RESEARCH ARTICLE

Comparative genomics of the pIPO2/pSB102 family of environmental plasmids: sequence, evolution, and ecology of pTer331 isolated from *Collimonas fungivorans* Ter331

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Abstract

Plasmid pTer331 from the bacterium *Collimonas fungivorans* Ter331 is a new member of the pIPO2/pSB102 family of environmental plasmids. The 40 457-bp sequence of pTer331 codes for 44 putative ORFs, most of which represent genes involved in replication, partitioning and transfer of the plasmid. We confirmed that pTer331 is stably maintained in its native host. Deletion analysis identified a mini-replicon capable of replicating autonomously in *Escherichia coli* and *Pseudomonas putida*. Furthermore, plasmid pTer331 was able to mobilize and retromobilize IncQ plasmid pSM1890 at typical rates of 10^{-4} and 10^{-8}, respectively. Analysis of the 91% DNA sequence identity between pTer331 and pIPO2 revealed functional conservation of coding sequences, the deletion of DNA fragments flanked by short direct repeats (DR), and sequence preservation of long DRs. In addition, we experimentally established that pTer331 has no obvious contribution in several of the phenotypes that are characteristic of its host *C. fungivorans* Ter331, including the ability to efficiently colonize plant roots. Based on our findings, we hypothesize that cryptic plasmids such as pTer331 and pIPO2 might not confer an individual advantage to bacteria, but, due to their broad-host-range and ability to retromobilize, benefit bacterial populations by accelerating the intracommunal dissemination of the mobile gene pool.

Introduction

Plasmids are extrachromosomal self-replicating DNA elements. One view on plasmids stresses the benefits to bacterial hosts. Heavy metal resistance genes, antibiotic resistance genes, or genes coding for degradative pathways are typically located on and mobilized by plasmids (Smalla *et al*., 2000a; Frost *et al*., 2005). Plasmids also catalyze retrotransfer or retromobilization (Szpirer *et al*., 1999; Ronchel *et al*., 2000), which can be thought of as 'gene-fishing' for their host, as they effectively increase the accessibility of the host to the mobile gene pool available in a given environment. The other view on plasmids represents them as selfish DNA (Doolittle & Sapienza, 1980; Orgel *et al*., 1980; Rensing *et al*., 2002), i.e. the presence of plasmids in a bacterial population is mainly due to their efficiency in spread and not to the reproductive success of the individuals carrying the plasmid (Lilley *et al*., 2000; Thomas, 2000; Velmurugan *et al*., 2003). Examples of selfish DNA are cryptic plasmids: they are stably maintained in the host population but do not confer any evident or demonstrable advantage to their hosts.

The increasing availability of completely sequenced plasmids (http://www.ncbi.nlm.nih.gov/genomes/static/o.html, http://www.ebi.ac.uk/genomes/plasmid.html) offers new excitement to the study of plasmids by allowing novel answers to questions regarding their biological role, coding potential, and contribution to host fitness. Furthermore, it opens the way for comparative genomics approaches to elucidate the mechanisms of plasmid evolution, i.e. the forces that drive plasmid divergence and diversity. The general consensus is that plasmids are subject to two parallel

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evolutionary processes: micro-evolution (Price *et al*., 2001; Joshi *et al*., 2005), e.g. accumulation of nucleotide substitutions and insertion/deletions (indels), and macro-evolution (Osborn & Boltner, 2002; Dennis, 2005), i.e. the acquisition of whole operons and the creation of 'mosaic' plasmids, which typically involves the activity of other mobile elements such as transposons and insertion elements.

All sequenced plasmids fall into one of two groups: one with known native host and one without. Typically, this latter group of 'orphan plasmids' features elements that have been captured by and maintained in a surrogate bacterial host through one of several available methods, including bi-and tri-parental mating and transposon-aided capture of plasmids (for an overview see Haagensen *et al*., 2002; Smalla & Sobecky, 2002; Jones & Marchesi, 2007). These methods have played an important role in broadening our knowledge on plasmid diversity as they opened the way for the exogenous isolation of plasmids from bacterial hosts that typically resist cultivation in the laboratory. However, knowledge and availability of a plasmid's natural host(s) is always desirable, as it allows for experimental testing of hypotheses on the plasmid and associated functions in its natural background.

The recently recognized pIPO2/pSB102 family of environmental broad-host-range plasmids consists both of plasmids with known hosts and orphan plasmids. Proposed members of this family include pIPO2 (Tauch *et al*., 2002), pSB102 (Schneiker *et al*., 2001), and pXF51 (Marques *et al*., 2001). The former two (39 815 and 55 578 bp, respectively) were isolated exogenously, while pXF51 (51 158 bp) was identified as an extrachromosomal element in the genome of *Xylella fastidiosa* strain 9a5c (Marques *et al*., 2001). All three plasmids originated from bacterial communities associated with the plant environment. Their complete nucleotide sequences are similar in gene content and synteny, and in all three cases the majority of the coding potential seems dedicated to 'selfish' traits including plasmid replication, maintenance and transfer. Only pSB102 harbors a set of genes with a demonstrable advantage to its host by conferring resistance to mercury. A likely fourth member of the pIPO2/pSB102 family is pES1 (Gstalder *et al*., 2003), isolated exogenously from a hydrocarbon-polluted soil and partially (10.2 kb) sequenced as a mini-Tn5-Km1 derivative named pMOL98 (Gstalder *et al*., 2003). It has been suggested that pFBAOT6 (Rhodes *et al*., 2004) also represents a member of the pIPO2/pSB102 family. With a size of 84 748 bp, this IncU plasmid resembles pIPO2 and pSB102 in replication, maintenance and transfer functions, but confers tetracycline resistance. Plasmid pFBAOT6 was isolated from a clinical isolate of *Aeromonas caviae*, which challenged the notion (Tauch *et al*., 2002) that the pIPO2/pSB102 family of environmental plasmids is exclusive to plant-associated bacteria (Rhodes *et al*., 2004).

Here, we report on the isolation of plasmid pTer331 from its natural host *Collimonas fungivorans* strain Ter331 (de Boer *et al*., 2004). This bacterium is one of 22 *Collimonas* strains originally isolated from the rhizosphere of Marram grass (*Ammophila arenaria*) as dominant microorganisms among the cultivable, chitinolytic bacterial population (de Boer *et al*., 1998). Strains of *C. fungivorans* also exhibit antifungal activity (de Boer *et al*., 2001), a mycophagous phenotype, i.e. able to use living fungi as a growth substrate (de Boer *et al*., 2001), biocontrol activity towards the plant-pathogenic fungus *Fusarium oxysporum* f. sp. *radicis lycopersici* (Kamilova *et al*., 2007), and efficient colonization of the tomato rhizosphere (Kamilova *et al*., 2007). We present the complete nucleotide sequence and coding capacity of pTer331, its ability to replicate, (retro)mobilize, and stably maintain itself in host *C. fungivorans* Ter331, and its contribution to the rhizosphere competence of this host. Furthermore, we exploit the high degree of identity between pTer331 and pIPO2 to reveal possible mechanisms of divergence since these plasmids split from their common ancestor and to hypothesize on the evolutionary events that shaped the diversity of known members of the pIPO2/pSB102 family of environmental plasmids.

Materials and methods

Strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table 1. Collimonads were grown at 25 °C in liquid or on solid 0.1x tryptic soybean broth (TSB) medium, pH 6.5 (de Boer *et al*., 2004) or on King's B (KB) medium (King *et al*., 1954). For solid TSB or KB medium, 15 g agar was added per liter. In mating experiments, Luria–Bertani (LB) medium (Bertani, 1951) was used to grow collimonads and pseudomonads at 28 °C and *Escherichia coli* at 37 °C.

Isolation of genomic and plasmid DNA from *C. fungivorans* Ter331

Genomic DNA was isolated from TSB-grown *C. fungivorans* Ter331 according to a protocol described elsewhere (Rondon *et al*., 1999) with minor modifications. In short, cells were centrifuged and washed in 1 volume of buffer A, concentrated in the same solution to an $OD_{600\,nm}$ of 10–20, mixed with 1 volume of 2% low melting point agarose (Bio-Rad, Veenendaal, the Netherlands), and poured into plug molds (Bio-Rad). Solidified agarose plugs were gently shaken at room temperature in buffer B* (buffer B lacking sodium deoxycholate and Brij-58). After 30 min, lysozyme was added to a final concentration of $2\,mg\,mL^{-1}$, and incubation was continued at 37 °C for 20 h. After two washes of 30 min each in solution B* at room temperature, the plugs were transferred to solution C (solution B* containing

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Table 1. Strains and plasmids used in this study

	Relevant characteristics	References
Strain		
Collimonas fungivorans Ter331	Betaproteobacterium isolated from the rhizosphere of marram grass, with demonstrated antifungal activity and rhizosphere competency; harbors plasmid pTer331	de Boer *et al*. (2004), Kamilova *et al*. (2007)
C. fungivorans Ter331R	Spontaneous rifampicin-resistant derivative of *C. fungivorans* Ter331	Leveau *et al*. (2006)
C. fungivorans Ter331PC	Plasmid-cured derivative of *C. fungivorans* Ter331	This study
Escherichia coli CV601	Strain used as donor in bi- and tri-parental matings	Smalla *et al*. (2000b)
Pseudomonas fluorescens R2f	Strain used as recipient in tri-parental matings; rifampicin-resistant	Smit *et al*. (1991)
P. fluorescens (pIPO2T)	Strain used as positive control instead of *C. fungivorans* Ter331 in bi- and tri-parental matings	van Elsas *et al*. (1998)
P. fluorescens PCL1285	Rhizosphere-competent, kanamycin-resistant derivative of *Pseudomonas fluorescens* WCS365	Lugtenberg *et al*. (2001)
Plasmid		
pTer331	Plasmid native to *C. fungivorans* Ter331	This study
pTer331Δ	Deletion derivative of pTer331, constructed by replacement of the 27.6-kb BsaI/SacI fragment with a kanamycin resistance marker	This study
pSM1890	Mobilizable but not self-transmissable plasmid, confers resistance to gentamycin and streptomycin	Haagensen *et al*. (2002)
pIPO2T	Mini-Tn*5-tet* derivative of pIPO2, a self-transferable plasmid isolated exogenously from the wheat rhizosphere; confers resistance to tetracyclin	Tauch *et al*. (2002), van Elsas *et al*. (1998)

0.2 mg proteinase K mL^{-1}) and incubated at 50 °C overnight. This step was repeated for an additional 5 h, after which the plugs were washed extensively in TE buffer.

Plasmid pTer331 was isolated from *C. fungivorans* Ter331 using a QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands). For this, cells were grown in a 20-mL TSB liquid culture to an $OD_{600\,nm}$ of 1.1, harvested and resuspended in 750 μL buffer P1 provided with the kit. Three aliquots of 250 μL were lysed and neutralized according to the manufacturer's instructions. After centrifugation, supernatants of the three aliquots were combined and loaded on a single spin column, followed by washing and elution of the plasmid DNA with 50 μL preheated (70 °C) elution buffer.

Analysis of genomic and plasmid DNA by gel electrophoresis

Genomic DNA in agarose plugs was loaded on a 1% Pulsed Field Certified (PFC) agarose gel (Bio-Rad) in 0.5x Tris-borate-EDTA (TBE) buffer, and separated on a Contour-Clamped Homogeneous Electric Field (CHEF)-Mapper III system (Bio-Rad) at 12 °C, with the following settings: 6 V cm^{-1}, 120° angle, pulse intervals of 6–60 or 0.98–12.91 s, with a linear ramping factor. For restriction analysis of the plasmid, 0.2 μg of purified DNA was digested with 10 U EcoRI, HindIII or PstI and analyzed on a regular 1% MP agarose gel (Roche, Almere, The Netherlands) in 0.5x TBE.

Sequencing of plasmid pTer331 and DNA sequence analysis

A shotgun approach was taken to determine the complete nucleotide sequence of plasmid pTer331 (Macrogen, Seoul, Korea). In short, random DNA fragments of 1.5–3 kb were cloned into pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA) and sequenced from both ends. In total, 344 shotgun sequences were assembled using Lasergene's Seqman (DNAstar, Madison, WI). Remaining gaps were filled in by primer walking, representing 18 additional sequence reads. The complete nucleotide sequence of pTer331 (40 457 bp) was searched for ORFs using FGENESB (www.softberry.com) and by the automated genome interpretation system GenDB (Meyer *et al*., 2003). Sequence similarity searches were performed using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information. BPROM (www.softberry.com) was used for prediction of σ^{70}-dependent promoters, and FindTerm (www.softberry.com) for finding σ-independent terminator sequences. Repeat regions within the pTer331 sequence were identified with Lasergene's Megalign (DNAstar). The annotated nucleotide sequence of pTer331 is available at the DDBJ/EMBL/Genbank database (accession number EU315244). To allow comparison to pIPO2 at the DNA level, we reconstructed the original pIPO2 sequence (39 815 bp) *in silico* from that of pIPO2 T (45 319 bp; accession number AJ297913) by removal of nucleotides 38 238–43 741.

Detection of pTer331 in other *Collimonas* isolates

To test the presence of plasmid pTer331 in other collimonads, we used a pTer331-specific PCR assay on 44 strains in our *Collimonas* collection. Twenty-one of these strains (Ter6, 10, 14, 72, 90, 91, 94, 113, 118, 146, 165, 166, 227, 228, 252,

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266, 282, 291, 299, 300, and 330) have been described before by de Boer *et al*. (2004) and 23 strains (R35505-R35513, R35515-R35518, R35520-R35526, R35529, LMG23976, and LMG23968) by Höppener-Ogawa *et al*. (2007, 2008). From each strain, genomic DNA was isolated with a MO BIO Soil DNA Extraction Kit (MO BIO laboratories; Carlsbad, CA) and used as template in a PCR using primers pIPO2 forward and pIPO2 reverse (van Elsas *et al*., 1998). This set was originally designed to be specific for pIPO2 but based on sequence identity also detects pTer331, producing a 307-bp PCR product (see Fig. 2 for location on pTer331). PCR amplification was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) in a total volume of 15 μL containing 50 ng template DNA, 1.5 μL primer mix (final concentration 12.5 μM), 7.5 μL 2x ABsolute qPCR mix (ABgene, Epsom, UK), and using the following temperature profile: 15 min at 95 °C, 40 cycles of 45 s at 95 °C, 45 s at 55 °C, and 90 s at 72 °C. End-point fluorescence measurements were used to establish the presence or absence of a pTer331-derived PCR amplicon, using genomic DNA from *C. fungivorans* Ter331 as a positive control.

Quantifying plasmid stability

To determine the stability of pTer331 in *C. fungivorans* Ter331, the latter was cultivated for 35 generations in liquid KB by daily dilutions into fresh medium. Samples from the last generation were diluted and plated on KB agar to obtain individual colonies which were tested for possession of pTer331 by PCR as described above. Plasmid stability was estimated from the fraction of CFU that had retained the plasmid and expressed as *M*, i.e. the frequency of plasmid loss per cell per generation, calculated as $M = (\pi - \pi_0)/\ln(p/p_0)$, where π_0 and p_0 are, respectively, the proportion of plasmid-free cells and the total number of cells at the start of the experiment, and π the proportion of plasmid-free cells after the total number of cells has risen to p (Ike *et al*., 1981). It should be noted that this formula does not take into account phenomena such as plasmid fitness cost and conjugation transfer rates. Because we tested 93 CFU (cells) from generation 35 for possession of plasmid pTer331, our detection limit for *M* was $(1/93\text{-}0)/\ln(2^{35} \cdot p_0/p_0) = 0.00044$.

Mobilizing and retromobilizing activity of pTer331

To assess the mobilizing and retromobilizing capacities of plasmid pTer331, we performed tri- and biparental mating experiments with *C. fungivorans* Ter331. The triparental mating mixture consisted of *C. fungivorans* Ter331 as helper, *P. fluorescens* R2f as recipient and *E. coli* CV601 (pSM1890) as donor. In the biparental mating, the mixture consisted of *C. fungivorans* Ter331R as recipient and *E. coli* CV601 (pSM1890) as donor. In control experiments, *P. fluorescens* (pIPO2 T) was used instead of *C. fungivorans* Ter331. Overnight cultures of donor, recipient and/or helper strains were washed twice in 0.85% NaCl, mixed in equal amounts, pipetted as a 100-μL drop on LB agar, and incubated overnight at 28 °C. Following incubation, 1-by-1 cm agar plugs containing the mating mixtures were cut out and vortexed for 5 min in 9 mL 0.85% NaCl. A ten-fold dilution series was plated on LB agar containing gentamicin ($25\,\mu g\,mL^{-1}$) and rifampicin ($15\,\mu g\,mL^{-1}$) to enumerate pSM1890-containing *P. fluorescens* R2f or *C. fungivorans* Ter331 transconjugants from the tri- and biparental matings, respectively. Transconjugants were verified by testing for growth on LB agar containing streptomycin ($20\,\mu g\,mL^{-1}$) and rifampicin ($15\,\mu g\,mL^{-1}$) and by PCR amplification targeting *oriV* of pSM1890 as described elsewhere (Gotz *et al*., 1996). Transfer frequencies were calculated as the ratio of transconjugants to recipients. We also tested for the presence of pTer331 in triparental transconjugants by PCR amplification using primers VirB10f (5′-CGSATCTT YGTGCTSTGG-3′) and VirB10r (5′-AGKGTTGGCGGAA TRTTGA-3′) (see Fig. 2 for location on pTer331).

Construction of a pTer331 deletion derivative

For the construction of deletion derivative pTer331Δ, the kanamycin resistance gene from pCR-TOPO (Invitrogen, Breda, The Netherlands) was amplified with primers Km_UP (5′-TTTTCGAGACCGGAAAACGCAAGCGCAAAGAGAAA-3′; BsaI recognition site underlined) and Km_LP (5′-GAGC TCGGGAATAAGGGCGACACGGAAATG-3′; SacI recognition site underlined), and ligated as a 1085-bp BsaI–SacI fragment to BsaI/SacI double-digested plasmid pTer331. The architecture of pTer331Δ was confirmed by restriction enzyme digestion.

Plasmid curing of *C. fungivorans* Ter331

We cured *C. fungivorans* Ter331 from plasmid pTer331 exploiting the principle of plasmid incompatibility (Uraji *et al*., 2002). For this, we introduced pTer331Δ as curative plasmid into *C. fungivorans* Ter331 by electroporation (Sambrook *et al*., 1989). Electrotransformants were selected for growth on KB agar supplemented with kanamycin at a concentration of $600\,\mu g\,mL^{-1}$. Plasmid DNA isolated from kanamycin-resistant transformants was identified as pTer331Δ by restriction analysis. Furthermore, the absence of pTer331 in these transformants was confirmed by PCR using primers 222f (5′-ACAAGGGCAAGCCAGTCAAG-3′) and 842r (5′-TCTGCCGACGAACGCTGTGT-3′), which amplify a 1.1-kb DNA fragment that is present on pTer331 but missing from pTer331Δ (Fig. 2). One *C. fungivorans* Ter331 (pTer331Δ) transformant was grown for several generations on KB in the absence of kanamycin to allow

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spontaneous curing of plasmid pTer331Δ. Plasmid-free derivatives were detected by their inability to grow on KB agar supplemented with kanamycin. The absence of plasmid pTer331Δ in these colonies was confirmed by our inability to (1) isolate plasmid DNA and (2) obtain a PCR product using primers Kan_UP and Kan_LP, which are specific for the kanamycin resistance locus on pTer331Δ. This plasmid-cured (PC) derivative of *C. fungivorans* Ter331 is referred to in the text as *C. fungivorans* Ter331PC.

Competitive root tip colonization assay

The ability of wild-type *C. fungivorans* Ter331 and plasmid-cured *C. fungivorans* Ter331PC to colonize tomato root tips was compared through competition experiments of each strain with rhizosphere-competent *P. fluorescens* PCL1285 using a previously described protocol (Simons *et al.*, 1996; Kamilova *et al.*, 2007). CFU were counted to calculate the ratios of PCL1285 (rifampicin-resistant) to either Ter331 or Ter331PC (both rifampicin-sensitive). From these, the relative rhizosphere competency of Ter331 and Ter331PC could be indirectly estimated. Data were analyzed statistically by the nonparametric Wilcoxon–Mann–Whitney test (Zar, 1999).

Results and discussion

Identification, isolation, and size estimation of plasmid pTer331

Pulsed-field gel electrophoresis (PFGE) of genomic DNA isolated from *C. fungivorans* Ter331 revealed two discrete bands (Fig. 1a, lane 1). The smaller one migrated to the same location on the gel as DNA that was prepared from *C. fungivorans* Ter331 using a QIAprep Spin Miniprep Kit for the isolation of plasmid DNA (Fig. 1a, lane 2). When different PFGE settings were applied, this band migrated differently relative to the linear marker fragments (not shown), suggesting (Brito & Paveia, 1999; Nakamura *et al.*, 2003) that the plasmid, which we designated pTer331, is circular. Digestion of purified pTer331 with HindIII or PstI revealed in both cases a single, linear fragment with an estimated size of 40 kb (Fig. 1b, lanes 4 and 5), while digestion with EcoRI produced eight fragments (Fig. 1b, lane 3) adding up to a plasmid size of *c.* 39.9 kb.

Complete nucleotide sequence of plasmid pTer331

Plasmid pTer331 has a size of 40 457 bp and a G+C content of 60.6% (Fig. 2). *In silico* digestion of pTer331 with EcoRI produced nine fragments consistent with the observed EcoRI banding pattern (Fig. 1b, lane 3). Also, as expected, we identified on pTer331 single recognition sites for HindIII

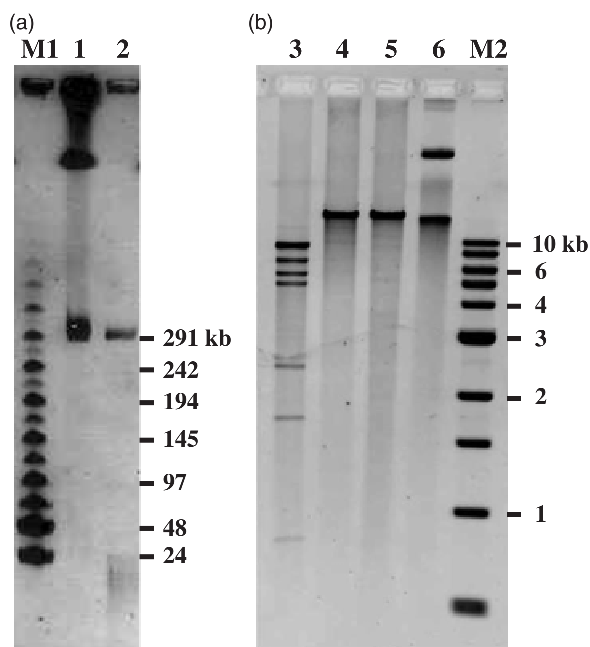

Fig. 1. Gel electrophoresis of genomic and/or plasmid DNA isolated from *Collimonas fungivorans* Ter331. (a) PFGE of *C. fungivorans* Ter331 genomic DNA prepared in agarose plugs (lane 1) and of plasmid pTer331 isolated with a QIAprep Spin Miniprep Kit (lane 2). PFGE conditions were as follows: 24 h run time with 6–60 s pulse times. Lane M1: MidRange II PFG Marker (New England Biolabs). (b) Regular agarose gel (1%) showing 0.2 μg of purified pTer331 DNA digested with EcoRI (lane 3), HindIII (lane 4), or PstI (lane 5). Lane 6: undigested plasmid DNA. Lane M2: 1-kb marker (New England Biolabs).

and PstI (Fig. 2). Analysis of the pTer331 DNA sequence revealed 44 ORFs, 39 of which were predicted to be organized in 11 operons of two or more genes. Table 2 lists all ORFs, their proposed gene names, locations, operonic organization, and G+C content, as well as the length and size of predicted gene products and highest similarity to proteins in the DDBJ/EMBL/Genbank databases.

The overall genetic organization of pTer331 (Fig. 2) was highly similar to that of plasmid pIPO2 (Tauch *et al.*, 2002). The percentage identity between shared homologs was remarkably high and varied from 77% (ORF15 or *virD*4) to 99% (ORF28b). No significant differences in GC3 content (Sueoka, 1988; Bellgard *et al.*, 2001) and synonymous/nonsynonymous substitution rates (Nei & Gojobori, 1986) were observed. Three of the seven ORFs that were identified as unique to either pTer331 (i.e. ORF43b) or pIPO2 (i.e. ORF28a and ORF38) were indeed specific for one plasmid only, because the DNA fragment corresponding to each of these ORFs was deleted at least partially in the other plasmid. Other discrepancies between plasmids pTer331 and pIPO2 could be attributed to differences in annotation. For example, Tauch *et al.* (2002) interpreted the region upstream of ORF44 on pIPO2 to contain a divergently transcribed

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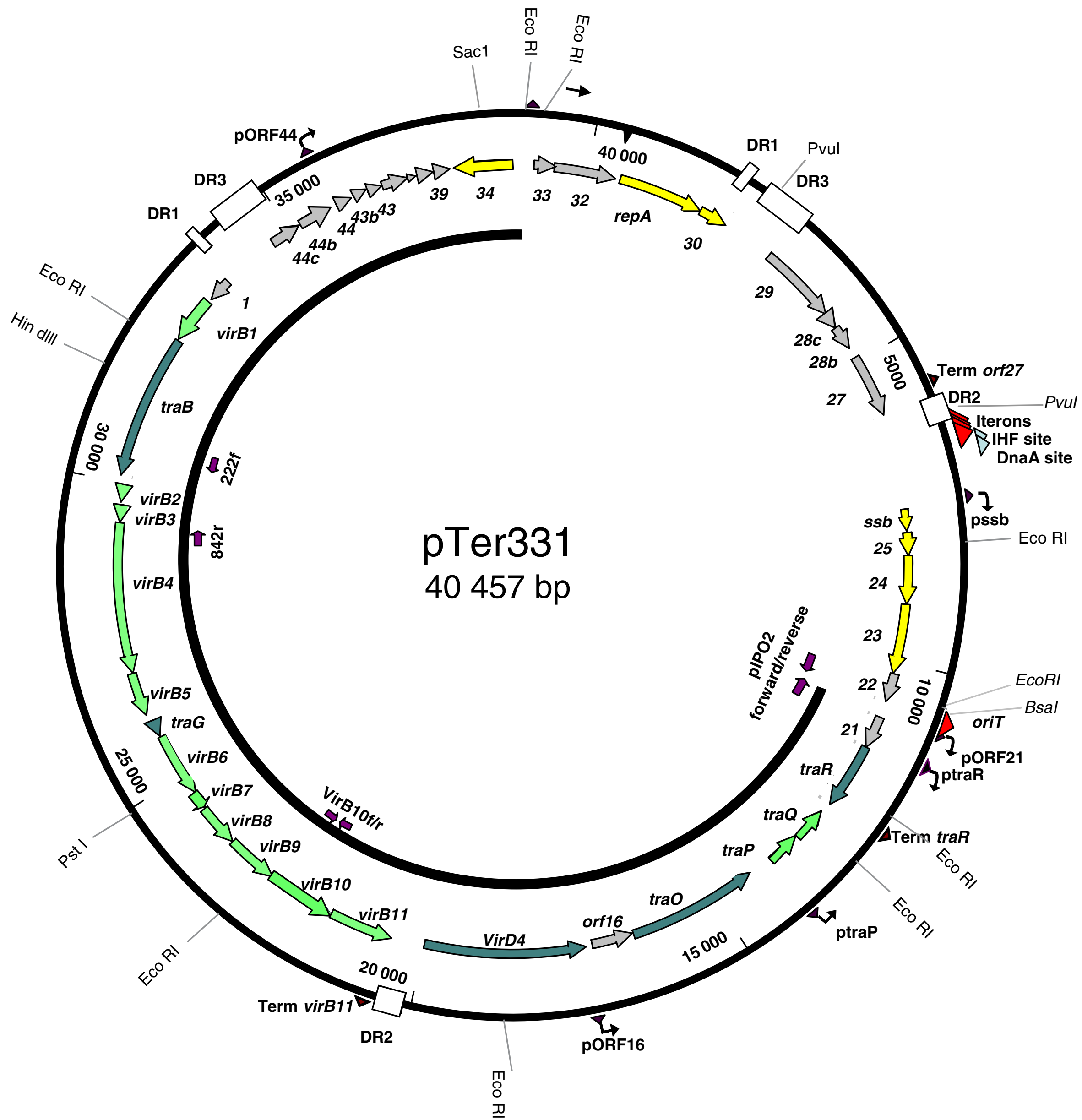

Fig. 2. Genetic map of plasmid pTer331. The arrows indicate the position and direction of transcription of the putative ORFs. Different colors indicate a presumed function in replication and maintenance (yellow), mating pair formation (light green), DNA processing (dark green). ORFs with unknown function are colored grey. Also indicated are the positions of the putative origin of transfer *oriT* (5′-GTGTGGGCTATTGCAGGAT-3′), putative promoter sequences (P), long range DRs (DR1–DR3), positions of primers 222f, 842r, pIPO2 forward, pIPO2 reverse, virB10f, and virB10r, the putative IHF site 5′-TTAAAGCCTTATGAATCAATGGCTTGCGCGCAAGA-3′; IHF consensus bases underlined), DnaA box (5′-CTATCCACA-3′), and four iterons (three identical DRs 5′-CACGCTGAAAGTGTCTTGCG-3′ and one imperfect repeat (5′-ACGCTGAAACTGTCTTGCG-3′).

ORF45, while we assigned two ORFs (i.e. 44b and 44c) on the opposite strand of the same region on pTer331. Also, a clear homolog of pTer331 ORF28c appears to exist on pIPO2 (positions 4172–4354), but it was not recognized earlier (Tauch *et al.*, 2002).

Pairwise comparison of pTer331 to plasmids of the pIPO2/pSB102 family showed various degrees of sequence conservation (Fig. 3). Evidently, plasmid pSB102 diverged more from pTer331 than did pIPO2 and pMOL98. Plasmid pSB102 also distinguished itself from pTer331 and pIPO2 by the acquisition of a transposon, Tn*5718*, which confers mercury resistance (Schneiker *et al.*, 2001). Clearly, sequence similarity between pTer331 and pFBAOT6 or pXF51 was much lower and was mostly restricted to

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Table 2. Putative coding regions of plasmid pTer331, their possible function and closest relationship to other proteins

ORF	Gene name*	Start position (nt)	Stop position (nt)	Predicted operon	G+C content (%)	Putative function	Protein length (aa)	Protein size (kDa)	Amino acid identity to pIPO2 homolog	Percentage identity to other plasmid gene products	Accession
31	repA	1	1389	1	62.2	Replication initiation	462	50.9	95% repA	38% to RepA, plasmid pSa17	S30121
30	parB	1389	1853	1	65.6	Involvement in plasmid partition	154	16.9	94% ORF30	57% to ParB, plasmid RP4	AAA26416
29	ardC	2699	4009	2	61.2	Antirestriction	436	47.7	93% ORF29	91% to ArdC, plasmid pSa	AAD52160
28c	orf28c	4097	4279	2	56.8	Probable transmembrane protein, function unknown	60	6.2	n/a	n/a	n/a
28b	orf28b	4283	4675	2	65.9	Unknown	130	14.5	99% ORF28b	61% to ORF5, plasmid pSB102	CAC79150
27	krfA	4849	5883	3	69.9	Regulation of plasmid segregation	344	36.9	90% ORF27	38% to KrfA, plasmid/ mobile genomic island pKLC102	AAP22622
26	ssb	7396	7800	4	56.3	Single stranded DNA binding activity, function unknown	182	20.3	97% ssb	33% to P116, plasmid RK2	CAD58038
25	orf25	7804	8202	4	55.9	Partition gene repressor	132	14.7	96% ORF25	48% to XACb0052, plasmid pXAC64	AAM39298
24	incC	8199	8990	4	55.9	IncC-like protein	263	28.7	98% ORF24	35% to IncC, plasmid pMBA19a	AAX19280
23	korB	8991	10 136	4	63.4	KorB-like transcriptional repressor	381	40.2	94% ORF23	35% to KorB, plasmid pBP136	BAF33443
22	orf22	10 100	10 609	4	57.1	Unknown	169	18.4	94% ORF22	43% to Neut_2600, plasmid 2	ABI60803
21	mobC	10 877	11 416	5	58.9	Unknown	179	20.2	97% ORF21	31% to MobC, plasmid pRA3	ABD64841
20	traR/ virD2	11 413	12 507	5	61.7	Nickase/relaxase activity	364	40.4	96% TraR	40% to Nic, plasmid pRA3	ABD64842
19	traQ	12 678	13 256	6	62	Type IV secretion channel, structural component	192	20.5	94% TraQ	63% to TrbM, plasmid pB3	CAG26010
18	traP	13 270	13 830	6	57.7	Outer membrane protein	218	23.9	95% TraP	52% to Upf30.5, plasmid pA1	BAE19699
17	traO	14 227	16 419	6	60.8	DNA primase activity	730	80.9	95% TraO	31% to TraC4, plasmid pRA3	ABD64845

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Table 2. Continued.

ORF	Gene name*	Start position (nt)	Stop position (nt)	Predicted operon	G+C content (%)	Putative function	Protein length (aa)	Protein size (kDa)	Amino acid identity to pIPO2 homolog	Percentage identity to other plasmid gene products	Accession
16	orf16	16 431	17 129	7	55.8	Unknown	232	25.3	95% ORF16	27% to Neut_2626, plasmid 2	ABI60828
15	traN/ virD4	17 230	19 932	8	58.9	ATPase activity, coupling the relaxosome with the transfer machinery	917	100.2	77% TraN	30% to VirD4, plasmid pTiA6NC	P09817
14	virB11	20 480	21 580	9	58.8	Mating pair formation, ATPase	355	39.9	97% TraM	55% to VirB11, plasmid pES100	AAW88285
13	virB10	21 531	22 691	9	62.4	Type IV secretion channel, structural component	386	39.4	87% TraL	32% to VirB10, plasmid pTiC58	P17800
12	virB9	22 691	23 563	9	60	Type IV secretion channel, structural component	290	31.6	98% TraK	28% to VirB9, plasmid pTi15955	P0A3W7
11	virB8	23 560	24 270	9	58.8	Type IV secretion channel, structural component	236	26.2	98% TraJ	29% to VirB8, plasmid pTiC58	P17798
10	virB7	24 276	24 440	9	56.9	Type IV secretion channel, structural component	54	5.6	98% TraI	36% to XF_a0011, plasmid pXF51	P58337
9	virB6	24 577	25 659	10	59.6	Type IV secretion channel, structural component	360	37.9	96% TraH	31% to VirB6, plasmid pES100	AAW88297
8	traG	25 671	25 967	10	60.9	entry exclusion	98	10.2	95% TraG	41% to BBta_p0253, plasmid pBBta01	ABQ39891
7	virB5	26 071	26 790	11	58.7	Type IV secretion channel, structural component	224	24.6	97% TraF	40% to VFB54, plasmid pES100	AAW88296
6	virB4	26 796	29 276	11	56.5	Mating pair formation, ATPase	826	93.9	98% TraE	46% to VFB39, plasmid pES100	AAW88281
5	virB3	29 283	29 606	11	58	Type IV secretion channel, structural component	107	12.1	98% TraD	34% to Neut_2637, plasmid 2	ABI60839

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4	virB2	29 616	30 086	11	56.9	Pilin precursor	152	16.1	93% TraC	44% to VFB38, plasmid pES100	AAW88280
3	traB	30 089	32 404	12	60.6	DNA topoisomerase activity	771	85.5	87% TraB	43% to ORF31, plasmid pRA3	ABD64859
2	virB1	32 414	33 184	12	63	Involved in the local enzymatic disruption of the peptidoglycan layer	256	26.6	94% TraA	56% to VirB1, plasmid pXcB	AAO72105
1	orf1	33 242	33 637	12	51.5	Unknown	131	14.9	93% ORF1	33% to XF_a0004, plasmid pXF51	AAF85573
44c	orf44c	34 789	35 097	13	56.6	Unknown	102	11.6	n/a	n/a	n/a
44b	orf44b	35 119	35 667	13	66.7	Unknown	182	20	n/a	72% to Neut_2597, plasmid 2	ABI60800
44	orf44	35 776	36 042	14	61	Unknown	88	9.8	98% ORF44	27% to Oant_4534, plasmid pOANT01	ABS17221
43b	orf43b	36 130	36 270	14	58.2	Unknown	46	5.2	n/a	n/a	n/a
43	orf43	36 327	36 563	14	65.4	Unknown	78	8.6	96% ORF43	48% to ORF41, plasmid pSB102	CAC79186
42	orf42	36 574	37 002	14	68.8	Unknown	142	16.5	93% ORF42	n/a	n/a
41	orf41	36 999	37 136	14	60.9	Unknown	45	5.2	97% ORF41	n/a	n/a
40	orf40	37 157	37 423	14	66.3	Unknown	88	9.5	97% ORF40	60% to Orf45	CAC79190
39	orf39	37 420	37 695	14	64.1	Unknown	64	9.7	95% ORF39	40% to ORF1, plasmid pBFp1	AAQ94180
34	parA	37 792	38 688	15	68.1	Resolvase activity	298	32	93% ORF 34	77% to ParA, plasmid pBP136	BAF33474
33	yacA	39 053	39 406	1	63.3	Regulation of plasmid replication	117	13.1	97% ORF33	60% to YacA plasmid pXcB	AAO72122
32	orf32	39 381	40 382	1	62.2	Cell filamentation	333	37.9	96% ORF32	30% to Ajs_4242, plasmid pAOVO01	ABM44342

*For the *virB* genes on pTer331, we followed the nomenclature proposed by Christie *et al*. (2005).

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the region with genes required for plasmid transfer. The pairwise comparison also revealed that pIPO2 and pMOL98 carry sequences with similarity to long direct repeats (DR) DR1, DR2, and DR3 found on pTer331 (Figs 2 and 3), whereas on pSB102, only two partially conserved homologs of DR3 were identified.

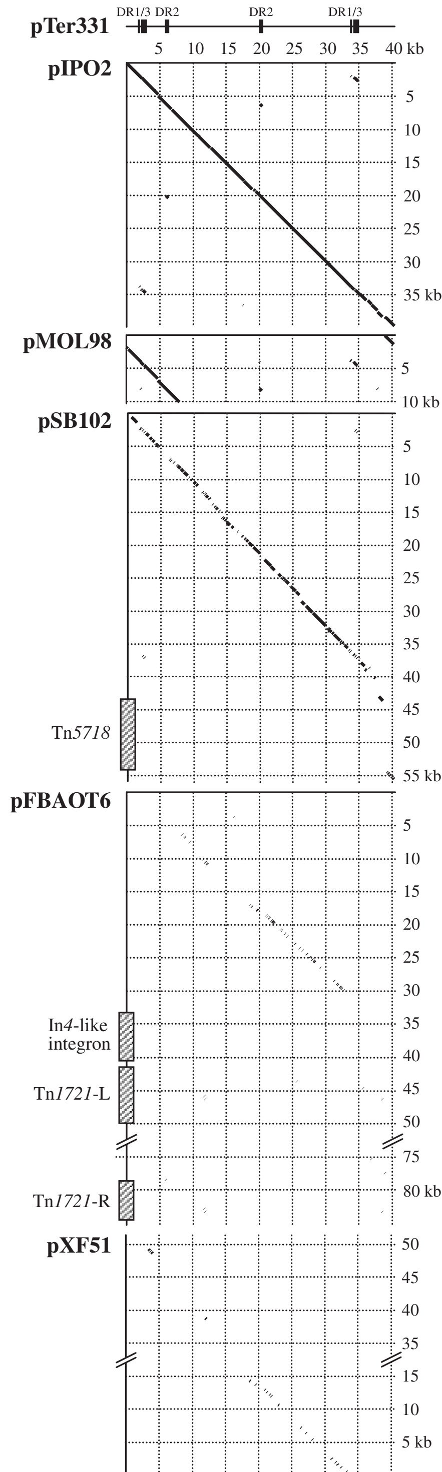

Fig. 3. Pairwise comparison of the pTer331 DNA sequence to those of pIPO2, pMOL98, pSB102, pFBAOT6, and pXF51. Dot plots were created using Lasergene's module MegAlign using a 65% match cut-off and a window size of 50. The alignment of pMOL98 only included the 10 264-bp sequence reported for this plasmid (Gstalder *et al.*, 2003). As reference points, the long DRs DR1, DR2 and DR3 are indicated on the linear representation of pTer331 at the top of the graph. The hatched bar on the Y-axis of the pSB102 alignment indicates the position of the 10 414-bp transposon Tn*5718* (Schneiker *et al.*, 2001), and those on the Y-axis of the pFBAOT6 alignment the location of an In*4*-like integron and the left and right ends of Tn*1721* (Rhodes *et al.*, 2004). Note that the Y-axis in the comparison with pXF51 has been reversed.

Functional annotation of plasmid pTer331

We grouped the predicted gene products of pTer331 into two categories: (1) replication and partitioning functions, (2) transfer functions, including mating pair formation and DNA processing. A third category consists of genes and their products for which no function could be predicted based on sequence homology (Table 2).

Replication and partitioning functions

Like other members of the pIPO2/pSB102 family (Gstalder *et al.*, 2003), plasmid pTer331 has several characteristics that suggest a Θ-type mode of replication (Summers, 1996; Espinosa *et al.*, 2000; Krüger *et al.*, 2004). First, ORF31 showed homology to several RepA replication initiator proteins. Second, in an *oriV*-like region *c.* 6 kb downstream of the *repA* gene, we identified four putative iterons, representing binding sites for RepA, that overlap in sequence and approximate location with iteron sequences identified on pSB102, pMOL98, and pIPO2 (Gstalder *et al.*, 2003). Furthermore, we located an AT-rich (83%) region (5′-TTTAGGTTTTTTTTCCCTTTAAAAATATA-3′) possibly representing the site of DNA strand opening (del Solar *et al.*, 1998), as well as a putative DnaA-binding site and a potential integration host factor (IHF) site (Fig. 2). The *repA* gene is the third in a putative operon that contains other genes with probable involvement in plasmid replication. The predicted product of ORF33 features a ribbon–helix–helix motif typical of CopG-like transcriptional repressor proteins involved in the regulation of plasmid replication (del Solar *et al.*, 2002). ORF32 revealed partial homology to the *fic* gene involved in cell cycling (Kawamukai *et al.*, 1989), suggesting that replication of pTer331 might be closely linked to host cell division.

To identify the origin of replication of pTer331, i.e. the minimal *cis*-acting region that can support its autonomous replication, we constructed a deletion derivative of pTer331.

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Plasmid pTer331Δ consists of a kanamycin resistance gene ligated to the 12.9-kb BsaI–SacI fragment of plasmid pTer331 (Fig. 2). This construct could be readily introduced and maintained in hosts *E. coli* EP-max 10B (Bio-Rad) and *P. putida* KT2440 (Nelson *et al*., 2003), suggesting that the 27.6-kb fragment deleted from pTer331 has no contribution in replication function. The remaining 12.9-kb fragment of pTer331 largely overlaps with a sequenced 10.2-kb region of pMOL98 which has previously been shown to be sufficient for autonomous replication of this plasmid (Gstalder *et al*., 2003). This further confirms the functionality of pTer331Δ as a mini-replicon. Additional deletion from pTer331Δ of an internal 4.1-kb PvuI fragment carrying ORFs 29, 28c, 28b and 27 (Fig. 2) diminished the plasmid's ability to replicate or be maintained in *E. coli* (results not shown). The functional annotation of these ORFs offers several possible explanations for the apparent requirement of this operon for plasmid replication/maintenance. The predicted product of ORF29 showed homology to ArdC from plasmid pSa (Belogurov *et al*., 2000) and might protect single-stranded pTer331 DNA from host endonuclease activity during replication. The ORF27 product resembled KfrA from plasmid pKLC102 (Klockgether *et al*., 2004), which has a role in plasmid segregation during cell division (Kornacki *et al*., 1993).

Plasmid stability is a measure of the likelihood with which a plasmid is inherited by daughter cells at cell division (Cooper & Heinemann, 2000). We estimated the stability of plasmid pTer331 in *C. fungivorans* Ter331 experimentally (see 'Materials and methods') and found a frequency of plasmid loss per generation (M) that was lower than the detection limit of 0.00044. This is typical for plasmids with an active stable partitioning system (Ike *et al*., 1981; Sanchis *et al*., 1997; Huang *et al*., 2006), and we identified several candidate genes on pTer331. ORFs 25, 24, and 23 are homologous to the KorA-IncC-KorB system which dictates the segregational stabilization of IncP-1 plasmids (Pansegrau *et al*., 1994; Bignell & Thomas, 2001), whereas ORF30 resembles ParB which has an essential role in the partitioning of plasmid RK4 (Gerlitz *et al*., 1990) and ORF34 codes for a putative multimer resolvase with homology to ParA of RP4 (Gerlitz *et al*., 1990).

Transfer functions: mating pair formation and DNA processing

In tri- and biparental mating experiments, we established that pTer331 possesses both mobilizing and retro-mobilizing properties. Triparental matings involved *C. fungivorans* Ter331 as a helper strain, facilitating through pTer331 the transfer of plasmid pSM1890 from *E. coli* CV601 to *P. fluorescens* R2f. Under the circumstances tested, this transfer occurred at a frequency (defined as the number of transconjugants per recipient) of 1.8×10^{-4}. In a control experiment with pIPO2, the rate was 233-fold higher at 4.2×10^{-2}. About 10% of the transconjugants in these mating experiments tested positive with primers for pTer331, indicating that cotransfer of pTer331 occurred during triparental mating. In biparental matings, we determined the rate with which pTer331 mediated the acquisition of plasmid pSM1890 by *C. fungivorans* Ter331 from *E. coli* CV601. With pTer331, the transfer frequency was 2.22×10^{-8} compared with 7.87×10^{-8} with pIPO2. Higher values of transfer rate have been reported for pIPO2 by Tauch *et al*. (2002) and for other plasmids (Top *et al*., 1992), but this discrepancy could be due to a difference in the mobilizing vector and in the experimental conditions.

The (retro)mobilizing activity of plasmid pTer331 can be attributed to two groups of genes: (1) those involved in mating pair formation (indicated in light green in Fig. 2) and (2) those involved in processing of the plasmid DNA for transfer to, and establishment in, the recipient cell (indicated in dark green in Fig. 2). On plasmid pTer331, these genes occur interspersed, which is characteristic for plasmids from the pIPO2/pSB102 family (Tauch *et al*., 2002). Similar to pIPO2 and pSB102, the mating pair formation genes of pTer331 showed a high degree of similarity in synteny and coding sequence to the type IV secretion system encoded by the *virB* gene cluster of several different *Brucella* species. Plasmid-encoded *virB* genes are responsible for assembling the secretion structure that allows the transfer of plasmid DNA (Llosa *et al*., 2002).

DNA processing in the donor cell involves nicking of the plasmid DNA at the origin of transfer (*oriT*) by a nickase that – together with accessory proteins and the plasmid DNA – constitutes the relaxosome. This protein-DNA complex is then presented by a so-called coupling protein to the membrane-located type IV secretion system (Llosa *et al*., 2002; Christie *et al*., 2005). Upstream of ORF21 on pTer331, we identified a putative *oriT* site with 100% identity to that described for pIPO2 (Tauch *et al*., 2002). The ORF downstream of ORF21 codes for a putative protein that is homologous to VirD2-like proteins with DNA relaxase/nickase activity (Avila *et al*., 1996), while ORF15 is the most likely candidate coding for a relaxosome-coupling function based on homology to *traN*/*virD4* genes from other plasmids (Porter *et al*., 1987). The predicted product of ORF17 (TraO) shows similarity to DNA primases and it has been suggested (Rees & Wilkins, 1990) that it is cotransferred with the plasmid DNA into the recipient to convert the single-stranded DNA to double-stranded DNA by lagging strand DNA synthesis. TraG, the product of ORF8, shows similarity to the entry exclusion protein from R388 (Llosa *et al*., 1994), which specifically prevents transfer to recipient bacteria that already carry the plasmid (Fernandez-Lopez *et al*., 2005).

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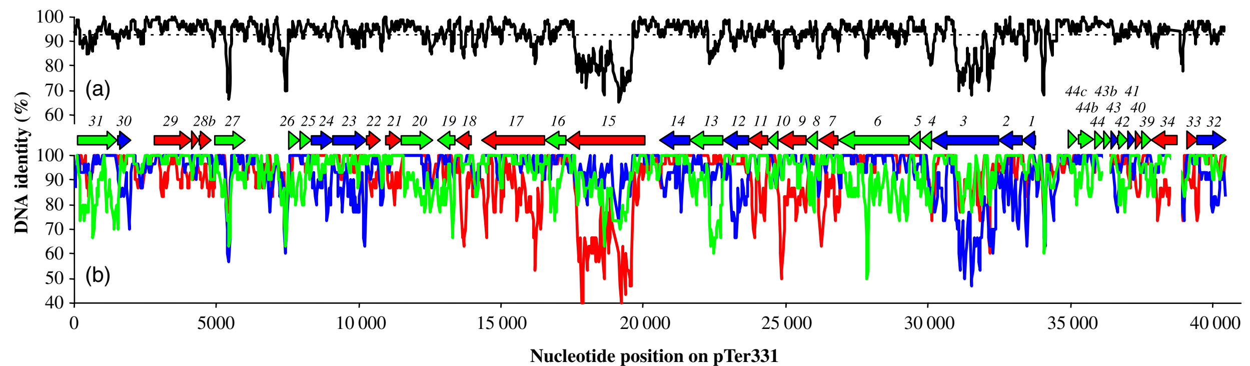

Fig. 4. Percentages of DNA identity along the aligned DNA sequences of plasmids pTer331 and pIPO2. (a) For this graph, pTer331 and pIPO2 were aligned, and with 10-bp steps the percentage of identical nucleotides in every 100-bp window of shared DNA sequence was plotted as a function of the position in the pTer331 sequence. (b) A similar analysis was done on three new alignments obtained after splitting the alignment of (a) three-ways as follows: frame 1: nucleotides 1, 4, 7, 10, etc. (red line in the graph), frame 2: nucleotides 2, 5, 8, 11, etc. (blue line), and frame 3: nucleotides 3, 6, 9, 12, etc. (green line). Also indicated are the position and orientation of each of the ORFs we identified on pTer331. The color of each ORF corresponds to its wobble frame.

Comparison of pTer331 and pIPO2 at the DNA level: evidence for past evolutionary events

A CLUSTALW alignment of the 40 457-bp pTer331 and 39 815-bp pIPO2 sequences revealed that the two plasmids share 36 411 identical nucleotides, corresponding to 90.0% and 91.5% identity, respectively. We identified a total of 2869 substitutions. In total, 1177 nucleotide positions on pTer331 were not found on pIPO2, and 535 nucleotides on pIPO2 were absent from pTer331. This assortment of substitutions and indels offered several lines of indirect evidence for the evolutionary forces that shaped pTer331 and pIPO2 since the presumed split from their common ancestor.

First, a comparison of nucleotide identity along the aligned plasmid sequences (Fig. 4a) revealed that the similarity between pTer331 and pIPO2 deviated significantly in some regions from the average along the aligned sequences. In several parts of the DNA alignment, identity dropped to below 70%, e.g. in the central part of ORF27, upstream of ORF26, and upstream of ORF1. The two most prominent stretches with reduced identity were within the genes *virD4* (*traN*, ORF15) and *traB* (ORF3). At the amino acid level, the products of these genes showed a low degree of identity with their pIPO2 counterparts (77% and 87%, respectively). In comparison, most other shared proteins feature identity scores of 90% or higher. One hypothesis is that VirD4 and TraB interact with each other directly and that the introduction of mutations in one of the proteins selected for compensatory mutations in the other and vice versa. As a coupling protein, VirD4 is responsible for recruiting the relaxosome and presenting it to the membrane-located proteins involved in mating pair formation (Christie *et al.*, 2005). The *traB* gene product is annotated as a DNA topoisomerase I with as-yet undetermined function in pTer331/pIPO2 biology, but it might well be part of the relaxosome and involved in nicking and/or relaxation of the plasmid DNA. Direct interaction between VirD4-like proteins and enzymes with relaxase/topoisomerase activity has been demonstrated for other plasmid systems (Szpirer *et al.*, 2000).

We also observed that substitutions in the pTer331/pIPO2 alignment were not randomly distributed between each of the reading frames (Fig. 4b). Instead, substitutions occurred with greater frequency in one particular frame over stretches that clearly colocalized with ORFs (Fig. 4b). In all cases, this frame corresponded to the wobble-frame of the corresponding ORF, i.e. the sequence containing every third nucleotide of the ORF. This suggests that there has been selection for functional conservation of all ORFs shared between both plasmids. Interestingly, this applies also to ORFs for which no clear function could be assigned, including the ORF44c-39 cluster of hypothetical genes. Wobble-frame analysis of two genes from this cluster, ORFs 44c and 44b, favored their annotation over an ORF45-like gene present on the reverse-complement strand, as has been done for pIPO2 (Tauch *et al.*, 2002) and pSB102 (Schneiker *et al.*, 2001). Further support for the authenticity of ORF44b comes from the high degree of identity (up to 72%) of its gene product to hypothetical conserved proteins encoded on other plasmids, including pAgK84 from *Agrobacterium* K84 (Kim *et al.*, 2006), pLPP from *Legionella pneumophila* str. Paris (Cazalet *et al.*, 2004), R721 from *E. coli* K-12 (Kim & Komano, 1992), and pEL60 from *Erwinia amylovora* LebB66 (Foster *et al.*, 2004). Interestingly, ORFs 44c through 39 all have the same orientation and as a cluster are preceded by a region upstream of ORF44c that overlaps with the large DR DR3 (Fig. 2) which is duplicated upstream of the putative operon consisting of ORFs 29 through 28b. The significance of this finding and whether ORFs 44c-39 and ORFs 29-28b

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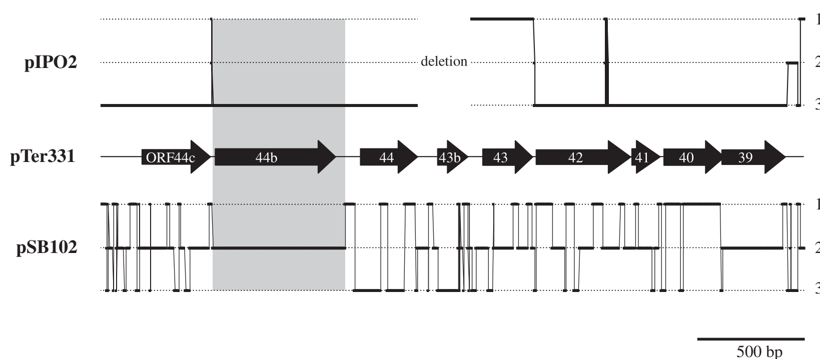

Fig. 5. Alignment of the ORF44c-39 clusters from pTer331, pIPO2, and pSB102. Shown are the locations of ORFs 44c, 44b, 44, 43b, 43, 42, 41, 40, and 39 on pTer331 in relation to 1-bp gaps and resultant frame shifts in the alignment to pIPO2 (top) and pSB102 (bottom). For pIPO2 and pSB102, each vertical line represents a shift from one frame (arbitrarily numbered 1, 2, or 3) to another. The largest section devoid of frame shifts in both pIPO2 and pSB102 is indicated by a grey box. The region encompassing ORF43b is absent from pIPO2 and has been marked as 'deletion'.

(and possibly ORF27) share an identical promoter and thus transcriptional profile remains to be elucidated.

A three-way comparison of pTer331 and pIPO2 to pSB102 revealed numerous frame shifts in the ORF44c-39 region of the latter (Fig. 5). This dictates that the coding potential of pSB102 in this region cannot be the same as that of pTer331 and pIPO2. By comparison, frame shifts in the pIPO2 sequence were much less frequent and furthermore restricted to three intergenic regions and an out-of-frame/back-in-frame shift in ORF42 (Fig. 5). It may be that the ORF44c-39 region on pSB102 is no longer functional and has started to rapidly diverge from the pTer331/pIPO2 sequences. Still, several regions in the pTer331/pSB102 alignment could be identified that are devoid of frame shifts (Fig. 5). The largest one of these covered exactly the length of ORF44b, suggesting that at least this gene escaped degeneration and was conserved between pTer331, pIPO2, and pSB102. This targeted conservation suggests an important, as-yet unknown role for this ORF.

The alignment of pTer331 and pIPO2 revealed 51 gaps of varying length. Eighteen of these gaps were located in ORFs (i.e. one in each of the ORFs 21, 23, 29, 42, and in *traA* and *traL*, two in ORFs 27 and 32, and eight in *traN*). Of these gaps, sixteen had a length that was divisible by three, which would add/delete amino acids from the encoded protein, but prevent a shift in the ORF. The other two gaps both occurred in ORF27 as 1-bp indels, one representing an out-of-frame shift and the other a back-in-frame shift. These results again suggest a positive selection for conservation of gene function during divergence from the pTer331/pIPO2 ancestor.

A third insight into the divergent evolution of plasmids pTer331 and pIPO2 comes from comparison of long and short DRs. The three pairs of long DRs on pTer331, i.e. a 649-bp perfect repeat (DR3 copies A and B), a 367-bp perfect repeat (DR2 copies A and B) and a 93-bp imperfect repeat with one mismatch (DR1 copies A and B) correspond to DRs DR3 (403 bp), DR2 (386 bp), and DR1 (96 bp), respectively, on plasmid pIPO2. Alignment of the DR3 sequences showed three distinct gaps on pIPO2 compared with pTer331 (Fig. 6a). Closer examination revealed the presence of short DRs (7, 10, and 8 bp) flanking these gaps on the corresponding DNA of pTer331 (Fig. 6b). In a plasmid ancestral to pIPO2, such repeats may have facilitated a 62-bp deletion from DR3 copy A and deletion of 60 and 79 bp from DR3 copy B. We found at least five other gaps in the pTer331/pIPO2 alignment which were flanked by such short DRs. In one case (Fig. 6b, example 4), the gap consisted of a 9-bp string that occurred immediately duplicated in *traA* on pTer331 and only once on pIPO2. A possible mechanism for deletion of DNA fragments that are flanked by short DRs is 'replication slippage' (Lovett, 2004), which involves mispairing between a replicating strand and its template at sites of repetitive DNA sequences.

We counted seven mismatches in the 403-bp overlap between the DR3 sequences of pTer331 and pIPO2 (Fig. 6a), and 10 mismatches in the 367-bp overlap between the DR2 sequences (not shown). We conclude for both DR3 and DR2 that (1) on either plasmid, copies A and B are identical, and (2) copy A on pTer331 differs from copy A on pIPO2 in exactly the same way as does copy B on pTer331 from copy B on pIPO2. This seems to suggest a mechanism that keeps the two copies of these long DRs on the same plasmid identical. The mechanism or function underlying this conservation remains to be elucidated. We note that the region of pIPO2 corresponding to subfragment *c* from pTer331 (Fig. 6a) is no longer considered part of DR3 on pIPO2 (Tauch *et al.*, 2002) and has diverged differently upstream of copy A compared with copy B (Fig. 6a). This would suggest that contiguity is a prerequisite for conservation.

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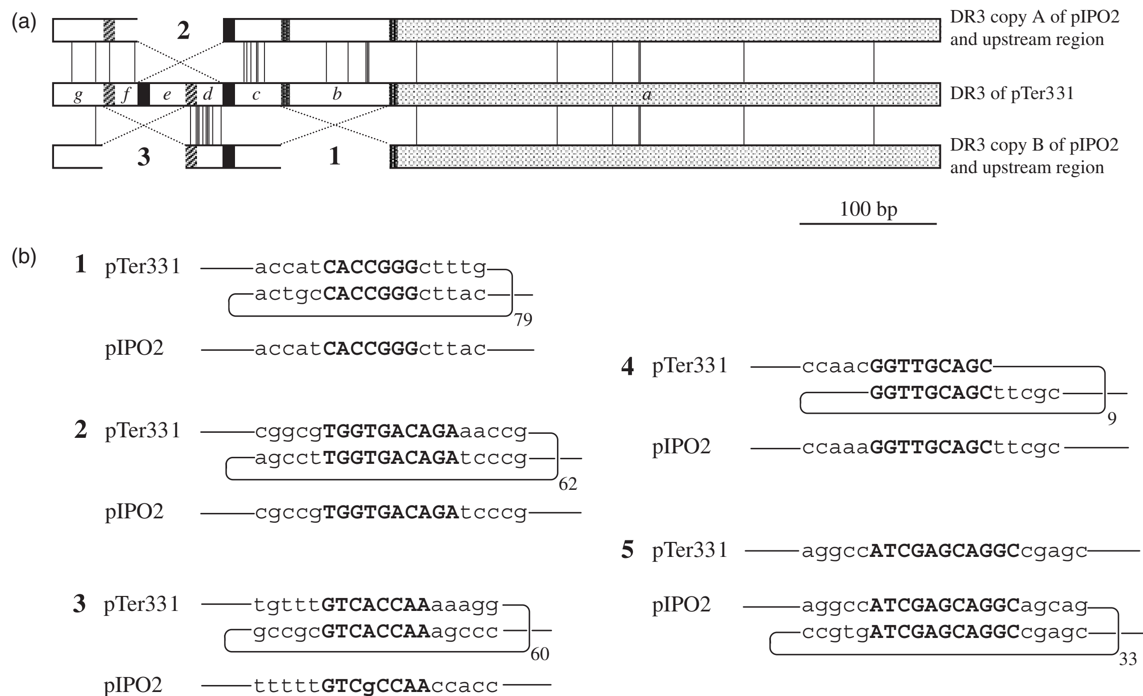

Fig. 6. Indels in the pTer331/pIPO2 alignment. (a) Shown is a schematic comparison of the DR3 long repeat of pTer331 with DR3 copies A and B of pIPO2 and their upstream regions. The DR3 region of pIPO2 is shaded and corresponds to the region on pTer331 labeled as fragment *a*. Short DRs in DR3 of pTer331 are indicated by boxes with similar shading and were transposed onto the DNA upstream of DR3 copy A and B of pIPO2 to reveal the absence of corresponding fragments *d* and *e* from the DNA upstream of DR3 copy A and of fragments *b*, *e*, and *f* from the DNA upstream of DR3 copy B. Indicated by vertical lines are single nucleotide differences between DR3 of pTer331 and copy A or B or their upstream regions on pIPOs. (b) Detail of short DRs flanking DNA fragments that are unique to pTer331 or pIPO2 and comparison with the corresponding region on pIPO2 or pTer331, respectively. Examples 1, 2 and 3 correspond to fragments/gaps *b*, *d-e* and *e-f* in (a), respectively. Examples 4 and 5 are discussed in the text. Repeats are shown in bold, together with five nucleotides upstream and 5 downstream of the repeat. The number in each example represents the length of the DNA fragment (in bp) that is unique to the respective plasmid (so for example 1, the alignment of pTer331 and pIPO2 features a 79-bp gap in the pIPO2 sequence).

Hypotheses on the ecological role of plasmid pTer331

We exploited the fact that pTer331 is available in its native host to test the contribution of the plasmid to some of the phenotypes that characterize *C. fungivorans* Ter331. One of these is the ability to efficiently colonize the root system of tomato (Kamilova *et al.*, 2007). We compared wild-type *C. fungivorans* Ter331 to an otherwise isogenic but plasmid-cured derivative, Ter331PC, in competition experiments with the established root colonizer *Pseudomonas fluorescens* PCL1285 (Lugtenberg *et al.*, 2001). In this indirect comparison, both *C. fungivorans* Ter331 and *C. fungivorans* Ter331PC competed equally well, colonizing root tips at log10 densities of 5.12 ± 0.2 $[(\mathrm{CFU}+1)]\mathrm{cm}^{-1}$ and 5.07 ± 0.4 $[(\mathrm{CFU}+1)]\mathrm{cm}^{-1}$, respectively. From this, we conclude that at least under the artificial conditions tested, pTer311 does not contribute significantly to the rhizosphere competence of its host. We also rule out an involvement of plasmid pTer331 in chitinolysis (de Boer *et al.*, 1998), fungistasis (de Boer *et al.*, 1998), mycophagy (de Boer *et al.*, 2004), and mineral weathering (Uroz *et al.*, 2007), given that 44 *Collimonas* strains in our collection with one or more of these phenotypes scored negative with a pTer331-specific PCR assay.

This leaves us to conclude that pTer331, like pIPO2, is a cryptic plasmid, as defined by our inability to assign, either based on analysis of gene content or on experimental evidence, an obvious advantage of the plasmid to its host *C. fungivorans* Ter331. Most, if not all, genes on pTer331 seem to be dedicated to the plasmid's spread and survival. Even the ORF44c-39 cluster, which has been suggestively linked to the plant-associated occurrence of plasmids pIPO2 (Tauch *et al.*, 2002) and pSB102 (Schneiker *et al.*, 2001), contains at least one gene (ORF44b) which is conserved in plasmids other than pIPO2 and pSB102, and which thus might not contribute to functions other than those related to plasmid biology.

Thus, plasmids like pTer331 (and pIPO2) can be considered canonical genetic parasites, with highly developed replication, maintenance and self-transfer systems to ensure

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their persistence in different bacterial hosts and natural environments. However, it has also been suggested (Bergstrom *et al.*, 2000) that cryptic plasmids eventually go extinct unless they acquire genes that benefit their host. Perhaps plasmids pTer331 and pIPO2 once, like pSB102 now, acquired useful genes, but recently lost them. This 'nonequilibrium' interpretation would predict that the ability of a plasmid to 'attract' conditionally useful genes would enhance survival of the plasmid. In this context, we note that plasmids pSB102, pIPO2, and pMOL98 apparently feature a putative, not previously recognized, hotspot for transposon insertions. Plasmids pIPO2 T and pMOL98 are mini-Tn*5*::luxABtet- and mini-Tn*5*-Km1-tagged derivatives of pIPO2 and pES1, respectively. In both cases, like in pSB102, the transposon occurs inserted upstream of the gene that corresponds to ORF33 on pTer331, i.e. ORF68 on pSB102, ORF33 on pIPO2, and *orf1* on pMOL98. It will be of interest to determine experimentally whether these hotspots are genuine and act as 'magnets' for the insertion of transposons and the genes associated with them. If this hypothesis would be confirmed, the pIPO2/pSB102 family would have a parallel in the IncP-1 family where it was recently shown that the presence of two hotspots for transposon insertion in combination with selection accounts for the common architectural feature of the family (Sota *et al.*, 2007). But even without accessory genes, plasmids such as pTer331 and pIPO2 may confer a clear benefit to bacteria. Through their (retro)mobilizing activity, they are potential catalysts of the dissemination of the mobile gene pool ('mobilome') within a bacterial community, effectively increasing its adaptability to a changing environmental condition. The rhizosphere, from which plasmids pTer331, pIPO2 and pSB102 were isolated, represents a microbial habitat that has been recognized as a natural hotspot for plasmid transfer (van Elsas *et al.*, 2000, 2003; van Elsas & Bailey, 2002; Espinosa-Urgel, 2004). It is unclear whether (retro)mobilizing, but otherwise cryptic, plasmids play a role in the acceleration of intracommunal gene transfer and of adaptation at the population level, but the pIPO2/pSB102 family presents an obvious and attractive model system to test this.

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