DNA Inversion on Conjugative Plasmid pVT745

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Plasmid pVT745 from *Actinobacillus actinomycetemcomitans* strain VT745 can be transferred to other *A. actinomycetemcomitans* strains at a frequency of 10^{-6} . Screening of transconjugants revealed that the DNA of pDMG21A, a pVT745 derivative containing a kanamycin resistance gene, displayed a structural rearrangement after transfer. A 9-kb segment on the plasmid had switched orientation. The inversion was independent of RecA and required the activity of the pVT745-encoded site-specific recombinase. This recombinase, termed Inv, was highly homologous to invertases of the Din family. Two recombination sites of 22 bp, which are arranged in opposite orientation and which function as DNA crossover sequences, were identified on pVT745. One of the sites was located adjacent to the 5' end of the invertase gene, *inv*. Inversion of the 9-kb segment on pVT745 derivatives has been observed in all *A. actinomycetemcomitans* strains tested except for the original host, VT745. This would suggest that a host factor that is either inactive or absent in VT745 is required for efficient recombination. Inactivation of the invertase in the donor strain resulted in a 1,000-fold increase in the number of transconjugants upon plasmid transfer. It is proposed that an activated invertase causes the immediate loss of the plasmid in most recipient cells after mating. No biological role has been associated with the invertase as of yet.

Conservative site-specific recombination is an important class of genetic rearrangements distinct from general recombination, as it requires only a short region of sequence identity. Site-specific recombinases can be grouped into two families (reviewed in references 4, 19, and 25). The integrase family is characterized by its ability to catalyze inversions, deletions, and intermolecular reactions with nearly equal frequencies. The resolvase/invertase family promotes the resolution of cointegrate intermediates and inversions. The frequency of recombination between two different molecules is extremely low in the resolvase/invertase family. Members of this latter family can be divided into three subfamilies: resolvases (Tn3 family), resolvase-invertases, and invertases (Din family). Resolvases and invertases employ similar reaction mechanisms during recombination. However, whereas resolvases bind to two directly repeated sites, invertases require the presence of two inverted repeats (IR) and a host factor that binds to a *cis*-acting, enhancer-type DNA sequence.

Resolvases and invertases have been identified on a variety of bacterial plasmids of gram-positive and gram-negative origins. They have been shown to play a role in plasmid partition (reviewed in reference 17) and plasmid replication (3, 7). In addition, phase variation in prokaryotes has been associated with DNA inversions catalyzed by site-specific recombinases encoded on phage, chromosomal, or plasmid DNA (for reviews see references 14 and 19). Complex DNA rearrangements in which multiple DNA segments can invert independently or in groups have been described for p15B (16), as well as for IncI1 and IncI2 plasmids (for review see reference 19).

One of the best-characterized multiple-DNA inversion systems is the shufflon of conjugative plasmid R64, which controls mating frequencies of the plasmid to different recipients by changing a component of the pilus (19).

Plasmid pVT745 is one of few plasmids isolated from the gram-negative periodontal pathogen *Actinobacillus actinomycetemcomitans* (20). DNA nucleotide base sequence analysis revealed functions related to conjugation, replication, and replicon stability on the 25-kb size plasmid (12). Conjugative transfer of pVT745 from its host, VT745, to other *A. actinomycetemcomitans* strains or *Escherichia coli* was shown at a low frequency of 10^{-5} to 10^{-6} (12). Despite its conjugative properties, the plasmid has not been found in any clinical isolates of *A. actinomycetemcomitans* other than VT745. However, remnants of pVT745 DNA were detected on the chromosomes of various *A. actinomycetemcomitans* strains (22). Analysis of the DNA sequence of pVT745 identified the presence of a gene, AA01, which showed a high degree of similarity to the Din family of site-specific recombinases (12). The objective of this study was to determine the functional and biological role of the pVT745-specific recombinase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. The *A. actinomycetemcomitans* recipient strain ATCC 700685Rif was isolated as a spontaneous rifampin (RIF)-resistant mutant of strain ATCC 700685. *A. actinomycetemcomitans* was grown in TSBYE (3% Trypticase soy broth, 0.6% yeast extract) at 37°C in 10% CO₂. *E. coli* strain JM109 was grown in yeast-tryptone medium (21). Where appropriate, antimicrobial agents were used at the following concentrations: ampicillin (AMP), 100 µg/ml; kanamycin (KAN), 50 µg/ml, except for strain ATCC 700685 at 100 µg/ml; RIF, 100 µg/ml; spectinomycin (SPT), 100 µg/ml; and streptomycin (STR), 50 µg/ml.

DNA preparations and recombinant DNA techniques. Plasmid DNA was isolated from *A. actinomycetemcomitans* and *E. coli* as described previously (11). Restriction endonucleases, Klenow polymerase I, and T4 DNA ligase were used in accordance with the manufacturer's instructions (Gibco-BRL). Standard re-

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>A. actinomycetemcomitans</i>		
ATCC 29522Rif	Rif	This work
ATCC 29522RifrecA	Rif Sm recA	This work
ATCC 700685	JP2-like strain; plasmid free	American Type Culture Collection
ATCC 700685Rif	Rif, JP2-like strain; plasmid free	This work
VT745	JP2-like strain; containing pVT745 or pVT745 derivatives	20
<i>E. coli</i>		
JM109	recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB)	26
Plasmids		
pDMG4	Sp; p15A-based cloning vector	10
pDMG20	Km; Sp; derivative of pVT745; contains pGB2; the invertible segment is either in the orientation found on pVT745 (A) or in the opposite orientation (B)	12
pDMG21	Km; derivative of pVT745; the invertible segment is either in the orientation found on pVT745 (A) or in the opposite orientation (B)	12
pDMG27	Km Sm; same as pDMG21A but with mutated site-specific recombinase	This work
pDMG28	Km Sp; same as pDMG21A but with SPT cassette in <i>Xba</i> I site	This work
pDMG30	Km Sm Sp; same as pDMG27 but with SPT cassette in <i>Xba</i> I site	This work
pDMG32	Km Sp; same as pDMG21A but with SPT cassette in <i>Eco</i> RI site	This work
pGB2	Sp; low-copy-no. cloning vector based on pSC101; nonmobilizable	9
pGEM7Zf(-)/spc	Ap Sp	8
pGEM-T Easy	Ap; cloning vector for PCR products	Promega
pJC20	Ap; pNP3 derivative, expresses invertase under control of <i>spc</i> promoter	This work
pMK3	Sp Km; pDMG4 derivative	13
pMK5	Sp Sm; pDMG4 derivative	13
pNP3	Ap; pMMB67-based expression vector with <i>spc</i> promoter	P. Permpnich
pUC19	Ap; high-copy-no. cloning vector	26

^a Rif, RIF resistant; Sp, SPT resistant; Km, KAN resistant; Ap, AMP resistant; Sm, STR resistant.

combinant DNA techniques were performed as described by Sambrook et al. (23). DNA-DNA hybridization conditions and transformation by electroporation have been described previously (11). Standard three-step PCR experiments were performed with *Taq* polymerase from Promega (Madison, Wis.). All PCR primers used are listed in Table 2. PCR amplification products were cloned into pGEM-T Easy (Promega) according to the manufacturer's instructions.

RNA isolation and RT-PCR. Total RNA was isolated from *A. actinomycetemcomitans* by use of the RNeasy kit (Ambion) according to the manufacturer's instructions. Residual DNA was removed by treatment with RNase-free DNase (Gibco-BRL) for 30 min at 37°C. Reverse transcriptase PCR (RT-PCR) was performed with the Qiagen OneStep RT-PCR kit (Qiagen) as described by the manufacturer. Reverse transcription was performed at 55°C with primers P11 and P12 (invertase gene) and at 65°C with primers P13 and P14 (leukotoxin gene) (Table 2). Amplification products were visualized after electrophoresis in 0.8% agarose gels.

DNA sequencing. Nucleotide sequencing was performed with custom-made primers (P1, P2, P3, and P4) (Table 2) flanking the junctions of the invertible element on pDMG21B by use of the femtomole DNA Cycle sequencing system (Promega).

Construction of a *recA*-deficient ATCC 29522 strain. The nucleotide sequence of the *recA* gene was retrieved from the genome of ATCC 700685 (<http://www.genome.ou.edu/act.html>) and was used to design two oligonucleotides flanking the gene on the 5' (P5) and 3' (P6) ends (Table 2). These primers were then used in a PCR experiment to amplify the corresponding gene in ATCC 29522. The amplification product of 4.2 kb was ligated to vector pGEM-T Easy (Promega) and was introduced into *E. coli* JM109. However, the intact gene recombined into the *E. coli* genome. Therefore, a 1.8-kb internal *Xho*II fragment of *recA* was removed from the 4.2-kb PCR product and was cloned into pUC19 linearized with *Bam*HI. The new construct was purified from *E. coli* JM109 and was digested with *Mlu*I. The latter restriction site was unique and was located in the center of the 1.8-kb PCR insert. The linearized vector was then blunt ended with the large fragment of Klenow polymerase I (Gibco-BRL) and was ligated to a ca. 2-kb fragment excised from pMK3, which carried the gene for STR resistance. The latter, a *Cl*aI fragment, was blunt ended as well. The resulting construct of 6.5 kb was purified from *E. coli* and was used to transform strain ATCC 29522Rif by electroporation. Since pUC19 does not replicate in *A. actinomycetemcomitans*, transformants resistant to RIF and STR indicated that all or part of the *recA*

construct had inserted into the *A. actinomycetemcomitans* chromosome via homologous recombination.

Construction of pVT745 derivatives: pDMG27, pDMG28, pDMG30, and pDMG32. All recombinant constructs used for allelic replacement were obtained in *E. coli* strain JM109. The following cloning strategy was used to construct pDMG27 in which the gene for the site-specific recombinase (*inv*) on pDMG21A is replaced with an STR resistance cassette via allelic exchange by homologous recombination (pDMG27). First, vector pUC19 was modified by eliminating its unique *Eco*RI and *Ava*I sites located in the multiple-cloning site. This was

TABLE 2. Primers used in PCR, RT-PCR, and sequencing reactions

Name	Primer sequence	Size (in bp) of PCR fragment
P1	5'-ATTCACCGCACACTTCCG-3'	NA ^a
P2	5'-CTAATTTCCATACTATGA-3'	NA
P3	5'-CCTTAGGCAACGCGAAAGAG-3'	NA
P4	5'-CAAGCCGCTAAAGAACTACG-3'	NA
P5	5'-TCAGGAACTCCCTCGCTGG-3'	4,203
P6	5'-CGAATCCGTGTTGCCTGTGCG-3'	
P7	5'-TGCGGTTTCGAGAATTCGAAGGACAGCGCCCGAATCTCC-3'	869
P8	5'-GCAACAATAAAAGCTTTACCTTTCTGCTAACGCCCTGC-3'	
P9	5'-GGTCAAGCGGAAGCTTCGAATCCAATAC-3'	2,017
P10	5'-AATTGTGTCCGGATCCTAACGAGGACTC-3'	
P11	5'-CCGTTCTAAAAAGTCTCGAGAAATATTTTGTGGAAGTGATTG-3'	815
P12	5'-AATTGAATATCAACGTGCGAGACGGTTATCAAGCTTGAATGC-3'	
P13	5'-ATCGATCCGGAGTAAATTAGTCTCC-3'	883
P14	5'-ATCGATGTTGCCATAATCTATTCTCC-3'	

^a NA, not applicable. These primers were used for sequencing.

FIG. 1. Physical and genetic map of pVT745 (A) and its invertible segment (B). *XbaI* and *EcoRI* are present more than once on the plasmid. (A) Map of pVT745 depicting the gene clusters *magA* and *magB* necessary for conjugative transfer of the plasmid (12) and the location of an invertible region flanked by IR22. Transcriptional orientation of genes or operons is indicated by an arrowhead. Small arrows point to target sites for the insertion of antibiotic resistance markers *kan* (KAN), *spc* (SPT), and *stm* (STR). The pVT745 derivatives resulting from the insertion of these markers are listed in brackets next to the appropriate resistance cassette. Note that all constructs carry more than one resistance gene. *inv*, invertase gene. (B) Genetic organization of the invertible region (isomeric form A). Genes and/or open reading frames are represented by boxes. For more specific information on the open reading frames shown, see Galli et al. (12). Hatched boxes indicate that the corresponding open reading frames are transcribed counterclockwise.

accomplished by digesting pUC19 with the corresponding restriction enzymes, treatment of the restriction sites with the large fragment of Klenow polymerase I (Gibco-BRL), and religation of the free ends of the vector. Subsequently the 2.9-kb *Bam*HI-*Hinc*II fragment of pVT745, which carried the gene for the site-specific recombinase (12), was cloned into the modified pUC19 vector digested with the same restriction enzymes. The resulting construct was double digested with *Eco*RI-*Ava*I to remove an internal 400-bp fragment from the target gene. The linearized construct was then blunt ended with Klenow polymerase I and was ligated with a 2-kb blunt-ended fragment carrying the STR resistance cassette from pMK5. This final pUC19 derivative was purified from *E. coli* JM109 and was transferred into VT745:pDMG21A via electroporation. Since pUC19 does not replicate in this host, only transformants that had the *stm* gene integrated into the resident plasmid, pDMG21A, by homologous recombination

were able to grow in the presence of SPT. The pUC19 construct allowed for a single- or a double-crossover event to occur. Plasmid DNA isolated from the transformants was analyzed by restriction enzyme digestion and Southern blot hybridizations using pUC19 or the STR resistance gene as probes. Results from these experiments confirmed that both, a single-crossover event and a double-crossover event, had occurred. One of the pDMG21A derivatives obtained by double crossover, pDMG27, was subsequently used in conjugation assays.

To construct pDMG28 and pDMG30, an additional selective marker, SPT (*spc*), was inserted into pDMG21A and pDMG27 at an *Xba*I site, which is located outside the invertible segment (Fig. 1A). This was accomplished as follows: primers were designed (P7 and P8 [Table 2]) and were used to amplify an 870-bp fragment of pVT745 by PCR. The target sequence corresponded to a region upstream of the putative *oriV* and contained the *Xba*I site into which *spc*

was to be inserted (12). To one of the primers the restriction site for *Eco*RI and to the other primer the restriction site for *Hind*III were added to allow for cloning of the PCR amplification product into pUC19 digested with both of these enzymes. The resulting pUC19 construct was digested with *Xba*I; the free ends were treated with the large fragment of Klenow polymerase I (Gibco-BRL) and were ligated with a 1.1-kb *spc* cassette. The latter had been retrieved from pGEM7Zf(-)/*spc* (8) as a 1.1-kb *Xba*I-*Bam*HI fragment and had been blunt ended as well. The final construct was purified from *E. coli* strain JM109 and used to transform strains VT745:pDMG21A and VT745:pDMG27 by electroporation. Plasmid DNA was isolated from SPT-resistant transformants and analyzed by restriction enzyme digest and Southern blot hybridization. Recombinant plasmids into which the *spc* gene had inserted into the target *Xba*I site via single crossover were labeled pDMG28 (for the pDMG21A construct) and pDMG29 (for the pDMG27 construct).

Construct pDMG32 was obtained similar to pDMG28 with the *spc* gene inserted into an *Eco*RI located 3' of *oriV* (Fig. 1A). Again, the regions adjacent to the target *Eco*RI site were amplified by PCR (primers P9 and P10 [Table 2]) and the 2-kb amplification product was cloned into a pUC19 derivative from which the *Eco*RI site had been removed by treatment with the large fragment of Klenow polymerase I. The new construct was then digested with *Eco*RI to cleave this unique site located in the center of the PCR product, blunt ended, and ligated with the *spc* cassette. Again, the final product was purified from JM109 and introduced into VT745 via electroporation. Recombinant SPT-resistant transformants were obtained in which the *spc* gene had inserted into the target *Eco*RI site of pDMG21A via single and double crossover. One of the pDMG21A derivatives obtained by double crossover, pDMG32, was used subsequently in mating assays.

Construction of invertase expression vector pJC20. The invertase gene was amplified from pVT745 by PCR with custom-made primers P11 and P12 (Table 2). The latter primer included a *Hind*III site. The amplification product of 815 bp was then cloned into pGEM-T Easy (Promega) and was subsequently retrieved as a *Sal*I-*Hind*III fragment. The latter fragment was cloned into expression vector pNP3 (P. Permpnich, personal communication) cut with the same enzymes. The final construct, pJC20, was purified from *E. coli* JM109 and used to transform various strains of *A. actinomycetemcomitans* via electroporation.

Analysis of segregational stability of plasmids. Plasmid stability was assessed as described by Galli et al. (10). In short, strains were grown in antibiotic-free TSBYE broth for 7 days (equivalent to 90 to 100 cell doublings) while being diluted 1:100 in fresh medium once a day to maintain the cultures in exponential growth phase. Thereafter, serial dilutions of each culture were spread on antibiotic-free TSBYE plates. Percentages of plasmid-containing cells were obtained by transfer of 100 colonies from these plates to selective and nonselective plates with sterile toothpicks. Experiments were performed at least twice.

Mating experiments. Conjugative surface matings were performed between *A. actinomycetemcomitans* strains as described previously for 6 h unless noted otherwise (12). Transfer frequencies were expressed as the number of transconjugants per donor cell. At least 10 selected transconjugants were examined for the presence of plasmid DNA after each mating experiment. Spontaneous RIF mutations occurred at a frequency of less than 10^{-9} and did not interfere with low-frequency matings.

Characterization of RecA⁻ transformants. Recombination-deficient mutants (RecA⁻ cells) were identified based on their sensitivity to UV light and methyl methanesulfonate (MMS). Parental ATCC 29522 cells and potential RecA⁻ transformants were grown in broth to late exponential phase and were then streaked in parallel onto TSBYE agar plates. Plates were irradiated with UV light with increasing doses (500, 1,000, 2,000 and 4,000 μ J) (UV Stratalinker 1800; Stratagene, La Jolla, Calif.), and their contents were then incubated for 2 days.

The sensitivity of RecA⁻ cells to the chemical MMS was tested by adding MMS to TSBYE agar plates in various concentrations, and a comparison of cell growth on these plates to that of parental strain ATCC 29522 grown under the same conditions was made.

RESULTS

DNA rearrangement of pVT745 derivatives. The conjugative transfer of pDMG20 and pDMG21, two KAN-resistant derivatives of pVT745, between different *A. actinomycetemcomitans* strains was reported previously (12). When ATCC 29522 and ATCC 700685 transconjugants were being screened for pDMG20 and pDMG21 content, it was observed that the plas-

FIG. 2. Analysis of *Eco*RI-generated patterns of plasmid pDMG21 by agarose gel electrophoresis. Lane 1, pDMG21A, as isolated from host VT745; and lane 2, pDMG21B, as isolated from transconjugants after transfer. Asterisks indicate the bands that have shifted due to DNA rearrangement of the plasmid. The third band from the top in lanes 1 and 2 represents a triplet and a doublet, respectively. The 1-kb ladder from Gibco-BRL was used as a molecular size standard. MWM, molecular weight marker.

mids showed restriction fragment length polymorphism for certain restriction enzymes such as *Eco*RI when compared to the original constructs (Fig. 2). Further analysis by restriction enzyme digestion confirmed that this polymorphism was due to the inversion of a DNA segment of approximately 9 kb. To distinguish both isomers of pDMG20 and pDMG21, the letter A was added to the pVT745 derivatives carrying the inverted segment in the same orientation as pVT745 in its original host, whereas plasmids harboring the inversion after conjugative transfer were assigned the letter B. Surprisingly, the 9-kb segment was always completely fixed in either the A or B orientation. Constructs pDMG20B and pDMG21B were never detected in the original host VT745, while pDMG20A and pDMG21A could not be retrieved from any of the transconjugants screened. Even when these transconjugants were used as donors in subsequent mating experiments (not shown), pDMG20B and pDMG21B did not appear to revert to the original structure. Also, DNA inversion had never been observed on pVT745 when residing in VT745.

The DNA inversion is *recA* independent. To determine if *recA* was involved in the structural rearrangement of pDMG21A, a recombination-deficient mutant of strain ATCC 29522Rif was constructed. Allelic replacement of *recA* via a double-crossover event with a mutated copy carrying an STR resistance cassette was confirmed by restriction enzyme analysis and Southern blot hybridization (not shown). Sensitivity to 0.001% MMS and UV light (2,000 μ J) confirmed the *recA*-deficient phenotype (not shown).

Strain ATCC 29522Rif*recA* was then used as a recipient in the conjugative transfer of pDMG20A and pDMG21A from VT745. Again, all transconjugants tested harbored the rearranged version of the transferred plasmids, indicating that the

TABLE 3. Rearrangement of pVT745 derivatives after conjugative transfer

Donor	Plasmid	Recipient	Inversion
VT745	pDMG20A	ATCC 29522Rif	+
VT745	pDMG21A	ATCC 29522Rif	+
VT745	pDMG20A	ATCC 29522Rif <i>recA</i>	+
VT745	pDMG21A	ATCC 29522Rif <i>recA</i>	+
VT745	pDMG27	ATCC 29522Rif	—
VT745	pDMG20A	ATCC 700685Rif	+
VT745	pDMG21A	ATCC 700685Rif	+
ATCC 700685Rif	pDMG21B	VT745:pDMG4	—

DNA inversion event was independent of the *recA* status of the recipient (Table 3).

The DNA inversion is caused by the pVT745-encoded site-specific recombinase. Nucleotide sequence analysis of pVT745 revealed that the plasmid carried a gene, AA01, which showed strong homology to site-specific DNA recombinases (12). Indeed, the AA01 gene product was 45% identical to Min, a DNA inversion enzyme of plasmid p15B, which is a member of the Din family of site-specific recombinases (Fig. 3A) (16). Conserved regions included the N-terminal end with the active

serine residue required for the formation of covalent intermediates with DNA at the crossover sites (Fig. 3A) (14). To elucidate the role of the pVT745-specific recombinase in the DNA inversion event, the gene was replaced with an STR resistance cassette as described in Materials and Methods. The resulting construct, a derivative of pDMG21A, was designated pDMG27. When pDMG27 was transferred from VT745 to ATCC 29522Rif via conjugation, rearrangements on the plasmid were no longer observed (Table 3). This was true for the *recA*⁺ and *recA* mutant recipient strain. This result clearly demonstrated that the site-specific recombinase was involved in the DNA inversion event. Therefore, it was decided to rename gene AA01 encoding the site-specific recombinase *inv* for DNA invertase.

Comparison of restriction enzyme profiles of pDMG21A and pDMG21B located the junctions of the invertible segment to a region just upstream of the site-specific recombinase and between AA10 and AA11 (Fig. 1A) (12). Custom-made oligonucleotide primers were used to sequence the junction fragments. Analysis of the resulting DNA nucleotide sequence revealed the presence of a 22-bp repeat (IR22) with dyad symmetry in opposite orientation at the junctions (Fig. 1A and

A

B

FIG. 3. Comparison of Inv of pVT745 and Min of p15B, a member of the Din family of invertases (16). (A) Sequence alignment of Inv and Min. Amino acids that are identical are boxed. The active serine residue (S) involved in the 5' phosphoryl linkage is boldfaced. (B) Alignment of the recognition sites for Inv (IR22), Min (*mix*) (24), and *dix*, the consensus sequence for the recombination sites of all members of the Din family (14). Areas of dyad symmetry are indicated by arrows.

TABLE 4. Segregational stability of pVT745 derivatives

Strain:plasmid	Stability (%) after:	
	100 generations	200 generations
VT745:pDMG21A	100 ^a	100
VT745:pDMG21B	50	2
VT745:pDMG20B	48	9
VT745:pDMG27	100	100
ATCC 29522Rif:pDMG21B	27	ND ^b

^a Percentage of plasmid-containing cells.^b ND, not determined.

3B). These two recombination sites (DNA crossover sequences) were similar but not identical to the conserved crossover sites of the Min and Din systems (Fig. 3B) (14, 24). The main difference was in the region containing the central asymmetric dinucleotide, where Min introduces a 2-bp staggered cut. The central nonsymmetric A-A dinucleotide is conserved in the family of Din invertases (14). Its absence in the Inv-specific crossover sites could indicate that, despite the high degree of homology, there is a difference in target specificity for Inv and Min. The inversion of the 9-kb segment did not result in the creation of new open reading frames or the connection or disconnection of parts of open reading frames (Fig. 1B) as described for other systems.

The DNA inversion is host dependent. Since no rearrangement had ever been observed on pVT745 or one of its derivatives in the original host, VT745, it was possible that the inversion was triggered by the mating event. Therefore, pDMG20A was introduced into ATCC 29522Rif by electroporation. Again, DNA inversion took place. The transformants screened all harbored pDMG20B. This raised the possibility that a host-specific factor was involved in the inversion event. The following possibilities were postulated: (i) an accessory factor is required for inversion in addition to the site-specific recombinase, and such a factor is present in all *A. actinomycetemcomitans* strains but VT745. (ii) An accessory factor is required for inversion in addition to the site-specific recombinase, and such a factor is present in all *A. actinomycetemcomitans* strains but JP2, of which VT745 is a member (15). (iii) VT745 or any JP2 strain harbors a factor that is absent in all other *A. actinomycetemcomitans* strains and that maintains the plasmid in the preferred arrangement "A." The second assumption could be eliminated when conjugative transfer of

pDMG20A and pDMG21A to ATCC 700685Rif, a JP2-like strain, resulted in the inversion of the 9-kb segment (Table 3). To test the third assumption strain, ATCC 29522Rif was used as a donor to transfer pDMG21B to VT745 (Table 3). The latter harbored pDMG4 (10), an SPT-resistant replicon that is neither conjugative nor mobilizable, to allow for selection of transconjugants. No reversion of pDMG21B to pDMG21A was observed in VT745:pDMG4. Also, transformation of VT745 by electroporation with pDMG20B did not result in the reversion of the plasmid to pDMG20A. Therefore, it was concluded that a specific host factor other than RecA is necessary in addition to the site-specific recombinase to catalyze the inversion. Such a factor is either missing or inactive in VT745. However, the absence of this specific host factor is not a distinct feature of JP2-like strains to which VT745 belongs, since DNA inversion was observed in ATCC 700685Rif, another JP2-like strain.

The invertase does not contribute to the segregational stability of pVT745. Strain VT745 carrying the various pVT745 derivatives was grown in the absence of selective pressure to determine if inversion of the 9-kb segment on pDMG21B or the loss of invertase activity on pDMG27 affected segregational stability of the plasmids. The percentage of cells within a given population that still harbored the plasmids after 100 and 200 generations, respectively, is shown in Table 4. Since pDMG27 was as stable as pDMG21A, it is unlikely that the site-specific recombinase is required for efficient partitioning of pVT745. However, the rearrangement of the invertible region seems to affect the segregational stability of the plasmid as demonstrated by the rapid loss of pDMG21B in the absence of selective pressure (Table 4). This was true for pDMG21B in VT745 as well as in ATCC 29522Rif.

The invertase affects pVT745 conjugation frequencies. While the various mating experiments described above were being conducted, it was observed that significantly higher frequencies of transfer were obtained for pDMG27 than for pDMG21A. Therefore, actual conjugation frequencies per donor cell were determined using VT745 as a donor strain and the ATCC 29522Rif *recA*⁺ and *recA* mutant recipient strains (Table 5). Surprisingly, pDMG27 appeared to transfer at a frequency several orders of magnitude higher than did pDMG21A. However, it was unlikely that the plasmids were transferred at different rates, since they differed only in their

TABLE 5. Transfer frequencies of various pVT745 derivatives

Donor	Plasmid	Recipient	No. of transconjugants/donor ^a after:			
			0.5 h on:		6 h on:	
			RIF, KAN	RIF, SPT	RIF, KAN	RIF, SPT
VT745	pDMG21A	ATCC 29522Rif	<10 ⁻⁷	NA	10 ⁻⁶	NA
VT745	pDGM21A	ATCC 29522Rif <i>recA</i>	ND	NA	10 ⁻⁶	NA
ATCC 29522	pDGM21B	ATCC 700685Rif	ND	NA	10 ⁻⁶	NA
VT745	pDMG27	ATCC 29522Rif	10 ⁻⁴	NA	10 ⁻³	NA
VT745	pDMG27	ATCC 29522Rif <i>recA</i>	10 ⁻⁴	NA	10 ⁻³	NA
VT745	pDMG28	ATCC 29522Rif	<10 ⁻⁷	<10 ⁻⁷	10 ⁻⁶	NA
VT745	pDMG30	ATCC 29522Rif	10 ⁻⁴	10 ⁻⁴	10 ⁻³	10 ⁻³
VT745	pDMG32	ATCC 29522Rif	10 ⁻⁶	10 ⁻⁶	10 ⁻⁵	10 ⁻⁵

^a The number of transconjugants per donor (average of at least two independent experiments) is shown. Selection of transconjugants was with RIF for the recipient strain and either KAN or SPT for the incoming plasmid. NA, not applicable; ND, not determined.

invertase activity and since electroporation experiments had indicated that *Inv* was neither active in the host VT745 nor activated by conjugation alone. Therefore, the difference in number of selectable transconjugants had to be associated with an event that took place in the recipient cells after completion of the actual plasmid transfer. A possible explanation for the low number of detectable pDMG21 transconjugants was that the invertible segment and its associated KAN-resistant phenotype had been excised from the plasmid in recipient cells after the mating event. The resulting truncated pVT745 derivative would then only be detectable if a second marker located outside the invertible region was present. Therefore pDMG28 and pDMG32 were constructed, both derivatives of pDMG21A with an additional *spc* cassette inserted 5' or 3' to *oriV* (Fig. 1A) to allow for replication and selection of any potential deletion derivative. Insertion of *spc* was in a noncoding region. An *spc* marker was also inserted into pDMG27 to confirm that the addition of a new cassette and the target location for this marker did not affect the mating frequencies of the *inv* mutant derivative. This last construct was designated pDMG30.

Mating experiments were then carried out with all new constructs. Selection for transconjugants was on either KAN or SPT. Again, mating frequencies for both pDMG21A derivatives, pDMG28 and pDMG32, were several orders of magnitude lower than for pDMG30, which carried a mutated *inv* gene in addition to the *spc* cassette (Table 5). However, whereas the numbers of transconjugants obtained for pDMG28 were similar to the ones obtained for pDMG21A, mating frequencies were increased for pDMG32 (Table 5). Doubling times for *A. actinomycetemcomitans* range between 1.5 and 2 h. Therefore, mating experiments were performed for 6 h as described previously (13) and for 0.5 h to confirm that the decrease in number of transconjugants was not the result of segregational instability of the plasmid. The significant decrease in actual mating times had no effect on the outcome of the experiment. Also, all KAN-resistant transconjugants were resistant to SPT and vice versa, indicating that neither marker had been lost as was to be expected with a deletion derivative. Results of restriction enzyme analysis with plasmid DNA isolated from selected transconjugants confirmed the absence of any truncated version of either pDMG28 or pDMG32 (not shown). Surprisingly, up to 30% of the pDMG32 transconjugants screened carried the invertible 9-kb region in orientation A (not shown).

Expression of the invertase gene alone does not trigger the inversion event. The fact that the invertible segment appeared to remain stable in orientation A or B once the pVT745 derivatives were established in the recipient cells suggested that the invertase gene was no longer expressed after the inversion event. Also, lack of inversion in VT745 could be explained by the absence of *inv* expression. To test these assumptions, the presence of *inv*-specific mRNA was determined in strains VT745:pDMG21A and -B, ATCC 29522Rif:pDMG21B, and ATCC 29522Rif:pDMG32A. Results of RT-PCR experiments confirmed the absence of an *inv*-specific transcript in all strains tested. In contrast, an RT-PCR product was detected in strain ATCC 29522Rif:pJC20, which expressed the invertase gene under the control of the *spc* promoter (not shown). Integrity of the RNA preparations was confirmed by the detection of the

A. actinomycetemcomitans-specific leukotoxin transcript in VT745:pDMG21A and -B by RT-PCR (data not shown).

If indeed inversion was regulated by *inv* expression, the presence of a constitutively expressed invertase would result in the rearrangement of pVT745 derivatives during normal cell growth. Such an event could then be detected by the presence of pVT745 derivatives in the A and B orientation in the same plasmid preparation. To test this assumption, pJC20, a pMMB67 derivative that expresses the invertase, was transferred into VT745:pDMG21A and -B, ATCC 29522Rif:pDMG21B, and ATCC 29522Rif:pDMG32A by electroporation. Plasmid DNA isolated from selected transformants was then screened for the orientation of the invertible segment. In all cases, DNA inversion was not observed for any of the pVT745 derivatives, although expression of *inv* from pJC20 was confirmed by RT-PCR.

DISCUSSION

Conjugative plasmid pDMG21A, a derivative of pVT745, undergoes a structural rearrangement when transferred to recipient strains. The rearrangement is the result of site-specific recombination between two 22-bp indirect repeats present at the ends of a 9-kb element and requires the activity of the plasmid-expressed invertase gene *inv*. It was shown that inactivation of *inv* blocked rearrangement. The pVT745-encoded invertase resembles the recombinases of the Din family (14). Functionally important regions are highly conserved between *Inv* and *Min*, a member of the Din invertase subfamily (Fig. 3A). Members of the Din family are known to complement one another due to the conserved protein sequences and recombination sites. However, significant differences in the IR sequences of *Inv* and *Min* suggest a possible difference in DNA binding specificity for both enzymes.

Once a pVT745 derivative was established within the recipient cell, subsequent DNA inversion was never observed. The possibility that inversion occurred in response to the mating event itself was ruled out since the transfer of a pVT745 derivative into ATCC 29522Rif via electroporation also resulted in rearrangement of the invertible segment. However, it is conceivable that DNA entry into the cell, regardless of entry mechanism, triggers the inversion. Regulation of invertase expression alone did not appear to be sufficient to turn the inversion event on or off. Constitutive expression of *inv* in *trans* during normal cell growth did not result in the inversion of the 9-kb segment regardless of its orientation on the various pVT745 derivatives used. Therefore, one or more additional factors that are either plasmid or host encoded are required to activate the inversion event. It is likely that such a factor(s) is expressed transiently when new DNA enters the cell.

Also, inversion of the 9-kb fragment on pVT745 was never observed in VT745 regardless of the transfer mechanism used. Again, the likeliest explanation is that one or more specific host factor(s) is required to activate the inversion event and that such a factor(s) is absent in the native strain. The Din family of recombinases requires *Fis* (factor for inversion stimulation), which binds to specific *cis*-acting enhancer sequences for efficient inversion (18). These conserved *Fis*-binding sites are located in the 5' end of the recombinase genes within the first 90 nucleotides of the coding frame. Consensus sequences

for Fis host factor binding sites are present in the 5' end of the pVT745-specific *inv* gene (J. Chen and D. M. Galli, unpublished data). Future work will include elucidation of the role of Fis or other host factors in the promotion of inversion on pVT745.

It is not yet known if Inv of pVT745 has a resolvase activity in addition to its function as an invertase. Such activity would play a role in plasmid partitioning by catalyzing the conversion of dimers and higher oligomers into monomers. It was shown in the present study that pDMG27, the pVT745 derivative with an inactivated *inv*, was still segregationally stable in the original host strain after 200 generations. This result is in line with the notion that recombination between two repeats in direct orientation, which would cause the deletion of the intervening DNA segment, is inefficient in the Din family of inversion systems (24).

In contrast to most other systems, at this point no biological function can be linked to the inversion event. Neither are new open reading frames created nor are existing open reading frames connected or disconnected. In future studies we will attempt to show if the transcriptional expression of the open reading frames flanking the two IR22 sites has been altered by the inversion of the 9-kb fragment. The only obvious consequence of invertase activity was a significant decrease in the number of selectable transconjugants upon transfer of the pVT745 derivatives. The reason for the loss of transconjugants in the presence of a functional invertase is not clear at this point. In two plasmid systems of *Clostridium perfringens*, the activity of a site-specific recombinase has been associated with the excision of a conjugative transposon, Tn4451, and associated resistance cassettes (1, 5, 6). The transposon was consequently lost in *C. perfringens* transconjugants or integrated into the chromosome of *E. coli* recipient cells at a low frequency (2). However, it is unlikely that a transposon or some other putative element present on pVT745 was excising after conjugative transfer. The insertion of a *spc* marker upstream (pDMG28) or downstream of *oriV* (pDMG32) would have permitted selection for deletion derivatives of either construct that might have lost the KAN cassette. However, the number of retrievable transconjugants did not increase when selection was for SPT. Also, the few SPT-resistant transconjugants obtained still carried the KAN resistance marker, suggesting that excision of only a defined fragment was not occurring. The loss of all resistance markers at a high frequency regardless of their location on the plasmids was an indication of the complete loss of the plasmid in recipient cells. Surprisingly, the transfer of pDMG32 increased numbers of transconjugants obtained by 1 order of magnitude when compared to that of pDMG21A and pDMG28, although all three constructs harbor an intact *inv* gene. Furthermore, during screening of selected transconjugants for plasmid content, pDMG32 was isolated with the invertible element in orientations A and B. Therefore, it appears that an additional plasmid-encoded element that acts either in *cis* or in *trans* affects inversion of the 9-kb segment. Such an element has to be located in the vicinity of the *EcoRI* site into which *spc* was inserted in the pDMG32 construct. Its interaction with the invertase gene or gene product appears to be associated with the plasmid loss observed in transconjugants. Likewise, this factor could be involved in regulating the

inversion event when pVT745 DNA enters the cell as discussed above.

At this point the presence of the *inv* gene on pVT745 would appear to be a disadvantage for the effective dissemination of the plasmid via conjugation. In addition, the rearranged version is segregationally unstable, as shown with pDMG21B. It remains to be seen if a true biological role can be associated with *inv* or if the gene is the remnant of a functional element, which once was part of pVT745 but subsequently lost.

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