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Genetic characterization of the conjugative DNA processing system of enterococcal plasmid pCF10

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Abstract

Conjugation is a major contributor to lateral gene transfer in bacteria, and pheromone-inducible conjugation systems in *Enterococcus faecalis* play an important role in the dissemination of antibiotic resistance and virulence in enterococci and related bacteria. We have genetically dissected the determinants of DNA processing of the enterococcal conjugative plasmid pCF10. Insertional inactivation of a predicted relaxase gene *pcfG*, via insertion of a splicing-deficient group II intron, severely reduced pCF10 transfer. Restoration of intron splicing ability by genetic complementation restored conjugation. The pCF10 origin of transfer (*oriT*) was localized to a 40-nucleotide sequence within a non-coding region with sequence similarity to origins of transfer of several other plasmids in gram positive bacteria. Deletion of the *oriT* reduced pCF10 transfer by more than five orders of magnitude without affecting pCF10-dependent mobilization of co-resident *oriT*-containing plasmids. Although the host range for pCF10 replication is limited to enterococci, we found that the pCF10 conjugation system promotes mobilization of *oriT*-containing plasmids to multiple bacterial genera. Therefore, this transfer system may have applications for gene delivery to a variety of poorly-transformed bacteria.

Keywords

Pheromone; Mobilization; Relaxase; Origin of transfer; Intron

1. Introduction

Since its identification in 1981 from an *Enterococcus faecalis* isolate from New York Hospital (Dunny et al., 1981), the 67.7 kb pCF10 plasmid has been a useful model for the study of conjugation in gram positive bacteria (Hirt et al., 2005). pCF10 belongs to a family of pheromone-inducible plasmids that include pAD1, pAM373, and pPD1 and which so far appears to be restricted to the genus *Enterococcus* (Clewett and Dunny, 2002). Members of this family are of clinical interest because of the virulence (Hirt et al., 2002) and antibiotic resistance determinants (Kak and Chow, 2002) they carry. In addition to promoting the spread of tetracycline resistance and virulence in *E. faecalis*, a pCF10-like plasmid was directly involved in conjugative mobilization of vancomycin resistance in *E. faecium* in a hospital environment (Heaton et al., 1996). *E. faecalis* strain V583, a multi-resistant strain whose complete genome sequence has been determined, contains a plasmid called pTEF2, with a high degree of sequence identity to pCF10 (Paulsen et al., 2003); no experimental analysis of pTEF2 conjugation has been reported. Although the pheromone-responsive regulation systems controlling expression of conjugation functions have been studied extensively, much less is

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known about the molecular mechanisms by which these plasmids are transferred from donor cells to recipients.

Based on our previously reported comparative sequence analysis (Hirt et al., 2005), the pCF10 and pTEF2 proteins involved in replication, pheromone sensing, and aggregation have significant identity to homologs encoded by other pheromone plasmids in *E. faecalis*, while the putative mating apparatus and conjugative DNA processing genes of pCF10 and pTEF2 have more similarity to genes encoded by conjugative elements of *Streptococcus agalactiae* and *Lactococcus lactis*. The *pcfG* gene of pCF10 (Fig. 1), predicted to encode a relaxase protein, is most closely related (at both the DNA and protein sequence levels) to the *ItrB* gene of the lactococcal conjugative element pRS01 (Mills et al., 1994). An interesting feature of *ItrB* is that the wild-type allele is interrupted by the self-splicing, mobile group II intron L1.ItrB, the first fully functional group II intron identified in bacteria (Mills et al., 1996). We recently showed that L1.ItrB efficiently targets DNA of a conserved relaxase domain in *pcfG* for insertion by a retro-transposition mechanism (Staddon et al., 2004). L1.ItrB splices out of *pcfG* mRNA, such that the host bacterium retains conjugation proficiency. In the present study, we inserted a splicing-deficient derivative of L1.ItrB into *pcfG*, which severely reduced conjugation proficiency. This defect was restored by introduction of a helper plasmid providing a protein that restores splicing of the mutant intron. We also localized the pCF10 *oriT* to a 40-nucleotide sequence between *pcfE* and *pcfF* through the use of deletion experiments and mobilization assays. Interestingly pCF10 mobilized plasmids containing the pCF10 *oriT* in matings with *L. lactis* and *S. agalactiae*, even though pCF10 is unable to be stably maintained in non-enterococcal host strains.

2. Materials and methods

2.1. General molecular techniques

Plasmid isolations were done using the Qiagen miniprep kits (Valencia, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI) and used according to the manufacturers' instructions. Oligonucleotides and primers were purchased from Integrated DNA Technologies (Coralville, Iowa). PCR was done using a Perkin-Elmer GeneAmp PCR System 2400 (Wellesley, MA), an Eppendorf Mastercycler (Hamburg, Germany), or a TechneTC-412 thermocycler (Burlington, New Jersey), generally with Bio-X-Act polymerase (Bioline, Randolph, MA). Buffer formulations were based on those of the PCR Optimization Kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA sequences were determined by automated dideoxynucleotide sequencing (Advanced Genetic Analysis Center and Microchemical Facility, University of Minnesota).

2.2. Bacterial strains and growth conditions

Escherichia coli DH5 α (Gibco-BRL) and *E. coli* EC1000, a strain that expresses the pWV01 RepA protein (Leenhouts et al., 1996), were used in cloning. All intron insertion experiments were conducted in *E. faecalis*. Conjugation assays of pCF10 and pCF10 derivatives utilized *E. faecalis* strains as donors and *L. lactis* LM2301RF, *S. agalactiae* COH31r, and several *E. faecalis* strains (see below) as recipients.

Enterococcus faecalis UV202 is resistant to rifampicin and fusidic acid and is recombination deficient (Yagi and Clewell, 1980). Four plasmid-free *E. faecalis* strains derived from OG1 were also used in this study. OG1ES is resistant to erythromycin and streptomycin (Staddon et al., 2004). Because of the high degree of natural resistance of other OG1 strains to streptomycin and their exquisite sensitivity to erythromycin, erythromycin alone was typically used to select for OG1ES in mating assays. OG1SSp is resistant to streptomycin and spectinomycin (Dunny et al., 1978). OG1RF is resistant to rifampicin and fusidic acid (Dunny

et al., 1991). CK104 is an OG1RF derivative with a deletion of *upp*, the gene that encodes uracil phosphoribosyltransferase (UPRT) (Kristich et al., 2005). UPRT is required for the metabolism of 5-fluoro-uracil (5-FU) to its toxic metabolite, 5-fluoro-deoxyuridylate. The deletion of *upp* from the chromosome allows for 5-FU counterselection of plasmids that express UPRT. *L. lactis* LM2301RF (obtained from L. McKay, Univ. of Minnesota) is resistant to rifampicin and to fusidic acid. *S. agalactiae* COH31r (obtained from C. Rubens, Children's Hospital, Seattle, WA) is resistant to rifampicin.

Enterococcus faecalis and *S. agalactiae* strains were grown in Todd-Hewitt broth (THB). *L. lactis* was grown in M17 supplemented with 0.5% glucose. *E. coli* was grown in Luria broth (LB) or in Brain Heart Infusion broth (BHI).

Antibiotics were used at the following concentrations, except when otherwise noted. For *E. faecalis*: erythromycin, 10 (μg/ml for the plasmid marker and 100 (μg/ml for the chromosomal marker; fusidic acid, 25 (μg/ml; kanamycin, 500–1000 (μg/ml; rifampicin, 200(μg/ml; spectinomycin, 500–1000 μg/ml for plasmid and 150–250 μg/ml for chromosomal marker; streptomycin, 767(μg/ml; tetracycline, 10(μg/ml. For *L. lactis*: rifampicin, 100(μg/ml; fusidic acid, 25 (μg/ml; spectinomycin, 300 (μg/ml. For *S. agalactiae*: rifampicin, 200(μg/ml; spectinomycin, 1000(μg/ml. For *E. coli*: erythromycin, 300 (μg/ml in LB, 75 (μg/ml in BHI; spectinomycin, 30–50 ng/ml. *L. lactis* was grown at 30 °C. All other strains were grown at 37 °C. *E. coli* strains were generally grown with aeration.

2.3. Plasmids used in this study

2.3.1. Self-transmissible plasmids—Two conjugative plasmids that encode tetracycline resistance, pCF10 (Dunny et al., 1981) and its derivative, pCF11 (Dunny et al., 1982), were used in this study. pCF11 has a constitutively elevated transfer phenotype, probably due to a point mutation in the pheromone-responsive regulatory region (Bae et al., 2004).

2.3.2. Plasmids used in *oriT* mobilization studies—Plasmids used in *oriT* mobilization assays were derivatives of pDL278 and pDL281, both of which encode spectinomycin resistance. Plasmid pDL281 is identical to the pDL278 shuttle plasmid (Dunny et al., 1991) except for the deletion of part of the pDL278 multicloning site between the *HindIII* and *SphI* sites (Zhou et al., 2000). Plasmid pMSP2281 was constructed by PCR amplifying the 3' portion of *pcfE* and much of the *pcfE*–*pcfF* intergenic region (pCF10 nucleotides 32638–32896) subcloned into the pGEMT-Easy vector (Promega) and then cloned into the *AhdI* site of pDL281. Primers used to amplify the pCF10 sequence were based on the sequence of pTEF2 (Paulsen et al., 2003), resulting in a guanosine instead of an adenosine at position 12 of the insert (pCF10 nucleotide 32,649). Other inserts were obtained by PCR of pCF10 (pORIT_1, nucleotides 32,638–32,896; pORIT_5, nucleotides 32,776–32,896), or by annealing single-stranded oligonucleotides (pORIT_14, nucleotides 32,820–32,840; pORIT_15, nucleotides 32,819–32,861; pORIT_16, nucleotides 32,819–32,858; pORIT_17, nucleotides 32,840–32,861; pORIT_18, nucleotides 32,819–32,849). These inserts were cloned into the multicloning sites of pDL278 or the pDL278 derivative, pORIT_5. DH5α was generally used as an intermediate host during cloning, although in some cases newly-constructed plasmids were directly electroporated into *E. faecalis* OG1RF. Insert-vector junctions with intervening inserts were sequenced and overall plasmid integrity tested by restriction enzyme digest. Restriction enzyme digests of pORIT_16 were consistent with the deletion of approximately 400 nucleotide in the vector sequence counterclockwise to the multicloning site. This region includes the T1T2 terminator region, which is composed of two tandem copies (pDL278 nucleotides 5063–5561, 5561–6059) of the 3' portion of the *rrnB* ribosomal RNA operon of *E. coli*. This deletion did not affect the pCF10 insert sequence, nor did it have any discernable effect on pORIT_16 replication.

2.3.3. pCF10 Δ oriT—Fifty-four nucleotides in the *pcfE*–*pcfF* intergenic region (pCF10 nucleotides 32,812–32,866) were deleted and replaced with a *Pst*I site (CTGCAG), using an improved allelic exchange system recently described in detail (Kristich et al., 2005). DNA from upstream and downstream of the region to be deleted was PCR amplified and cloned into pCJK2, a pORI280 (Leenhouts et al., 1996) derivative. Primers used to amplify the upstream fragment incorporated *Xma*I and *Pst*I restriction enzyme sites while primers used to amplify the downstream fragment incorporated *Pst*I and *Xba*I sites, simplifying stepwise cloning into pCJK2. The resulting plasmid, pCJK2:: Δ oriT, was electroporated into EC1000, followed by plasmid isolation, confirmation of the DNA sequence by dideoxynucleotide sequencing, and electroporation into *E. faecalis* CK104/(pCF10, pVE6007). Transformants (CK104/(pCF10, pVE6007, pCJK2:: Δ oriT)) were selected at 30°C in 200 μ l THB supplemented with 0.5M sucrose and erythromycin (induction at 50ng/ml for 1.5h; selection at 5 μ g/ml for 1.5h). A shift in temperature to 37°C for 4h (abolishing pCJK2:: Δ oriT replication) in the continued presence of erythromycin selected for integration of pCJK2:: Δ oriT into pCF10 by single cross-over recombination. Transformants were plated out on THB plates supplemented with X-Gal (250 μ g/ml) and erythromycin (10 μ g/ml) and incubated overnight. A resulting blue colony was grown overnight in THB supplemented with erythromycin. The culture was then diluted by a factor of 10⁸–10⁹ and incubated at 37°C for 30 h in BHI supplemented with tetracycline in the absence of erythromycin to allow for excision of the integrated pCJK2:: Δ oriT from pCF10. The resulting culture was then plated out on BHI plates supplemented with tetracycline and 5-FU (130 μ g/ml). Bacteria from these plates were subsequently replated on BHI plates, supplemented with tetracycline, 5-FU (130 μ g/ml), and X-Gal (250 μ g/ml). White colonies were picked and plated on BHI plates that also contained tetracycline, 5-FU, and X-Gal. PCR was used to screen for the deletion of *oriT* in pCF10. The precise deletion, with replacement by the *Pst*I site, was confirmed by automated DNA sequencing of PCR product from the CK104/(pCF10 Δ oriT) strain used in subsequent experiments. Plasmid pORIT_15 was electroporated into both CK104/(pCF10) and CK104/(pCF10 Δ oriT) strains for use in the experiments presented in Table 2.

2.3.4. Plasmids used in *pcfG* functional analysis—Plasmid pCF10::L1.ltrB Δ ORF-Kan consists of a splicing-deficient L1.ltrB derivative inserted into the L1.ltrB target site in *pcfG*. This plasmid was constructed by introducing pLEIIt⁺KR⁺ Δ ORF (Staddon et al., 2004) and pCOM Δ A13.16 (Klein et al., 2004) into OG1RF/(pCF10) by electroporation. pLEIIt⁺KR⁺ Δ ORF encodes for the splicing-deficient L1.ltrB Δ ORF-Kan intron (Staddon et al., 2004). Plasmid pCOM Δ A13.16 confers production of LtrA for L1.ltrB Δ ORF-Kan splicing and insertion into *pcfG*. Following L1.ltrB Δ ORF-Kan insertion into *pcfG*, pCF10::L1.ltrB Δ ORF-Kan was conjugated to the plasmid-free OG1SSp strain. Insertion of L1.ltrB Δ ORF-Kan into pCF10 and the absence of pLEIIt⁺KR⁺ Δ ORF were confirmed by PCR. The sequence of the 3' intron–exon junction between L1.ltrB Δ ORF-Kan and pCF10 was confirmed by automated sequencing (Advanced Genetic Analysis Center, University of Minnesota). Plasmid pMSP3545ltrA contains a complete wild-type copy of *ltrA* cloned under the control of the nisin promoter of pMSP3545 (Bryan et al., 2000) and was used to complement splicing of L1.ltrB Δ ORF-Kan expressed from pCF10::L1.ltrB Δ ORF-Kan.

2.4. Electroporation

Electroporation of plasmids into *E. coli* and *E. faecalis* utilized the Bio-Rad Gene Pulser, Philadelphia, Pennsylvania (1.7kV, 200 Ω , and 25 μ F; 1 mm diameter cuvettes) as previously described (Staddon et al., 2004).

2.5. Conjugation and mobilization assays

Cultures of donor and plasmid-free recipient *E. faecalis* strains were grown overnight in THB + antibiotics. Donor and recipient cultures were washed in THB to remove antibiotics before

being mixed for matings. Overnight cultures were generally diluted 1:9 or 1:10 in fresh THB and pre-incubated to ensure that cultures were in exponential phase during the matings. In experiments involving pCF10, donor cells were induced with cCF10 (20–50 ng/ml). Pre-incubation of the slower-growing UV202/(pCF11) strain was generally not done, nor was cCF10 induction performed with pCF11. Donor and recipient bacteria were combined in THB with an approximate 1:10 donor to recipient ratio and incubated at 37°C. Quantitation of colony forming units (CFU) on THB agar with relevant selective antibiotics followed. Transconjugant to donor ratios were calculated by dividing the number of CFU/ml resistant to both a recipient chromosomal marker and a plasmid-mediated marker by the number of CFU resistant to donor antibiotic markers. Since the transfer frequency of pCF10 can vary more the 10-fold in replicate experiments carried out under identical conditions, we normalized the frequency of mobilization of *oriT*-containing non-conjugative plasmids to that of pCF10 transfer from the same donor strain in the mobilization experiments reported here. Microsoft Excel was used to calculate geometric means and standard deviations in genetic assays utilizing conjugation.

Conjugative transfer of pCF10::Ll.ltrBΔORF-Kan was tested in the presence and absence of pMSP3545ltrA, which was introduced by electroporation into the donor strain. pMSP3545ltrA was induced with nisin, 25 ng/ml. Induction of the donor strains with cCF10 (±nisin) was done for 3 h prior to a 30-min broth mating with recipients and the transfer levels of pCF10::Ll.ltrBΔORF-Kan compared to the transfer levels of wild-type pCF10. All pCF10::Ll.ltrBΔORF and pCF10 mating assays utilized OG1SSp donor hosts and OG1RF recipients.

Plasmid pMSP2281 (and the vector-only pDL281 control) were tested for their ability to be mobilized in 1–2h matings by pCF11 in matings between UV202 donor strains and OG1ES. Donor to recipient CFU ratios in these assays ranged from 1:17 to 1:241. Two independent experiments were done for each mating. Plasmids in the pORIT series and their pDL278 control were tested for their ability to be mobilized by pCF10 in matings between OG1RF donor strains and OG1ES. In these experiments, donor and recipient overnight cultures were diluted 1:9 or 1:10 in fresh medium and incubated for 3–3.5 h at 37°C. Donor strains were supplemented with cCF10 (50 ng/ml). Cultures were then mixed at a 1:9 or 1:10 donor to recipient ratio by volume and incubated for 30 min at 37°C. Donor to recipient CFU ratios ranged between 1:10 and 1:38. To control for possible variations in pCF10 induction levels, mobilization levels were normalized by dividing the transconjugant to donor ratio of the mobilized plasmid by the transconjugant to donor ratio of pCF10 in the same experiment. Three independent mobilization assays were done with each member of the pORIT series and with pDL278.

Surface matings of pORIT_16 from *E. faecalis* OG1ES/(pCF10, pORIT_16) to *E. faecalis* OG1RF, *L. lactis* LM2301RF, and *S. agalactiae* COH31r were done as follows. One milliliter aliquots of overnight cultures was washed thoroughly to remove antibiotics, diluted 1:9 with fresh media, and incubated for 3h. OG1ES/(pCF10, pORIT_16) was supplemented with cCF10 (50 ng/ml) during the 3-h incubation. Next, 20 µl donor culture and 200 µl recipient culture were mixed on antibiotic free plates and incubated overnight at 37°C for 20 h. Plates were washed with potassium phosphate-buffered saline (KPBS), followed by dilutions in KPBS and enumeration of CFUs on the selective plates. GM17 (M17 medium (Becton Dickinson) + 0.5% glucose) plates were used in matings that involved *L. lactis* and when selecting for *L. lactis*. THB agar plates were used with all other species. Two independent experiments of each mating pair were done. Enumerations were done by making parallel triplicate serial dilutions of each culture and plating three replicate plates from the appropriate dilutions of each dilution series. Suspected transconjugant colonies were patched serially to eliminate contamination. Purified colonies were submitted to PCR analysis (using Bio-X-Act polymerase in Techne TC 412 thermocycler). Primers that anneal to the pUC multicloning site flanking the *oriT* in pORIT_16 (5' CCCAGTCACGACGTTGTAAACG 3', 5' GGA AACAGCTATGACCATGATTAC 3')

were used. Donor strain OG1ES/(pCF10, pORIT_16) and the recipient plasmid-free strains were used as positive and negative controls, respectively. Plasmid content of three transconjugants showing positive PCR reactions was confirmed by restriction enzyme digests of plasmid preparations from pure transconjugant cultures. Single vector-only plate matings from *E. faecalis* OG1ES/(pCF10, pDL278) to *L. lactis* LM2301RF and to *S. agalactiae* COH31r were done as negative controls.

2.6. Computer analyses

Protein and DNA searches and sequence alignments (Altschul et al., 1997) utilized online software at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). Multiple sequence alignments of DNA and protein utilized a Seqweb version of GCG (Wisconsin Package) and online alignment tools available at the Baylor College of Medicine website (<http://searchlauncher.bcm.tmc.edu/multi-align>), the Canadian Bioinformatics Resource website (<http://www.cbr.nrc.ca>), and/or the multialign sequence program by Florence Corpet (<http://prodes.toulouse.inra.fr/multalin/>) (Corpet, 1988). Searches for inverted repeats utilized the DNA Strider program (Douglas, 1995).

3. Results

3.1. *pcfG* is essential for conjugation

pcfG encodes a 561 amino acid protein with sequence similarity to relaxases (Staddon et al., 2004). We used a splicing-deficient version of the L1.ltrB group II intron, L1.ltrBΔORF, to insertionally inactivate *pcfG*. L1.ltrBΔORF is splicing-deficient due to the deletion of most the L1.ltrB intron-encoded protein gene *ltrA* (Cousineau et al., 1998). The resulting plasmid, pCF10::L1.ltrBΔORF, was reduced in conjugation efficiency by more than five orders of magnitude compared to wild-type pCF10 (Table 1). The effect on conjugation was specific to the insertion event since the presence in trans of plasmid pMSP3545ltrA, which encodes LtrA from a nisin-inducible promoter and allows the defective intron to splice, restored conjugative transfer (Table 1).

3.2. The *pcfE*-*pcfF* intergenic region contains a functional *oriT*

Origins of transfer are often located in intergenic regions upstream of their cognate relaxase genes (Francia et al., 2004). There is a 245 bp intergenic region between *pcfE* and *pcfF* (Fig. 1A). We cloned a 259-bp segment corresponding to pCF10 nucleotides 32,638-32,896 (Hirt et al., 2005) most of this region (as well as the 3' portion of *pcfE*) into shuttle vectors pDL278 and pDL281. The resulting plasmids, pORIT_1, and pMSP2281, were both mobilized at high frequency in broth matings between enterococcal strains (Fig. 1B). Mobilization was independent of homologous recombination, as demonstrated by the high-frequency transfer of pMSP2281 in experiments using the recombination-deficient UV202 as the donor strain (Fig. 1).

Deletion analysis demonstrated that the minimum functional *oriT* resides within the 40 bp sequence contained in pORIT_16 (Fig. 1B). A 20 bp sequence containing a perfect 10-nucleotide inverted repeat is necessary but not sufficient for mobilization, as demonstrated by the inability of pCF10 to mobilize pORIT_17 and pORIT_14. Additional nucleotides to the right of the inverted repeat are also required for high frequency mobilization, as demonstrated by the low transfer frequency of pOR-IT_18 (Fig. 1B).

3.3. Deletion of the functional *oriT* abolishes pCF10 conjugation

The enterococcal pheromone plasmid pAD1 has two origins of transfer (Francia and Clewell, 2002). To determine whether the *pcfE*-*pcfF* intergenic region contains the sole functional

oriT in pCF10, a 54-bp region encompassing the entire minimum *oriT* described above (Fig. 1) was replaced by a *Pst*I restriction enzyme site (Section 2.). The resulting plasmid, pCF10Δ*oriT*, was reduced in conjugation frequency by more than five orders of magnitude compared with pCF10 (Table 2). This reduction was not due to polar effects on other genes since the deletion had no effect on pORIT_15 mobilization (Table 2). Therefore, the *pcfE*–*pcfF* intergenic region contains the sole functional *oriT* for the pCF10 pheromone-inducible conjugation system.

3.4. pCF10 mobilizes pOBIT plasmids to *Lactococcus lactis* and *Streptococcus agalactiae*

Plate mating assays were performed to determine whether pCF10 is capable of mobilizing *oriT*-containing plasmids to related bacterial species; we carried out plate matings because the pheromone-inducible aggregation system required for efficient mating in liquid cultures only functions with enterococcal recipient strains. *E. faecalis* OG1ES/(pCF10 pORIT_16) was mated with *L. lactis* LM2301RF and *S. agalactiae* COH31r. Mobilization frequencies to *L. lactis* recipients were about 1%–10% those of plate matings using *E. faecalis* recipients. *S. agalactiae* showed a recipient ability of about 1% relative to *L. lactis*. In all cases the *oriT*-containing plasmid was transferred at frequencies two to five orders of magnitude greater than that of the pDL278 vector control (Table 3). Mobilization was confirmed by colony PCR and restriction enzyme digests of *oriT*-containing plasmid DNA isolated from transconjugant bacteria (data not shown).

4. Discussion

We used a splicing-deficient version of LI.ltrB deleted for *ltrA* to create an insertion mutation in *pcfG* that was complemented by a co-resident plasmid that expresses LtrA. LI.ltrB insertion into DNA target sites depends upon a combination of LtrA-target site and intron RNA-target site interactions (Singh et al., 2002). EBS (exon binding site) and δ nucleotides within the intron base pair with IBS (intron binding site) and δ' nucleotides flanking the intron insertion site (Mohr et al., 2000). Since LI.ltrB targets a conserved DNA motif found in *pcfG* and several other relaxase genes (Staddon et al., 2004), we used an intron with the wild-type EBS and δ nucleotide sequence to inactivate *pcfG*. Splicing-deficient versions of LI.ltrB have been engineered by the modification of EBS and δ nucleotides to knock out specific chromosomal genes in *E. coli* (Perutka et al., 2004) and *L. lactis* (Frazier et al., 2003). Similar techniques could be applied for mutagenesis in other gram positive bacteria, perhaps using LI.ltrB derivatives cloned into pORIT plasmids for delivery by conjugation.

The sharp reduction in conjugation caused by LI.ltrBAORF insertion in *pcfG* and its restoration by expression of *ltrA* in trans indicates that *pcfG* plays a central role in conjugation. The PcfG sequence is related to relaxases of the IncP type, with the highest similarity to relaxases in gram positive bacteria, such as the pRS01 relaxase, ltrB (Staddon et al., 2004). The pCF10 *oriT* is 75% identical to a sequence found in the pRS01 *oriT* (Mills et al., 1998). There is also nucleotide identity with sequences of other mobile genetic elements, particularly in a highly conserved stretch of six nucleotides to the right of inverted repeats (Fig. 2). Interestingly, relaxase *nic* sites in pC221, pC223 (Caryl et al., 2004), and RP4 (a plasmid found in gram negative bacteria; Pansegrau et al., 1988) are located within homologs of this conserved sequence (Fig. 2). Perhaps functional constraints imposed by protein–DNA interactions to the right of the inverted repeat have selected for conservation of this sequence in pCF10. Additional nucleotide(s) to the right of the conserved GCTTGC sequence are clearly required for optimal pCF10 *oriT* function since pORIT_18, whose *oriT* insert includes but does not extend beyond the conserved sequence, was poorly mobilized by pCF10 (Fig. 1). In spite of the high degree of conservation between the pCF10 and pRS01 relaxases and *oriT* sequences, preliminary results from our laboratory (Y. Chen and G. Dunny, unpublished) indicate that the two *oriT*

regions do not function efficiently with the heterologous DNA processing proteins. Thus, comparative analysis of the conjugative DNA processing systems of pRS01 and pCF10 may yield interesting insights into the molecular basis for the specificity of relaxosome formation.

Mobilization of *oriT*-containing plasmids to *E. faecalis*, *L. lactis*, and *S. agalactiae* recipient strains suggests that the pCF10 *oriT* system may be generally useful as a gene delivery system. Plasmid pORIT_16 transfer to *L. lactis* LM2301RF corresponded to approximately 5×10^5 transconjugants per plate mating, 10–100× greater than the electrotransformation efficiency of 1 µg of purified pDL278 into *L. lactis* (Dunny et al., 1991). The improved efficiency that conjugation offers over electrotransformation is even more dramatic in *E. faecalis*, where pORIT_16 mobilization resulted in over 10^8 transconjugants per plate mating. This is three to four orders of magnitude greater than the electrotransformation efficiency in *E. faecalis* of another plasmid with the same replicon (pVA380-I) (Dunny et al., 1991). The improved efficiency of conjugation over electroporation will be particularly helpful in the delivery of suicide vectors for mutagenesis of gram positive bacteria. Allelic exchange in gram positive bacteria often requires a two-step process (Biswas et al., 1993; Leenhouts et al., 1996). The first step involves selection of plasmid transformants under conditions that permit replication of the plasmid. In the second step conditions, such as temperature, is changed so that the transformed plasmid can no longer autonomously replicate, allowing for the selection of single cross-over co-integration. Highly efficient conjugation systems may obviate the need for this laborious two-step process for allelic exchange, as well as facilitating the efficient delivery of transposons for random mutagenesis.

The mobilization of *oriT*-containing plasmids to *L. lactis* and *S. agalactiae* demonstrate that the pCF10 mating apparatus can support DNA transfer to these species. Interestingly, several putative mating pore and DNA processing proteins encoded by pCF10 have greater sequence similarity to proteins encoded in *L. lactis* and *S. agalactiae* than to their counterparts encoded by other enterococcal pheromone plasmids (Hirt et al., 2005). Our observation that pCF10 mobilizes *oriT* plasmids to other species is consistent with a role for cross-species gene transfer in the evolution of pCF10 and suggests that lateral gene transfer is responsible for the widespread distribution of *pcfG-like* relaxase genes in gram-positive bacteria (Staddon et al, 2004).

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Fig. 1.

(A) Linear map of the conjugative DNA processing region of pCF10. For a complete map of this plasmid, see (Hirt et al., 2005). (B) Determination of the minimum pCF10 *oriT*. Cloned segments of pCF10 DNA are represented by horizontal bars. The position of these segments relative to *pcfE* and *pcfF* is shown at the top; note the expanded scale of the lower half of this illustration. The horizontal bar representing the insert in pORIT_16 is magnified, with arrows indicating a perfect 10bp inverted repeat present within the sequence. The boundaries of progressively smaller inserts are shown relative to the insert in pORIT_16. pDL281 is the vector-only control for pMSP2281 while pDL278 is the vector-only control for all other constructs. Transconjugant per donor (T/D) ratios, normalized to pCF10 transfer in the same experiment, and transconjugant per recipient (T/R) ratios are given on the right. Geometric means $\pm$ standard deviations for two (pMSP2281 and pDL281) or three (pORIT series and pDL278) independent experiments are given on the right. *Mobilized by pCF10 from OG1RF to OG1ES. †Mobilized by pCF11 from UV202 to OG1ES. **When this region was deleted from pCF10, transfer was abolished.

Fig. 2.

Alignment of the minimal pCF10 *oriT* with other origins of transfer. The pCF10 *oriT* sequence corresponds to the insert in pORIT_16. Horizontal arrows indicate inverted repeats, vertical arrows (↓) show experimentally demonstrated nick sites. Nucleotide conservation with the pCF10 sequence is indicated in bold. The pRS01 sequence is part of a 446 bp functional *oriT* sequence (Mills et al., 1998), while the Tn5252 sequence is part of a 2-kb sequence that includes the relaxase substrate (Srinivas et al., 1997). Nick sites have been experimentally demonstrated in pC221, pC223, and RP4 (Pansegrau et al., 1988; Caryl et al., 2004). The single asterisk is explained in the legend to Fig. 1.

Table 1Effect of *pcfG* insertional mutagenesis on pCF10 transfer

Plasmid	T/D ratio
pCF10	4.0×10^{-3} ; 5.7×10^{-2}
pCF10::LLItrBAORF-Kan	$<4.6 \times 10^{-8}$; $<1.1 \times 10^{-7}$
pCF10::LLItrBAORF-Kan, pMSP3545ItrA (nisin induction)	1.1×10^{-3} ; 2.6×10^{-2}

Transconjugant per donor (T/D) ratios are given from two independent experiments.

Table 2Ability of pCF10ΔoriT to mobilize an *oriT*-containing plasmid

Donor genotype	Plasmid selected	T/D ratio
CK104(pCF10ΔoriT, pORIT_15)	pCF10ΔoriT	$<7.1 \times 10^{-8}$; $<6.1 \times 10^{-8}$
	pORIT_15	2.1×10^{-2} ; 6.2×10^{-2}
CK104(pCF10, pORIT_15)	pCF10	7.5×10^{-3} ; 9.7×10^{-2}
	pORIT_15	1.5×10^{-2} ; 2.2×10^{-2}

Transconjugant per donor (T/D) ratios of conjugation experiments, given as the result of each independent experiment. OG1ES was the recipient strain in all cases.

Table 3

pCF10-mediated mobilization in plate matings within and between species

Mobilized plasmid	Recipient	T/R ratio
pORIT_16	<i>L. lactis</i> LM2301RF	4.4×10^{-3} ; 2.2×10^{-4}
pDL278	<i>L. lactis</i> LM2301RF	4.9×10^{-9a}
pORIT_16	<i>S. agalactiae</i> COH3lr	3.8×10^{-6} ; 7.7×10^{-6}
pDL278	<i>S. agalactiae</i> COH3lr	9.6×10^{-9a}
pORIT_16	<i>E. faecalis</i> OG1RF	1.7×10^{-2} ; 8.7×10^{-3}

Donor strains were OG1ES/(pCF10, pORIT_16) or OG1ES/(pCF10, pDL278). Transconjugant per recipient (T/R) ratios are given for each individual experiment.

^aPlasmid content of these putative transconjugants not confirmed by PCR; they may represent background spontaneous antibiotic resistance mutations.