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 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 30 599 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 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 conjugative plasmids from various α -Proteobacteria (Schulein *et al.*, 2005). P_{tra} 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 *orf11*, 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, 2004; Horii *et al.*, 2002; for reviews see Clewell & Dunny, 2002; Chandler & Dunny, 2004).

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-*traAN*₂₄₆ (Kopeć *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 RapiDzym. The enzymes were used as specified by the suppliers. PCR fragments for cloning experiments were purified by Wizard PCR Preps (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} . P_{tra} was amplified as a 179 bp PCR fragment with the oligonucleotide primers P_{tra_fw} and P_{tra_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 *Bgl*II-*Bgl*II fragment was ligated with *Bgl*II-cut and dephosphorylated pQF120 (9200 bp). *E. coli* JM109 cells harbouring recombinant plasmids (pQF120- P_{tra})

Fig. 1. Organization of the pIP501 *tra* region. The overlapping *oriT* and P_{tra} 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 ₅₀₉DEFHLL₅₁₅), of proteins belonging to the VirB4 family of NTP-binding proteins (COG3451). The ORF10 protein is a member of the pfam02534 family of TraG/TraB/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 acetylmuramic acid and *N*-acetylglucosamine in the cell wall.

Table 1. Oligonucleotides used in this work

Name	Sequence (5'→3')	Position	Experiment
orf1 fw	GATCAATCCCAAGAATTGGATA	3185–3206*	Growth phase (<i>orf1</i> –2)
orf2 rev	CTTCGCACAAGCAATCCCACC	3571–3591*	Growth phase (<i>orf1</i> –2)
orf3 fw	GGTACGGCATTAATAAGCGT	3901–3020*	Growth phase (<i>orf3</i> –4)
orf4 rev	CGGTTTCATATAAGAACGTTTTGAG	4401–4423*	Growth phase (<i>orf3</i> –4)
orf6 fw	CCAATGACTTGTAGAAAAGA	8048–8066*	Growth phase (<i>orf6</i> –7)
orf7 rev	CCGTCATATTGTCTAGCGTAC	240–260†	Growth phase (<i>orf6</i> –7)
pIPSeq10	GAGAGTTGGTACACCTG	4187–4203†	Co-transcription (<i>orf11</i> –12)
orf13 rev	GTTGTCTTATTGGC	4915–4929†	Co-transcription (<i>orf11</i> –12)
Seq13	TTCTTTGCCTTTTCCATTGCC	5302–5322†	Co-transcription (<i>orf12</i> –13)
orf14 rev	AATAGTAGAAAAGAATCACTATC	6135–6153†	Co-transcription (<i>orf12</i> –13)
orf15 fw	AAGTAGATATATCCGATTGG	6437–6457†	Co-transcription/growth phase (<i>orf13</i> –14)
orf15 rev	TCTTTCCTAACAACCTGGCTT	6893–6912†	Co-transcription/growth phase (<i>orf13</i> –14)
Seq16	CAGCAAAAGAAATGAGTGAAAGG	6718–6740†	Co-transcription (<i>orf14</i> –15)
orf16 rev	CTAACAAAGTGGGGCTG	7164–7179†	Co-transcription (<i>orf14</i> –15)
orf16 fw	GACGGAACAGTAACGG	7823–7838†	Co-transcription (<i>orf15</i> – <i>copR</i>)
copR rev	CTCTAAATGCTAGTTCAAAG	8284–8303†	Co-transcription (<i>orf15</i> – <i>copR</i>)
GAP-DH fw	GGCACCTATCCTGGCACC	1484236–1484253‡	Growth phase (standard)
GAP-DH rev	GGGAACACGTTGGGCATGG	1484772–1484790‡	Growth phase (standard)
prom-10 fw	GCACTTATACGCAGTAACTT	1264–1283*	Bandshift assay
prom-10 rev	AAGTTACTGCGTATAAGTGC	1264–1283*	Bandshift assay
prom-35 fw	TATTAAGGATTCCCTTAGATTATTTA-CTAA	1230–1259*	Bandshift assay
prom-35 rev	TTAGTAAATAATCTAAGGGAATCCTT-AATA	1230–1259*	Bandshift assay
promoter fw	ATTCCTTAGATTATTTACTAAGGGC-GCACTTATACG	1238–1274*	Bandshift assay
promoter rev	CGTATAAGTGCGCCCTTAGTAAATAA-TCTAAGGGAAT	1238–1274*	Bandshift assay
42control fw	AGAATGTTGAACGACTAACTAAAAAT-AGACTAGCGGTTGAAT	2179–2220§	Bandshift assay
42control rev	ATTCAACCGCTAGTCTATTTTGTAGTT-AGTCGTTCAACATTCT	2179–2220§	Bandshift assay
FP_forward	TTCCATGAAATACGCCTCCT	1097–1116*	DNase I footprint
FP_reverse	CAAACACGCTTGACGATAAAA	1326–1346*	DNase I footprint
pGEX-2TSphI fw	GGGCATGCATCATAACGGTTC	146–158	Cloning of $P_{tac}::GST$, $P_{tac}::GST-traA$
pGEX-2TSalI rev	GGGTCGACTGACGTCTAAGA	1141–1152	Cloning of $P_{tac}::GST$, $P_{tac}::GST-traA$
Ptra fw	CTCAGATCTATTAAGGATTC	1231–1241*	Cloning of P_{tra}
Ptra rev	CTCAGATCTTGATCACCTC	1382–1392*	Cloning of P_{tra}

Accession nos: *L39769, †AJ505823, ‡NC_004668 (locus EF1526), §AJ301605, ||U13850. Restriction sites are underlined.

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 *SalI/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

induction 4 h) to *E. coli* JM109 cultures harbouring pACYC184-*P_{lac}::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 μ g 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/60 °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 *Ent. faecalis* 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 μ l DNase-treated RNA was incubated with 0.5 μ g random hexamer primer (Promega) in a volume of 5 μ l for 5 min at 70 °C, and then stored on ice. dNTPs (10 pmol), 2 mM $MgCl_2$, 4 μ l 5 $\times$ M-MLV buffer and 1 μ l M-MLV (Moloney murine leukaemia virus) reverse transcriptase and diethyl pyrocarbonate-treated water were added to a final volume of 20 μ l 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_{260} measurement and calculated by using the online help (www.promega.com/biomath) for single-strand DNA calculations. ii) PCR: 1 μ g cDNA was applied to PCRs containing 2 mM $MgCl_2$, 10 pmol dNTPs, 5 μ l 10 $\times$ PCR buffer, 1.5 U Genterm DNA polymerase and 20 pmol of each primer in a total volume of 50 μ l. 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 (1.11×10^{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.

EMSAs. TraAN₂₄₆ was overexpressed and purified as described in Kopeć *et al.* (2005). All oligonucleotides used to generate double-stranded DNA fragments are shown in Table 1. Binding mixtures (20 μ l) 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 Kopeć *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 μ M. 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 *P_{tra}* and *oriT* 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 Kopeć *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 μ M in binding buffer (20 mM Tris/HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA) in a total volume of 10 μ l. DNase I (0.01 Kunitz units, Fermentas) was added to each sample. Ten microlitres of stop buffer (100 μ g yeast tRNA ml $^{-1}$, 30 mM EDTA, 1% SDS, 200 mM NaCl) (Leblanc & Moss, 1994) were added to naked DNA after 1 min, and to reactions with TraA after 3 min. DNA was extracted with 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 $\times$ 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 *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 ($\text{OD}_{600}=0.2$), the mid-exponential ($\text{OD}_{600}=0.6$), and the stationary growth phase ($\text{OD}_{600}=1.0$). 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 A_{260} 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 $\text{OD}_{600}=0.2$, 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 ($\text{OD}_{600}>1.1$; 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 P_{tra} promoter

The compact organization of the pIP501 *oriT* region (Fig. 4), in the sense that the P_{tra} -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 (Kopeć *et al.*, 2005), makes autoregulation of the *tra* operon by the TraA relaxase likely. To study relaxase binding to the P_{tra} promoter, we selected three DNA fragments, the first comprising the whole promoter (-35 and -10 region), the second the -35 region alone, and the

Fig. 2. Co-transcription of *orf11* to *orf15* of the *tra* region of pIP501. PCR products were loaded onto a 2% agarose gel. The expected product size is given below in parentheses. Lane 1, *orf11* to *orf12* (690 bp); lane 2, *orf12* to *orf13* (852 bp); lane 3, *orf13* to *orf14* (474 bp); lane 4, *orf14* to *orf15* (461 bp); lane 5, *orf15* to *copR* (480 bp); M, 1 kb Plus DNA ladder (Invitrogen); lane 7, *orf15* to *copR*, PCR with lysed cells of *Ent. faecalis* JH2-2 (pIP501); lane 8, *orf15* to *copR*, PCR with lysed cells of *Ent. faecalis* JH2-2 (plasmid free); -, negative control, *orf15* to *copR* (without template).

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.

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).

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 *oriT*_{PIP501} region, containing the *P*_{tra} 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.

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 P_{tra} regions are marked by filled rectangles. The open and solid arrows 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.

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 (Kopeć *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 P_{tra} 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 (Kopeć *et al.*, 2005).

DNase I footprinting analyses with a 250 bp DNA fragment composed of P_{tra} and the complete IR-1 and IR-2 structures showed protection of both the P_{tra} -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 P_{tra} promoter

To prove that TraA binding to the P_{tra} promoter region affects promoter activity, we put the promoterless *lacZ* gene in pQF120 under the control of the P_{tra} promoter. *E. coli* JM109 cells harbouring this construct, pQF120- $P_{tra}::lacZ$, 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 P_{tra} activity was tested by co-transformation of *E. coli* JM109 cells with pQF120- $P_{tra}::lacZ$ and pACYC184- $P_{tac}::GST-traA$ 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- $P_{tac}::GST$ on the P_{tra} activity of

pQF120-*P_{tra}::lacZ* was tested. No significant change in β -galactosidase activity (407 Miller units) was observed. The copy number of the pACYC184 derivatives (pACYC184-*P_{tac}::GST* and pACYC184-*P_{tac}::GST-traA*) is considerably smaller (10–12 copies per cell) than that of pQF120-*P_{tra}::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 (Fariás *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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