for 5 min at 70°C, and then stored on ice. cDNTPs (10 pmol), 2 mM MgCl$_2$, 4 μl 5x M-MLV buffer and 1 μl M-MLV (Moloney murine leukemia virus) reverse transcriptase and diethyl pyrocarbonate-treated water were added to a final volume of 20 μl and incubated for 5 min at 25°C, 60 min at 42°C and 15 min at 70°C; cDNA was purified by ethanol precipitation. The DNA concentration was determined by A$_{350}$ measurement and calculated 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 MgCl$_2$, 10 pmol dNTPs, 5 μl 10x PCR buffer, 1.5 U Genscript 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/66°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 $[^{32P}]$ATP ($1.1 \times 10^{8}$ Bq mmol$^{-1}$) by T4 polynucleotide kinase (Roche Diagnostics). Unbound $[^{32P}]$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.** TraAN$_{246}$ was overexpressed and purified as described in Kopeč 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 TraAN$_{246}$ 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 Kopeč 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 TraAN$_{246}$ 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 fragment, which was labelled and annealed to generate double-stranded 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 Kopeč 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 beaded DNA 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 x 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 the Typhoon 9400 imaging system (Amersham Biosciences).

**RESULTS AND DISCUSSION**

The pIP501 tra region is transcribed as a single operon in *Enterococcus 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 *Enterococcus 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 auto-regulation 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 (Kopeč 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).

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**Fig. 3.** PCR with cDNA from *Ent. faecalis* JH2-2 (pIP501). PCR product (2 µl) was loaded onto a 1·5% agarose gel. cDNA (1 µg) was used in two tra-specific PCRs (orf1 to orf2, 404 bp product; orf13 to orf14, 474 bp product) and a PCR specific for the GAP-DH of *Ent. faecalis* (555 bp product). RNA was isolated at OD600 = 0·2, 0·6 and 1·0. M, 100 bp DNA ladder (Invitrogen); +, positive control with lysed cells of *Ent. faecalis* JH2-2 (pIP501); −, negative control without template.

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**Fig. 4.** EMSA of the −10 promoter region fragment with TraAN246. Increasing TraAN246 concentrations (0, 5, 50, 200, 350, 500, 650, 800 nM, 1 µM) were incubated with 10 fmol DNA of the −10 promoter region fragment at 42 °C for 30 min, and loaded onto a 10% native polyacrylamide gel. The oriTpIP501 region, containing the Ptra promoter, a perfect inverted repeat (IR-1) (open horizontal arrows), an imperfect inverted repeat (IR-2) (solid horizontal arrows) and the nic site (vertical arrow), is indicated. The promoter fragment and the −35 DNA fragment are also shown.
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.

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 pQF210-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::pmz::lacZ was tested. No significant change in β-galactosidase activity (407 Miller units) was observed. The copy number of the pACYC184 derivatives (pACYC184-PM: GST and pACYC184-PM::GST- traA) is considerably smaller (10–12 copies per cell) than that of pQF120::pmz::lacZ (500–700 copies per cell, pMB1 ori of pUC18). The β-galactosidase activities were corrected for apparent copy-number variations. The data (mean values of three independent measurements) clearly indicate that the tra operon is regulated at the level of transcription by the TraA relaxase.

The compact organization of the pIP501 oriT region: partial overlapping of relaxase promoter and 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 nic site of which is identical with that of pMV158, autoregulation by the binding of Mob to its promoter region overlapping with oriT has been demonstrated (Szpirer et al., 2001).

Autoregulation of 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 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 G− bacteria appear to be designed to achieve an optimum balance between the maximum transfer potential and the lowest metabolic burden for the host.

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