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Mapping of the nick site on conjugative plasmid pVT745 by interrupted mating

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Abstract

Conjugal transfer of plasmid DNA initiates and terminates at a specific non-coding site called the origin of transfer (*oriT*). Previous analysis of conjugative plasmid pVT745 from *Aggregatibacter actinomycetemcomitans* suggested that *oriT* was located adjacent to the operon responsible for initiation of ssDNA transfer. The location of *oriT* was confirmed by assaying both subclones of the region as well as a pVT745 deletion derivative for mobilization. The precise DNA nick site (*nic*) and polarity of DNA transfer were identified by use of interrupted mating assays, a technique originally used for the mapping of bacterial chromosomes. Nucleotide sequence analysis revealed that the pVT745-specific nick region was similar to the consensus nick sequence of the IncP family albeit the actual cleavage site differed. Functionality of *nic* was confirmed by point mutations.

Keywords

Aggregatibacter actinomycetemcomitans; conjugation; *oriT*; interrupted mating

1. Introduction

Comparative sequence analyses of oral bacterial genomes seem to suggest that horizontal gene transfer is rather common in oral bacteria [1]. However, not much is known about conjugative gene transfer in the oral cavity, particularly among Gram-negative bacteria. Replicons that are transferable by conjugation need to be identified to obtain information about their transfer dynamics. Previous work identified the presence of a conjugative plasmid, pVT745, in the periodontal pathogen *Aggregatibacter actinomycetemcomitans* [2,3]. The observation that remnants of the plasmid were detected in the chromosome of various clinical isolates of *A. actinomycetemcomitans* are suggestive of conjugative transfer among strains of this oral pathogen [4].

Transmission of conjugative and mobilizable plasmids is initiated within a *cis*-acting site, the origin of transfer (*oriT*) where a plasmid-encoded endonuclease (called relaxase or nickase) cleaves a single strand of DNA at a specific site [5]. This nick site, *nic*, is often flanked by inverted repeats and an AT-rich region [6]. Usually, the relaxase works in concert with one or

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more plasmid-encoded proteins. Nicking at *oriT* determines which strand of DNA will be transferred to the recipient cell. The relaxase remains covalently attached to the 5' end of the nicked strand while the strand is unwound and transferred unidirectionally. The termination of strand transfer is also *oriT* site-dependent and requires the enzymatic ligation of the linear strand into a circular form. Both initiation and termination of conjugal transfer, i.e. nicking and religation, are carried out by the relaxase [7,8].

Previous nucleotide sequence analysis suggested that the *magA* operon on pVT745 is involved in DNA strand processing with protein MagA2 showing significant homology to known relaxases, while MagA1 is believed to be an accessory protein supporting MagA2 activity [3]. Typically, *oriT* is located next to the DNA processing genes within conjugative systems. The intergenic region 5' of *magA1* exhibits features characteristic of *oriTs* such as a high % A +T content and the presence of inverted and directed repeat sequences [3]. The goal of the current study was to confirm the location of *oriT* and to determine the pVT745-specific nick site by functional and mutational analysis.

2. Material and Methods

2.1. Bacterial Strains and Plasmids

Strains and plasmids used in this study are listed in Table 1. *A. actinomycetemcomitans* strains were grown in TSBYE (3% trypticase soy broth, 0.6% yeast extract) at 37°C in 10% CO₂. A variant of VT745 without its resident plasmid, pVT745, was obtained as described previously [9]. *Escherichia coli* strain JM109 was grown in YT (0.8% tryptone, 0.5% yeast, 0.5% NaCl) medium [10]. Where appropriate, antimicrobial agents were added at the following concentrations: ampicillin (Ap), 100 µg/ml; erythromycin (Em), 20 µg/ml; kanamycin (Km), 100 µg/ml; rifampin (Rf), 100 µg/ml; spectinomycin (Sm), 100 µg/ml; and streptomycin (Sp), 50 µg/ml.

2.2. DNA Preparations, Recombinant DNA Techniques, DNA sequencing, and PCR

DNA manipulations were carried out in *Escherichia coli* strain JM109 [11]. Plasmid DNA was isolated from *A. actinomycetemcomitans* and *E. coli* as described previously [12]. Restriction endonucleases and T4 DNA ligase were used in accordance with the manufacturer's instructions (Invitrogen). Standard recombinant DNA techniques were performed as described in Sambrook et al. [13]. Transformation of *A. actinomycetemcomitans* by electroporation has been reported previously [14]. Standard three-step PCR experiments were performed with Taq polymerase from Promega (Madison, WI). DNA sequencing was carried out by GENEWIZ, Inc. (South Plainfield, NJ).

2.3. Mating Experiments

Surface mating experiments were performed as described previously [3]. Mobilization of recombinant clones pJC1, pJC2, and pJC3 was assessed between *A. actinomycetemcomitans* strains and *A. actinomycetemcomitans* and *E. coli* JM109 with mating times of 6 h and 4 h respectively. *A. actinomycetemcomitans* transconjugants were selected on plates containing Sp and Em. Subsequently, at least 100 colonies were transferred in parallel to plates containing Sp or Km to determine co-transfer of the pVT745-derivatives. *E. coli* transconjugants were selected on YT plates containing Sp and grown at atmospheric CO₂ levels. Transfer frequencies were expressed as the number of transconjugants per donor cell. At least 20 selected transconjugants were examined for the presence of plasmid DNA.

Interrupted mating assays were performed as follows. Donor and recipient strain were mixed together in broth at a ratio of 1:1, centrifuged, washed, and centrifuged a second time. Mating was allowed to occur in the pellet for 2, 3, 4, 5, 6, 8 and 10 minutes respectively. After the

times indicated the pellet was resuspended in 1 ml of medium, the suspension vortexed to separate mating pairs, and 100 μ l aliquots spread onto selective agar plates containing Km/Rf or Sm/Rf. Subsequently, colonies obtained were transferred in parallel to plates containing either Sm or Km to identify transconjugants that were susceptible to one of the two antibiotics.

2.4. Construction of pJC1, pJC2, and pJC3

A 2.8-kb *EcoRI* fragment spanning *magA* and its adjacent non-coding region was derived from pVT745 and cloned into the non-transmissible plasmid pDMG4 digested with the same enzyme. Plasmid pDMG4 carries a spectinomycin resistant determinant and replicates in both *E. coli* and *A. actinomycetemcomitans*. The resulting construct was designated pJC1. Subsequently, pJC1 DNA was digested with *HincII/EcoRI* and *HincII/SmaI* to retrieve fragments of 1.1-kb and 0.5-kb respectively. These fragments were cloned into pDMG4 digested with the corresponding enzymes resulting in clones pJC2 and pJC3. All three recombinant clones were then introduced into the *A. actinomycetemcomitans* donor strain ATCC29522Rif^{recA} to test for *oriT* functions.

2.5. Construction of pDMG37

The deletion mutant pDMG37, which is *oriT*⁻, was constructed via a double crossover event. In a previous study a 4.6-kb *BamHI/AccI* fragment from pVT745, which carries the *magA* operon and its adjacent intergenic region, had been cloned into pGB2 [3,15]. The *oriT* region was deleted from this clone by two PCR reactions using circular DNA as a template. First, a 192-bp non-coding region extending from nucleotide 2779 to 2971 (GeneBank accession no. AF302424) was removed by using outward-pointing primers BB1 and BB2 (Fig. 1, Table 2). Both primers contained a *ClaI* restriction site. The resulting linear PCR fragment was subsequently digested with *ClaI* and religated. In a second PCR reaction this recircularized construct was amplified with outward-pointing primers BB3 and BB4 both containing a *BamHI* site (Fig. 1, Table 2). The PCR product was then digested with *BamHI* and religated, resulting in the deletion of a ca 1.6-kb region 3' to the *magA* operon. This step ensured that the DNA sequences that flanked the mutated *oriT* site were of similar length to facilitate a double crossover event. Finally, a *kan* gene cassette was retrieved from pMK3 as a *ClaI* fragment and inserted into the unique *ClaI* site created at the deleted *oriT* site. This last construct was introduced into strain VT745, which carries the wild-type plasmid pVT745. Since pGB2 does not replicate in *A. actinomycetemcomitans*, only transformants that had the *kan* gene integrated into the resident pVT745 via homologous recombination were able to grow in the presence of Km. Results from plasmid DNA isolated from selected transformants and analyzed by restriction enzyme digest demonstrated that a double crossover event had occurred. The resulting pVT745-derivative was designated pDMG37.

2.6. Construction of pDMG38

A point mutation at the nick site of pVT745-derivative pDMG27 was produced by PCR and splicing overlap extension [16] as follows. Two fragments of 375-bp (PCR A) and 770-bp (PCR B) with overlapping extensions that included the nick site were amplified via PCR using pVT745 DNA as a template. Location and sequence of primers used are shown in Fig. 1 and Table 2 respectively. Primers BB6 and BB7 were complementary to each other and contained a 2-bp mutation that changed the cleavage site from AC to CG. Additionally, the third nucleotide 3' of the cleavage site was changed from a T to a G to create a novel *XhoI* site within the modified nick region. In the next step, a recombinant fragment of 1.1-kb (PCR C) was created by first mixing equimolar amounts of PCR A and PCR B fragments to allow annealing of the two pieces during three PCR cycles in the absence of external primers. Subsequently, the external primers BB5 and BB8 were added to the PCR reaction and the longer template of PCR C was amplified. Next, PCR C was cut with *ClaI* and *EcoRI* and cloned into vector pGEM

7Zf(-) (Promega) cleaved with the same restriction enzymes. The insert was then retrieved as an 1.1-kb *SphI/BamHI* fragment and cloned into pDK810, a plasmid that contains the counter selectable marker *sacB* and is used for marker exchange- eviction mutagenesis [9]. This latter clone was ultimately transferred into VT745:pDMG27 via electroporation. Single crossover and subsequently double crossover recombinants were selected as described by Galli and Chen [9]. The presence of the mutated nick site in the pDMG27-derivative, pDMG38, was confirmed by restriction enzyme digest with *XhoI*.

3. Results and Discussion

3.1. Localization of *oriT*

DNA nucleotide analysis suggested that an intergenic region located 5' to the *magA* gene cluster harbored the pVT745-specific *oriT* [3]. Thus, a 192-bp DNA segment (nucleotide 2779 to 2971) of the intergenic region was replaced with a Km resistance marker resulting in pVT745-derivative pDMG37. The deletion was designed to avoid interruption of *magA1* expression. Transfer of pDMG37 was assessed in mating experiments using ATCC29522Rif as the recipient. No self-transfer of the deletion derivative pDMG37 was observed ($<10^{-9}$) which indicated that this 192-bp non-coding region is essential for DNA transfer. To confirm that the intergenic region harbored *oriT*, fragments of varying lengths adjacent to or overlapping with *magA* were cloned into non-transmissible plasmid pDMG4 resulting in clones pJC1, pJC2, and pJC3 (Fig. 2A). These pJC-clones were then assessed for *oriT* function in mobilization assays. Donor strains ATCC29522RifrecA:pDMG27 and ATCC29522RifrecA:pDMG37 were used to support the transfer of the pJC-clones to *A. actinomycetemcomitans* ATCC29522:pDMG1 or *E. coli* JM109. Conjugative plasmid pDMG27, a pVT745-derivative harboring *oriT*, is known to yield high transfer rates due to a mutated invertase [17], whereas pDMG37 is lacking *oriT*. Plasmid pDMG1, which is nonmobilizable, provided the selectable antibiotic resistance marker for the recipient *A. actinomycetemcomitans* strain. No mobilization was detected for any of the clones in the absence of pDMG27 or pDMG37 in donor cells. Plasmid pDMG37 promoted the transfer of pJC1 and pJC2, which is consistent with the presence of a functional *oriT* sequence on both clones. Transfer frequencies to *A. actinomycetemcomitans* were in the range of 10^{-6} , thus matching frequencies obtained with pVT745 [3]. Analysis of plasmid content in selected *A. actinomycetemcomitans* transconjugants confirmed the presence of pJC1 and pJC2 and the absence of pDMG37.

When using ATCC29522RifrecA:pDMG27 as a donor strain transfer frequencies were much higher for pJC1 and pJC2 as was expected (Fig. 2B). Detectable, but poor mobilization to *E. coli* was observed for pJC3 (Fig. 2B), which suggested that the 500-bp cloned fragment carried the *oriT* core but that *cis*-acting auxiliary sequences 3' to *SmaI* were missing for full transfer efficiency. Indeed, nucleotide sequence analysis shows the presence of two 17-bp inverted repeats flanking *SmaI*, which may play a role in initiation of transfer (Fig. 2C). Surprisingly, all *A. actinomycetemcomitans* transconjugants tested appeared to be resistant to Sp (associated with pJC-clones) and Km (associated with pDMG27), which suggested that mobilization of pJC1 and pJC2 did not occur independent of pDMG27 transfer. Screening of selected transconjugants for plasmid content confirmed the presence of both plasmids. When isolating plasmid DNA from *E. coli* transformants, only the pJC-clones were present. However, in several instances the mobilized plasmids pJC1, pJC2 and pJC3 were significantly larger than the original construct although the increase in plasmid sizes varied. This observation suggested that despite the use of a *recA*⁻ donor strain a transfer of pJC-pDMG27 cointegrates had occurred with subsequent deletion of major portions of the pVT745-specific DNA prior to resolution of the recombinant molecule. Structural instability of a pVT745-based *A. actinomycetemcomitans*/*E. coli* shuttle-vector resulting in the loss of 13 to 18-kb when transferred to *E. coli* has been reported previously [3] and suggests that numerous genes on

pVT745 could potentially be lethal to *E. coli* host cells. Formation of a recombinant intermediate could also explain the co-transfer observed in *A. actinomycetemcomitans* transconjugants in the presence of pDMG27, although these recombinants appeared to be resolved efficiently since both plasmids were recovered in recipient cells. It is postulated that mobilization by cointegration can be ascribed to conjugation-dependent *oriT*-specific recombination of the pJC-clones with pDMG27. This would also explain the lack of co-transfer when pDMG37, which carries the 192-bp *oriT* deletion, was used to mobilize the pJC clones. Formation of *recA*-independent cointegrates has been reported for ColE1 and related plasmids, F and R1162, where it is promoted by the relaxase in the presence of two copies of *oriT* [18, 19,20,21].

3.2. Identification of the *oriT*-specific nick site

DNA transfer from the nick site at *oriT* occurs in a 5' to 3' direction. Plasmid transfer typically terminates with the relaxase joining the 5' end of the transferred strand to the 3' end generated by a second cleavage at *oriT*, which has been reconstituted as duplex DNA in the donor cell by rolling circle replication [6]. It was speculated that disruption of the mating event prior to a complete round of transfer, would require the relaxase to rejoin the 5' end obtained by proper nicking with a free 3' end generated by physical strand breakage. Thus, recipient cells would carry a truncated version of the conjugative plasmid with a hybrid *oriT* site. Nucleotide sequence comparison of these junction points would then allow for the identification of the wild-type nick site. Hence, to determine the precise location of *nic*, interrupted mating assays were performed with plasmid pDMG27, which harbors two antibiotic resistance markers, *stm* and *kan*, both located on opposite sites of *oriT* (Fig. 3). Transconjugants harboring truncated derivatives of pDMG27 due to incomplete transfer would be detectable by the loss of either the *stm* or the *kan* marker. Mating was performed with ATCC29522:pDMG27 as the donor and ATCC29522Rif as the recipient strain with the standard mating time of 6 h drastically reduced to minutes in an attempt to prevent complete transfer of pDMG27. Despite the drastically shortened mating time the majority of transconjugants obtained still displayed resistance to both selective markers. Nonetheless, a total of six transconjugants that exhibited the phenotype of Km resistance and Sm susceptibility were recovered in two independent rounds of mating assays when plate mating was interrupted after 5 min or less. The number of Sm susceptible clones obtained per total number of transconjugants was as follows: 1/20 at 2 min, 2/51 at 3 min, 1/71 at 4 min, and 2/125 at 5 min. Longer mating times did not result in Sm sensitive transconjugants. Also, no transconjugants resistant to Sm and susceptible to Km were detected. Restriction enzyme digest analysis of plasmids isolated from the six Sm susceptible transconjugants showed the presence of the *SmaI* site within *oriT*, but the absence of the *HincII* site located in *magA1* (Fig. 2), thus confirming that the nick site was located in the intergenic region adjacent to the *magA* operon. Nucleotide sequence analysis of these pDMG27-derivatives revealed that plasmid transfer had been interrupted at nucleotides 13654, 14595, 14734, 15612, 17908, and 19259 respectively (Fig. 3), which resulted in the loss of DNA up to nucleotide 2865 (Fig. 4). Hence, cleavage on pDMG27 had occurred between nucleotide 2864, an A, and 2865, a C, which denotes the nick site (Fig. 4). The identification of *nic* also elucidated the polarity of strand transfer confirming that the *magA* transfer genes enter the recipient last as shown for other conjugative plasmids [6]. A comparison of the pVT745-specific nick region to core consensus sequences of the five known *oriT* families revealed that the highest similarity was with the IncP family, which reads YATCCTGY, and thus matches pVT745 in six out of eight nucleotide positions [22]. The main distinction was the actual cleavage site, which is GY for IncP and AC for pVT745. Interestingly, all truncated plasmids derived from the interrupted mating assays exhibited the dinucleotide GC and not AC at the site of religation (Fig. 4). This observation would indicate that the pVT745-specific relaxase, MagA2, cleaves AC but preferably rejoins free G and C ends. Additionally, the C at nucleotide 2862 on the wild-type plasmid was also found in all the truncated derivatives (Fig.

4). The truncated versions of pDMG27 obtained via interrupted mating lost their ability for self-transfer and mobilization (data not shown).

It has been shown that the existence of two directly repeated *oriT*s on the same plasmid will produce transfer of truncated plasmid versions. DNA mobilization will initiate at one site and terminate at the other site due to the similarity in the DNA region, thereby generating a hybrid *oriT* through intramolecular site-specific recombination that is accompanied by the deletion of the intervening DNA [21,23,24,25]. However, we do not believe that the junction points generated in the truncated pDMG27-derivatives were the result of *oriT*-specific recombination events. Nucleotide sequence comparison of the wild-type nick site and the termination sites created by interrupted mating revealed only very limited sequence similarity (Fig. 4). Thus, it is highly unlikely that these other sites would be recognized and cleaved by the relaxase. Furthermore, if these sites were to represent secondary *oriT*s that cause premature termination of transfer, similar pDMG27-recombinants would have been obtained at the longer mating times of 6, 8, and 10 min as well. Instead, it is proposed that truncated pDMG27-strands were derived from the disruption of the mating bridge prior to complete plasmid transfer resulting in the physical breakage of the transferred *ssDNA* strand. Subsequently, the relaxase religated free ends with a preference for *ssDNA* strands that carried G at the free 3' hydroxyl end and C as the nucleotide second to last. All other strands generated by physical breakage were most likely degraded in the recipient. Further work will be necessary to delineate which nucleotides within *oriT* are crucial for the cleaving and joining reaction of *ssDNA* by MagA2.

3.3. Functionality of the *oriT*-specific nick site

To confirm the functionality of *nic* a point mutation was introduced that resulted in a change of the cleavage site from AC to CG to construct pDMG38. An added benefit of this mutation was the creation of a new *XhoI* site, which allowed for an easy confirmation of the modified nick site. The impact of the mutation on pDMG38 transfer was assessed in mating assays using the wild-type strain VT745 as a donor and ATCC29522Rif as a recipient. Whereas control plasmid pDMG27 transferred at a rate of 10^{-5} transfer frequencies for pDMG38 dropped to 10^{-8} . Hence, the point mutation at *nic* drastically impaired self-transfer of the plasmid.

In conclusion, the location of the pVT745-specific *oriT* was identified directly upstream of the operon associated with DNA strand processing, which is in agreement with findings for other Gram-negative plasmids [22]. We determined the pVT745-specific nick site in *oriT* and the polarity of DNA transfer by use of an interrupted mating assay. Although interrupted mating has been successfully applied to the mapping of bacterial genomes, this is the first report of using such technique to map the initiation site of conjugal plasmid transfer. Of particular interest is the fact that *oriT* is located on a DNA inversion element present on pVT745. DNA inversion appears to occur only during conjugation and results in structural rearrangement of the plasmid in recipient cells but not in donor cells [17]. Since nicking at *oriT* will disrupt the inversion element it is unlikely that such DNA rearrangement occurs during *ssDNA* transfer. Instead, it is hypothesized that inversion on pVT745 is initiated in the transconjugants during recirculation of the *ssDNA* at *oriT*. Future work will focus on this potential association of DNA inversion and religation at *oriT*.

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Abbreviations

| | |
|-------------|--------------------|
| Ap | ampicillin |
| Em | erythromycin |
| Km | kanamycin |
| <i>oriT</i> | origin of transfer |
| Rf | rifampin |
| Sm | spectinomycin |
| Sp | streptomycin |

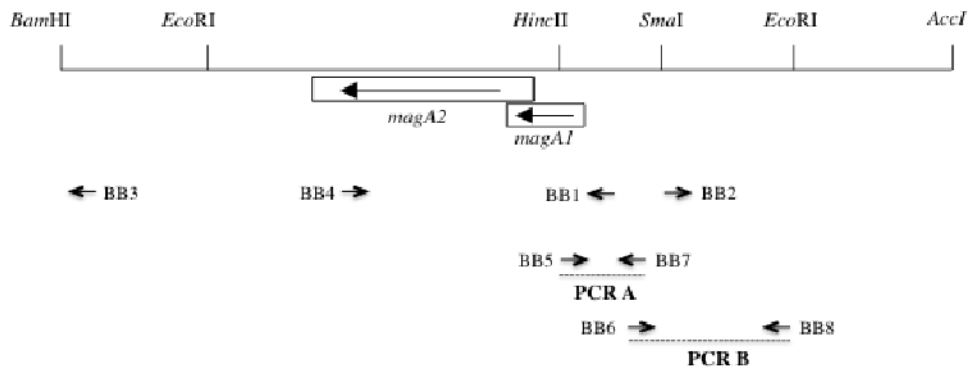


Fig. 1. Map of a 4.6-kb fragment from pVT745 that harbors the *magA* operon and flanking regions. The locations of primers BB1 to BB8 used in PCR reactions for the construction of pDMG37 and pDMG38 are shown. Refer to Material and Methods for specific details.

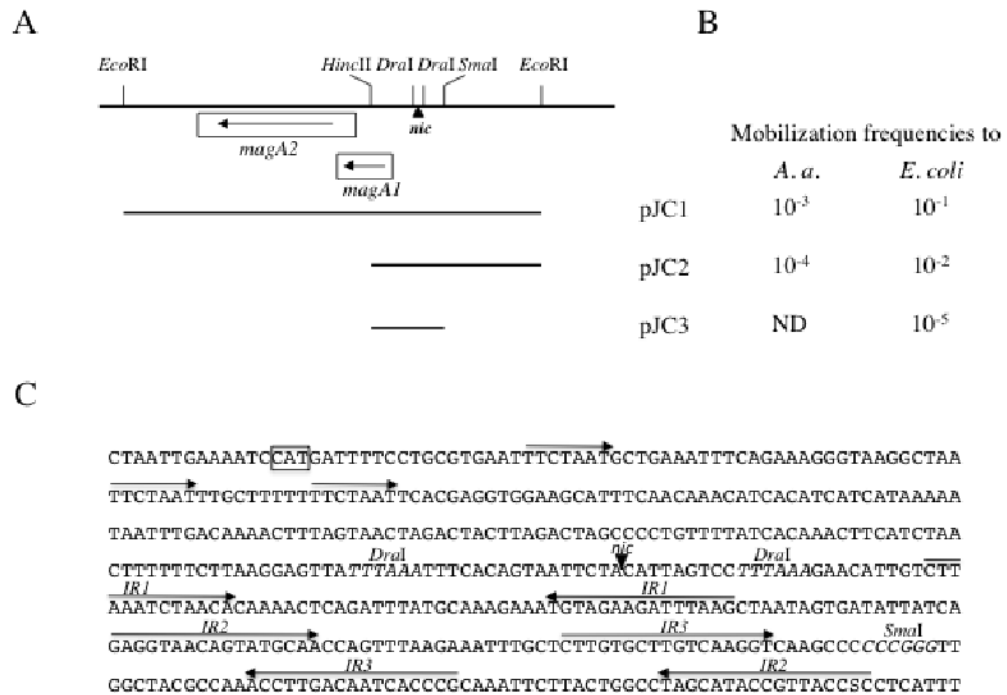


Fig. 2. Localization of the origin of transfer on pVT745. (A) Physical and genetic map of the *magA* operon and the adjacent intergenic region. Position and transcriptional orientation of genes are shown. The black triangle indicates the location of the nick site, *nic*, as determined by interrupted mating assays. The long horizontal lines represent the 2.8-kb *EcoRI/EcoRI* fragment, 1.1-kb *HincII/EcoRI* fragment, and 0.5-kb *HincII/SmaI* fragment cloned into pJC1, pJC2, and pJC3 respectively. (B) Mobilization frequencies of the three pJC-clones expressed as number of transconjugants per donor cell (ATCC29522Rif^{recA}:pDMG27). Transfer frequencies to the recipients *A. actinomycetemcomitans* and *E. coli* are not comparable due to the different growth rate of the two species. (C) Nucleotide sequence of the *oriT* region from nucleotide 2620 to 3095 (GenBank accession no. AF302424) encompassing the region 3' to *SmaI* that is present on pJC2 and missing on pJC3. Direct and indirect repeat (*IR*) sequences are highlighted by arrows. Restriction enzyme sites are shown in italics. The location of the putative start codon for *magA1* on the opposite strand is boxed.

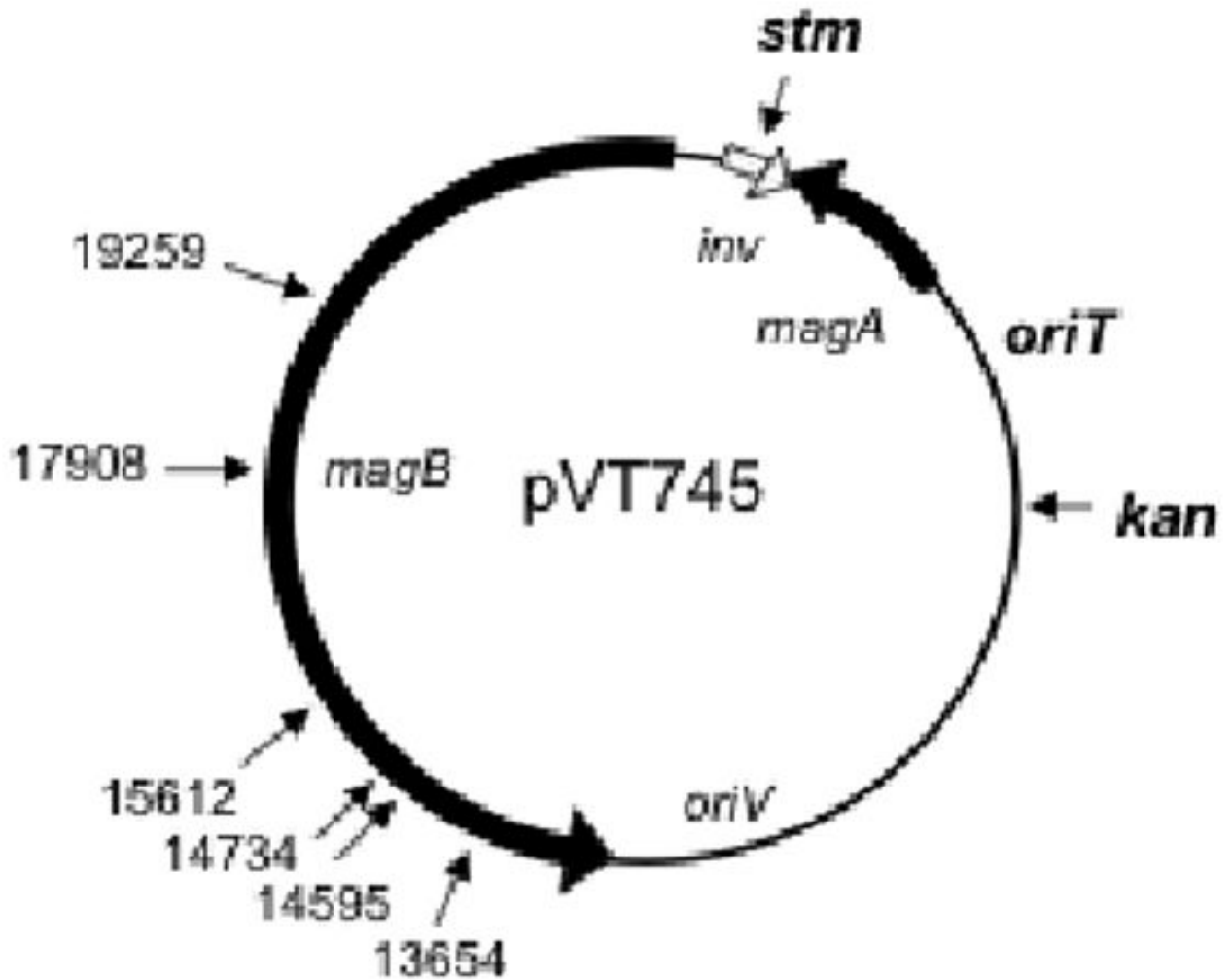


Fig. 3. Physical map of pVT745. The insertion sites of the streptomycin (*stm*) and kanamycin (*kan*) resistance genes on pVT745-derivative pDMG27 are shown. The locations of the free 3' ends generated by interrupted mating are indicated by arrows and the respective nucleotide positions on pVT745 are shown. *Inv*, gene for invertase; *magA*, operon for DNA processing; *magB*, genes for mating bridge formation; *oriT*, origin of transfer; *oriV*, putative origin of replication [3].

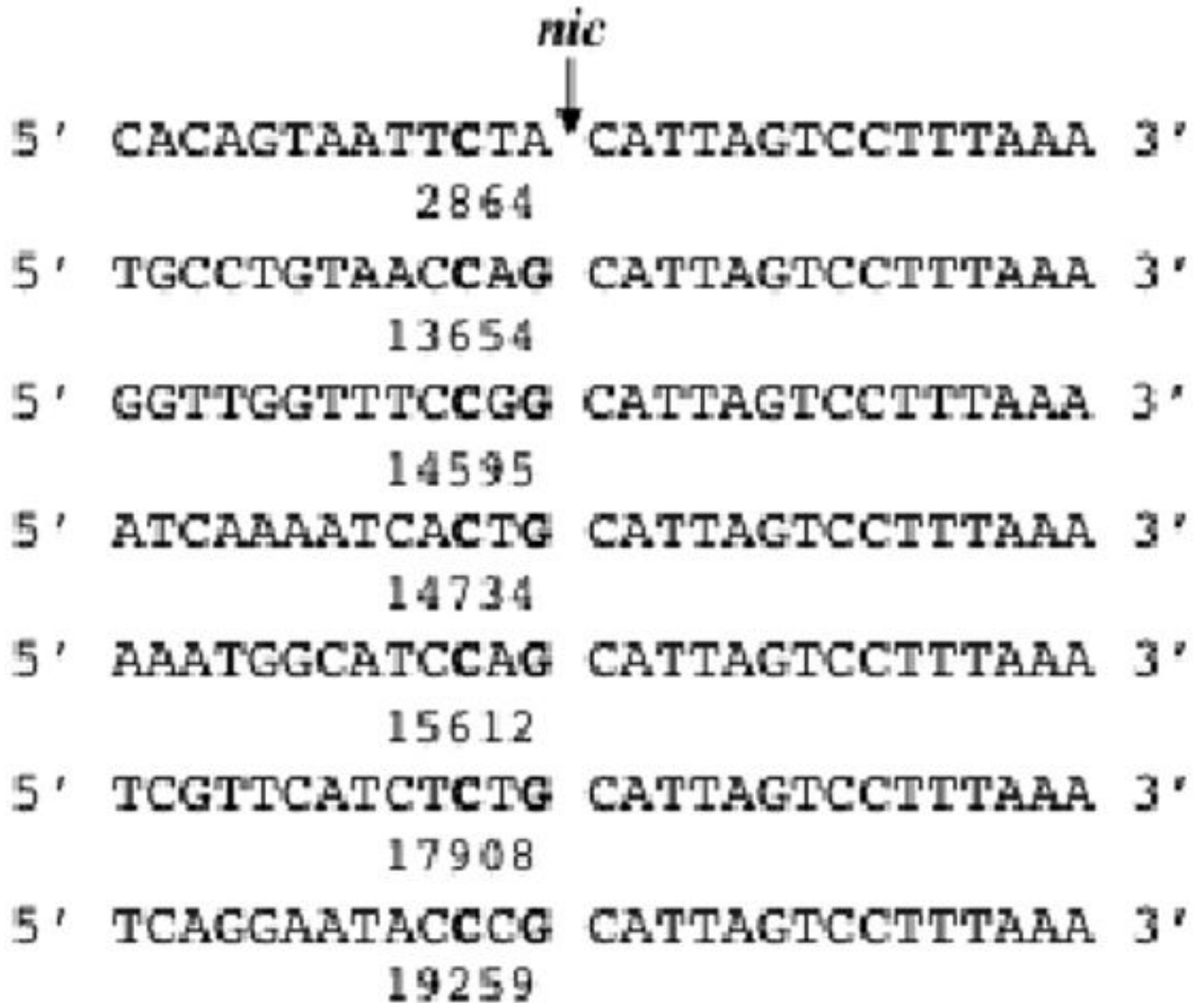


Fig. 4. DNA sequence comparison of the pVT745-specific nick region (line 1) and the six different junction points obtained by interrupted transfer of pDMG27. The nick site was identified at nucleotides 2864/2865. Nucleotides shown in bold upstream of *nic* were present in all the 3' ends of the truncated *ssDNA* derivatives.

TABLE 1

Strains and plasmids used.

| Strain or plasmid | Description | Reference |
|---------------------------------|---|-----------|
| <i>A. actinomycetemcomitans</i> | | |
| ATCC29522 | | ATCC |
| ATCC29522Rif | Rf | [3] |
| ATCC29522RifRecA | Rf; Sm; <i>recA1</i> | [17] |
| VT745 | JP2 strain | [2] |
| <i>E. coli</i> | | |
| JM109 | <i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB)</i> | |
| Plasmids | | |
| pDK810 | Ap, Sp; 6.1-kb; pUC-derivative harboring <i>sacB</i> | [17] |
| pDMG1 | Em; 3.7-kb; derivative of <i>A. actinomycetemcomitans</i> native plasmid pVT736-1 | [12] |
| pDMG4 | Sp; 3.4-kb; p15A-based cloning vector | [12] |
| pDMG27 | Km, Sm; 28.5-kb; pVT745-derivative; invertase mutated by <i>stm</i> gene; <i>kan</i> gene inserted in unique <i>ScaI</i> site; increased transfer frequencies | [17] |
| pDMG37 | Km; 26.5-kb; pVT745-derivative; 192-bp of <i>oriT</i> replaced with <i>kan</i> gene | This work |
| pDMG38 | pDMG27 with point mutation at <i>nic</i> | This work |
| pGB2 | Sp; 4.0-kb; pSC101-based cloning vector; low copy number | [15] |
| pJC1 | 6.2-kb; pDMG4 with 2.8-kb <i>EcoRI/EcoRI</i> insert from the pVT745- <i>oriT</i> region | This work |
| pJC2 | 4.5-kb; pDMG4 with 1.1-kb <i>HincII-EcoRI</i> insert from the pVT745- <i>oriT</i> region | This work |
| pJC3 | 3.9-kb; pDMG4 with 0.5-kb <i>HincII-SmaI</i> insert from the pVT745- <i>oriT</i> region | This work |
| pMK3 | Sp, Km; 4.7-kb; pDMG4 with <i>kan</i> gene | [26] |
| pVT745 | 25.4-kb; conjugative; <i>A. actinomycetemcomitans</i> native plasmid | [2] |

TABLE 2

Primers used in PCR.

| Name | Primer Sequence ^a |
|------|---|
| BB1 | 5'-GGGCTAGTCTAAGTAATCGATTACTAAAGTTTGTGTC-3' |
| BB2 | 5'-TCAGAGGTAACAGTATCGATCCAGTTTAAGAAATTTG-3' |
| BB3 | 5'-GTGGATACAAAAGAGATTTTCGAGGATCC-3' |
| BB4 | 5'-TAAGCGATATAGGATCCCCGCGTTTAAC-3' |
| BB5 | 5'-AAAAACCAAATCGATTTAATGCTGTTCTTAACTTTATG-3' |
| BB6 | 5'-AGTTATTTAAATTTACAGTAATTCTCGAGTAGTCCTTTAAAGAACATTGTCTTA-3' |
| BB7 | 5'-TAAGACAATGTTCTTTAAAGGACTACTCGAGAATTACTGTGAAATTTAAATAACT-3' |
| BB8 | 5'-CTTTAAAATCATCATTCTTGAAATTATTAATCAAGAATTCTCTAAAATCA-3' |

^aRestriction enzyme sites are underlined