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Streptococcal Plasmid pIP501 Has a Functional *oriT* Site

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DNA sequence analysis suggested the presence of a plasmid transfer origin-like site (*oriT*) in the gram-positive conjugative plasmid pIP501. To test the hypothesis that the putative *oriT* site in pIP501 played a role in conjugal transfer, we conducted plasmid mobilization studies in *Enterococcus faecalis*. Two fragments, 49 and 309 bp, which encompassed the *oriT* region of pIP501, were cloned into pDL277, a nonconjugative plasmid of gram-positive origin. These recombinant plasmids were mobilized by pVA1702, a derivative of pIP501, at a frequency of 10^{-4} to 10^{-5} transconjugants per donor cell, while pDL277 was mobilized at a frequency of 10^{-8} transconjugants per donor cell. These results indicated that the *oriT*-like site was needed for conjugal mobilization. To demonstrate precise nicking at the *oriT* site, alkaline gel and DNA-sequencing analyses were performed. Alkaline gel electrophoresis results indicated a single-stranded DNA break in the predicted *oriT* site. The *oriT* site was found upstream of six open reading frames (*orf1* to *orf6*), each of which plays a role in conjugal transfer. Taken together, our conjugal mobilization data and the in vivo *oriT* nicking seen in *Escherichia coli* argue compellingly for the role of specific, single-stranded cleavage in plasmid mobilization. Thus, plasmid mobilization promoted by pVA1702 (pIP501) works in a fashion similar to that known to occur widely in gram-negative bacteria.

Conjugation is believed to be the most common means of genetic transfer in the microbial world. Bacterial conjugation mechanisms in gram-negative organisms have been intensely studied, and the molecular mechanisms associated with transfer are well characterized (16–18). Conjugative plasmid DNA transfer from the donor to the recipient cell involves the processing of DNA by transfer-specific gene products. This subject has been recently reviewed and may be summarized as follows (14, 18, 29, 30). A strand- and site-specific endonuclease (nickase) makes a single-stranded nick at the plasmid transfer origin (*oriT*) locus. Depending on the plasmid system, this nickase works in concert with other proteins, and the DNA-protein complex consisting of these various components is commonly called the relaxosome or relaxation complex. The nickase protein forms a covalent bond with the terminus of the nicked strand, and strand displacement occurs accompanied by transport of a single strand of DNA from the donor to the recipient cell in a 5′→3′ direction. The termination of strand transfer is *oriT* site dependent and requires the enzymatic ligation of the linear molecular into a circular form, which is then replicated into duplex DNA. The single-stranded, circular molecule remaining in the donor cell is also replicated, restoring a copy of the conjugative plasmid. The site- and strand-specific nicking event at the *oriT* site can be induced in vitro by protein denaturants or proteolysis (18, 29, 30). "Relaxed"-form plasmid DNA containing the site-specific nick at *oriT* can also be separated from the supercoiled form following CsCl-ethidium bromide equilibrium centrifugation (6).

Comparable processing of conjugally transferred DNA in gram-positive systems has not been demonstrated. In the streptococci and enterococci, there are three types of conjugative elements: broad-host-range plasmids, pheromone-responding plasmids, and conjugative transposons (3, 5, 23). There is an emerging appreciation of the genetic organization of the regions involved in conferring conjugation in these differing systems. Studies on a pheromone-responding plasmid, pAD1, defined several genes involved in the conjugation process, and the existence of an *oriT* site has been proposed (3, 4, 7, 11, 12, 25). Genes involved in the conjugative activity of Tn*916*, a conjugative transposon, have been identified, but their precise functions are not known (5, 13).

In the case of the streptococcal broad-host-range conjugative plasmids, we have used pIP501 as a model to study plasmid transfer (15, 19, 20). We have identified two conjugative regions, A and B, in a derivative of pIP501 called pVA1702. The nucleotide sequence of the A region was determined, revealing six open reading frames (ORFs) (*orf1* to *orf6*) resembling an operon. Here we report a sequence immediately upstream from these genes with striking similarity to the *oriT* loci of gram-negative plasmids. Next to the pVA1702 *oriT*-like site, we found a gene (*orf1*) which encoded a putative protein similar in sequence to the site-specific endonucleases (nickases) of gram-negative plasmids. Our data demonstrate that the *oriT* site of pVA1702 is essential to the mobilization of non-self-transmissible plasmids. Moreover, we present evidence that in *Escherichia coli*, nicking at the predicted *oriT* site occurs and this nicking is catalyzed by the product of the *orf1* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Plasmids and bacterial strains used in this study are described in Tables 1 and 2. A derivative of pIP501 was used in this study. This plasmid, called pVA1702, is a conjugation-proficient deletion derivative of pIP501 whose smaller size has facilitated transposon mutagenesis studies (19). *Enterococcus faecalis* strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), and *E. coli* strains were grown in Luria broth (GIBCO-BRL Inc., Gaithersburg, Md.). Agar (1.5%) was included when solid medium was desired. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations: carbenicillin, 50 μg/ml; kanamycin, 1,000 μg/ml; spectinomycin, 600 μg/ml for *E. faecalis* and 50 μg/ml for *E. coli*; rifampin, 20 μg/ml; fusidic acid, 20 μg/ml; and erythromycin, 20 μg/ml.

Plasmid DNA isolation, manipulation, and nucleotide sequence analysis. The isolation of plasmid DNA from *E. faecalis* followed the methods of Clewell et al. (8). Large-scale preparation of *E. coli* plasmid DNA used for nucleotide sequence analysis and cloning was done with Qiagen columns according to methods described by the manufacturer (Qiagen Inc., Chatsworth, Calif.). Restriction endonucleases, T4 DNA ligase, and Klenow fragment of DNA polymerase I were purchased from New England Biolabs, Inc., Beverly, Mass., or Bethesda Re-

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TABLE 1. Plasmids used in this study

Plasmid	Size (kb)	Replicon	Drug resistance[a]	Comments	Source or reference
pIP501	30.1	pIP501	Cm^r, Em^r	Parental plasmid	15
pVA1702	25.2	pIP501	Km^r	pIP501 derivative	19
pVA1857	11.8	ColE1	Cb^r	*Eco*RI-*Bam*HI fragment of pVA1702::Tn*917lac* insert 104 cloned into pGEM3	19
pVA1860	7.5	ColE1	Cb^r	*Eco*RI-*Bam*HI fragment of pVA1702::Tn*917lac* insert 33 cloned into pGEM3	19
pVA1861	6.2	ColE1	Cb^r	*Eco*RI-*Sph*HI fragment of pVA1860 cloned into pGEM3	19
pVA1862	7.0	ColE1	Cb^r	*Sph*I-*Bam*HI fragment of pVA1858 cloned into pGEM3	19
pDL277	6.6	pVA380-1 (*E. coli*)	Sp^r	*E. coli-Streptococcus* shuttle plasmid	22
pVA2241	6.9	pVA380-1 (*E. coli*)	Sp^r	A 309-bp fragment of pVA1702 (from *Sau*3AI to *Mbo*II) which encompassed the *oriT* region cloned into pDL277 *Bam*HI and *Hin*cII sites	This study
pVA2243	7	pVA380-1 (*E. coli*)	Sp^r	A 479-bp fragment of pVA1702 (from *Hin*dIII to *Sau*3AI) located downstream of the *oriT* region cloned into pDL277 *Hin*dIII and *Bam*HI sites	This study
pVA2245	10.6	pVA380-1 (*E. coli*)	Sp^r	A 4-kb fragment of pVA1862 (from *Sph*I to *Bam*HI) which contained 3,799 bp of the A region sequence of pVA1702 cloned into pDL277 *Sph*I and *Bam*HI sites	This study
pVA2261	6.6	pVA380-1 (*E. coli*)	Sp^r	A 49-bp fragment which encompassed the *oriT* region of pVA1702 cloned into pDL277 *Bam*HI and *Eco*RI sites	This study

[a] Drug resistance phenotypes: Cb^r, carbenicillin; Cm^r, chloramphenicol; Em^r, erythromycin; Km^r, kanamycin; Sp^r, spectinomycin. Concentrations used are described in Materials and Methods.

search Laboratories, Inc., Gaithersburg, Md., and used as specified by the manufacturers. Restriction enzyme digestion, ligation, transformation, PCR, and agarose gel electrophoresis were performed as previously described (27).

Plasmids containing various regions of the pVA1702 A region were constructed to determine the functionality of the putative *oriT* region. Regions containing the *oriT* with various flanking DNA or other fragments from the A region not encompassing the *oriT* region were introduced into pDL277 as PCR-amplified fragments or purified restriction fragments obtained directly from a pVA1702 derivative. Plasmid constructions described in Table 1 are illustrated in Fig. 2. The DNA insert carried by pVA2261, containing just the 49-bp putative *oriT* site from pVA1702 (see Fig. 3), was constructed by annealing two single-stranded oligonucleotides.

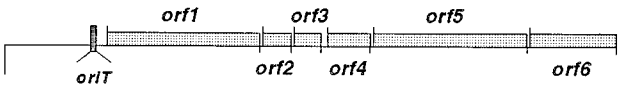
Plasmid mobilization assays were performed with isogenic strains of *E. faecalis* OG1. Each donor cell contained pVA1702 and a pDL277 derivative plasmid carrying a specific sequence of the A region (Table 2; also see Fig. 2). Plasmids were introduced into host cells by electroporation (10). The transfer frequency was expressed as the number of transconjugants divided by the number of input donor cells at the initiation of the mating. The conjugation frequency of pVA1702 was considered the baseline control for all matings (approximately 10^{-3} transconjugants per donor cell). Matings were conducted for 10 h. To evaluate comobilization of the nonconjugative plasmid, 100 colonies obtained following mating were scored for appropriate antibiotic resistance markers.

Nucleotide sequence analysis was performed by the Sanger dideoxy-chain termination method with double-stranded DNA as a template. The reactions were run as recommended by the manufacturer (U.S. Biochemicals, Cleveland, Ohio). For automated sequencing, the reactions were run on a Taq DyeDeoxy Terminator Cycle Sequencing Kit as specified by the manufacturer (ABI, Foster City, Calif.). The complete nucleotide sequence of both strands of the pVA1702 A region was determined. Sequencing data were subjected to computer analysis

TABLE 2. Bacterial strains used in this study

Species and strain	Description[a]	Source or reference
E. faecalis		
OG1	Host strain	D. Clewell, University of Michigan
OG1-RF	Rf^r Fs^r mobilization recipient	D. Clewell, University of Michigan
V1702	OG1 carrying pVA1702	19
V2213	OG1 carrying pVA1702 and pDL277	This study
V2242	OG1 carrying pVA1702 and pVA2241	This study
V2244	OG1 carrying pVA1702 and pVA2243	This study
V2246	OG1 carrying pVA1702 and pVA2245	This study
V2262	OG1 carrying pVA1702 and pVA2261	This study
E. coli		
HB101	*hsdS20 recA13 proA2 endA leu-6 thi-1 supE44 rpsL20 ara-14 galK2 lacYI xyl-5 mal-1*	B. A. D. Stocker, Stanford University
DH5α	F^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_k^- m_k^+) *supE44*λ^- *thi-1 gyrA96 relA1*	Bethesda Research Laboratories
V1857	HB101 carrying pVA1857	19
V1860	HB101 carrying pVA1860	19
V1861	HB101 carrying pVA1861	19
V1862	HB101 carrying pVA1862	19
V2241	DH5α carrying pVA2241	This study
V2243	DH5α carrying pVA2243	This study
V2245	DH5α carrying pVA2245	This study
V2261	DH5α carrying pVA2261	This study

[a] Drug resistance phenotypes: Rf^r, rifampin; Fs^r, fusidic acid.

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pVA1702      ATACGAAGTAACGAAGTTACTGCGTATAAGTGCGCCCTTAGT
             ----- -----> <----- -----
                  *****       ************

RSF1010        CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCCC
               -- ------> <------ --
                  *****       ************

pTF1           GGGTAATCTCGAAGAGATTACTCTAAGTGCGCCCTTGC
               - -------> <-------- -
                  *****       ************
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FIG. 1. The *oriT*-like site of pVA1702. The molecular arrangement of the A region of pVA1702 is shown at the top. The putative *oriT* site is found upstream of the clustered genes labelled *orf1* to *orf6*. Coordinates of the 8,128-bp A region are as follows: *oriT*, 1259 to 1296; *orf1*, 1415 to 3377; *orf2*, 3403 to 3733; *orf3*, 3754 to 4135; *orf4*, 4211 to 4781; *orf5*, 4794 to 6753; and *orf6*, 6769 to 8121. The nucleotide sequence of the putative *oriT* site of pVA1702 aligned with similar sequences found in the gram-negative plasmids pTF1 and RSF1010 is shown below. Palindromic sequences are marked by dashed lines and converging arrows. Identical sequences among these three plasmids are indicated by the asterisks.

with the GCG Computer Analysis package (Genetics Computer Group, Inc., Madison, Wis.) or the MacVector software package (IBI).

Localization of the nick site. Preparation of plasmid DNA for identification of the *oriT* site followed the procedure described by Clewell and Helinski (6, 9). Following gentle cell lysis with the nonionic detergent Brij 58, cleared lysates were subjected to two cycles of CsCl-ethidium bromide equilibrium ultracentrifugation (40,000 rpm at 19°C for 24 h in a TV865B rotor). DNA extraction from such gradients followed standard methods (27). The recovered plasmid DNA then was treated with 2.5% sodium dodecyl sulfate for 10 min and pronase (0.5 mg/ml) for 30 min at 37°C and extracted with phenol-chloroform (1:1, vol/vol) twice.

A 3-μg sample of plasmid DNA corresponding to the upper fluorescent component of a second equilibrium centrifugation (open circular [relaxed] form) was linearized with an appropriate restriction enzyme. A sample of one-fifth volume of digested plasmid DNA was electrophoresed on a 1% standard agarose gel, and the remaining material was electrophoresed on a 1.2% alkaline gel (24). To localize the nick site more accurately, chain termination DNA sequencing was applied by using a Sequenase kit (U.S. Biochemicals). Primers (Oligos Etc. Inc., Wilsonville, Oreg.) that specifically annealed about 100 bp upstream and downstream of the nick site in the opposite directions were used to carry out DNA sequencing. The template plasmid DNA was treated the same as that in alkaline gel analysis before the nucleotide sequencing reactions were carried out. To minimize compression problems, both dITP and 7-deaza-dGTP were used.

Nucleotide sequence accession number. The nucleotide sequence data reported here may be found under GenBank accession number L39769.

RESULTS

Mobilization assays to test *oriT* functions. The nucleotide sequence of the A region of pVA1702 was determined, and the arrangement of six ORFs found in this region is shown in Fig. 1. Interestingly, we identified a sequence upstream of the A region *orf* genes which was similar to *oriT* regions from certain plasmids of gram-negative origin (Fig. 1). To test the hypothesis that the putative *oriT* sequence in pVA1702 played a role in conjugal transfer, we introduced various sequences from pVA1702 into an *E. coli-Streptococcus* shuttle plasmid, pDL277. pDL277 is a nonconjugative plasmid which carried a spectinomycin resistance determinant (21a, 22) (Fig. 2). When pDL277 was electroporated into *E. faecalis* containing pVA1702, it was poorly mobilized by pVA1702 in isogenic matings. Such mobilization was within 1 order of magnitude of the lowest limit of detection of this mating system. The recombinant plasmids constructed with pDL277 carried pVA1702

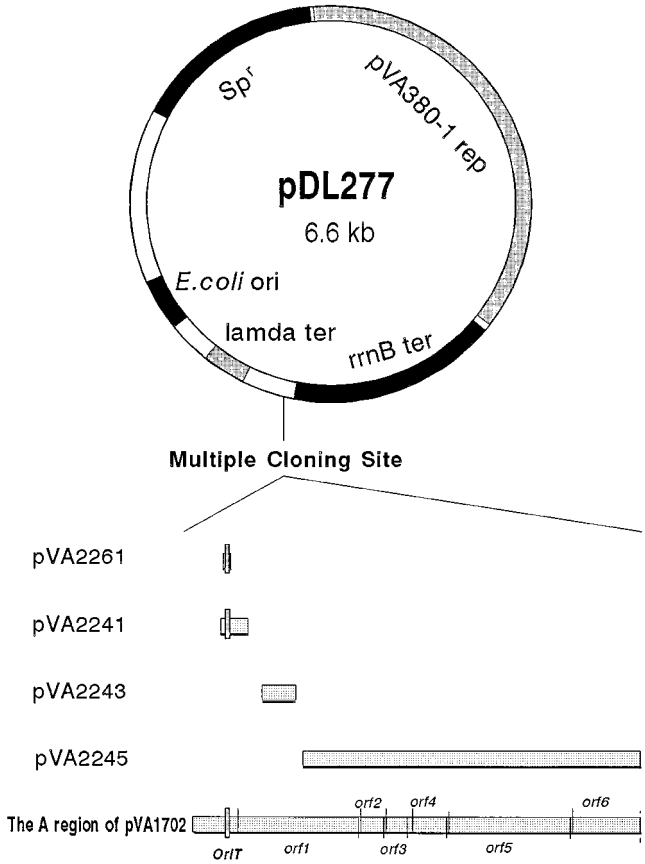

FIG. 2. Plasmid constructs used in the mobilization assays. The various lengths of the A region DNA sequence of pVA1702 were cloned in the multiple-cloning site of pDL277 to construct pVA2261, pVA2241, pVA2243, and pVA2245. pDL277 (6.6 kb) carries a spectinomycin resistance (Sp^r) marker and a multiple-cloning site bordered by λ and *rrnB* transcription terminators. The A region sequence of pVA1702 is schematically presented with the *oriT* site and its downstream ORFs (*orf1* to *orf6*) at the bottom of the figure. The DNA fragments cloned in each plasmid are positioned corresponding to the A region sequence of pVA1702 with sizes to scale. pVA2261 and pVA2241 contained the *oriT* site, designated by the vertical dark-stippled rectangle. pVA2243 and pVA2245 contained only sequences downstream of the *oriT* site.

fragments ranging from 49 bp to 4 kb. Mobilization of these derivatives and pVA1702 was detected by selection of colonies on the appropriate antibiotic-containing media (Table 3). Colonies which grew on rifampin and fusidic acid plates (chromosomal markers in recipient strain OG1) and kanamycin plates (pVA1702 marker) corresponded to transconjugants that received pVA1702, while the colonies recovered on rifampin, fusidic acid, and spectinomycin (pDL277 marker) plates indicated the mobilization of pDL277 derivative plasmids. The transfer frequencies of pVA1702 were about 10^{-3} transconjugants per donor in such crosses. pDL277 served as a control plasmid in mobilization assays. It was mobilized by pVA1702 at frequencies about 6.8×10^{-8} (donor, V2213). pVA2261, containing a fragment of 49 bp which included the nick site, was mobilized at frequencies around 4.6×10^{-5} transconjugants per donor (donor, V2262). The mobilization frequency of pVA2241, containing a fragment of 309 bp around the nick site, was about 4.8×10^{-4} transconjugants per donor (donor, V2242). These results demonstrated that the nonconjugative pDL277, which contained a fragment of 49 bp encompassing the putative *oriT*, could be mobilized by pVA1702. The sequence of the synthetic 49-bp fragment used in this work is

TABLE 3. Plasmid mobilization assay

Donor strain[a]	Plasmids contained	*oriT* in pDL277 derivatives[b]	Size (bp) of DNA from pIP501 cloned in pDL277[c]	Frequency of transfer of pVA1702[d]	% Cotransfer[e]	Frequency of transfer of pDL277 derivatives	% Cotransfer[f]
V2262	pVA2261	Yes	49	7.2×10^{-3}	ND[g]	4.8×10^{-5}	ND
	pVA1702			4.0×10^{-4}	2	3.5×10^{-5}	26
				1.4×10^{-3}	0	4.4×10^{-5}	30
				8.0×10^{-4}	1	6.1×10^{-5}	30
Mean ± SD				$1.3 \times 10^{-3} \pm 3.4 \times 10^{-3}$	1 ± 1	$4.6 \times 10^{-5} \pm 1.2 \times 10^{-5}$	28.7 ± 2.3
V2242	pVA2241	Yes	309	1.6×10^{-3}	ND	3.6×10^{-4}	ND
	pVA1702			1.1×10^{-3}	8	3.7×10^{-4}	43
				5.0×10^{-3}	7	8.3×10^{-4}	44
				1.9×10^{-3}	9	4.7×10^{-4}	44
Mean ± SD				$2.0 \times 10^{-3} \pm 1.9 \times 10^{-3}$	8 ± 1	$4.8 \times 10^{-4} \pm 1.5 \times 10^{-4}$	43.7 ± 0.6
V2246	pVA2245	No	3,799	9.5×10^{-4}	ND	1.9×10^{-5}	ND
	pVA1702			5.4×10^{-5}	53.3	3.8×10^{-5}	98
				2.5×10^{-4}	45	1.3×10^{-4}	100
				1.4×10^{-4}	67.2	7.3×10^{-5}	100
Mean ± SD				$2.1 \times 10^{-4} \pm 3.3 \times 10^{-4}$	55.2 ± 11.2	$5.1 \times 10^{-5} \pm 2.3 \times 10^{-5}$	99.3 ± 1.2
V2244	pVA2243	No	479	1.5×10^{-3}	ND	2.0×10^{-7}	ND
	pVA1702			9.2×10^{-4}	0	6.0×10^{-8}	76.5
				2.6×10^{-3}	0	2.3×10^{-7}	66.1
				2.3×10^{-3}	0	1.3×10^{-7}	72
Mean ± SD				$1.7 \times 10^{-3} \pm 1.6 \times 10^{-3}$	0 ± 0	$1.4 \times 10^{-7} \pm 1.8 \times 10^{-7}$	71.5 ± 5.2
V2213	pDL277	No	0	1.4×10^{-3}	ND	1.6×10^{-7}	ND
	pVA1702			1.1×10^{-3}	1	1.8×10^{-8}	72.7
				3.3×10^{-3}	0	1.3×10^{-7}	88.2
				1.2×10^{-3}	0	6.0×10^{-8}	86.7
Mean ± SD				$1.6 \times 10^{-3} \pm 1.7 \times 10^{-3}$	0.3 ± 0.6	$6.8 \times 10^{-8} \pm 2.7 \times 10^{-8}$	82.5 ± 8.5

[a] V1103 served as a recipient in all mating. For all donor cells, see Tables 1 and 2.
[b] *oriT* site of pIP501 contained in pDL277 derivatives.
[c] The size of the DNA fragment from pIP501 cloned into pDL277 derivatives: pVA2261, pVA2241, pVA2245, and pVA2243.
[d] The transfer frequencies were calculated as the number of transconjugants per donor cell. This column shows the results from four independent experiments.
[e] Transconjugants containing pVA1702 received pDL277 derivatives.
[f] Transconjugants containing pDL277 derivatives received pVA1702.
[g] ND, not done.

shown in Fig. 3. The mobilization frequencies of pVA2243 (donor, V2244), which contained a 479-bp fragment downstream of *oriT*, were close to that of pDL277 (1.4×10^{-7} compared with 6.8×10^{-8} transconjugants per donor). pVA2245 (donor, V2246), which carried a 3,799-bp fragment from pVA1702 without the putative *oriT* site, was mobilized at a frequency around 5.1×10^{-5} transconjugants per donor cell.

Mapping of the nick site within the *oriT* region. When cleared lysates are subjected to CsCl-ethidium bromide equilibrium centrifugation, open circular plasmid DNA accumulates in gradients and may be found at a position above native, covalently closed, supercoiled plasmid DNA (6). Under such conditions, the high solute concentration of the gradient probably perturbs the protein structure, generating open circular plasmid DNA. Thus, we used equilibrium centrifugation to isolate nicked plasmid DNA for examination of specific cleavage at *oriT*. pVA1857, an *E. coli* recombinant plasmid, contained the entire A region sequence of pVA1702 and was used as a model plasmid in these studies. The upper fluorescent components in CsCl-ethidium bromide gradients contained open circular plasmid DNA, and the lower components corresponded to closed circular plasmids. Open circular plasmid DNA was subjected to nick site analysis. After electrophoresis under alkaline denaturing conditions, the appropriate linearized plasmid DNA with a specific, single-strand break at the *oriT* site generated three single-stranded DNA species while those that were randomly nicked showed only one single-stranded DNA species (i.e., unit-length DNA was revealed as a result of random single nicking events on the two strands). The predicted *oriT* nick on each of these specifically linearized fragments is shown by the arrows in Fig. 4A. In all cases, the standard agarose gel showed a uniform single component of 11.8 kb (Fig. 4B, lanes B, C, and D). However, on alkaline gels, the double-stranded DNA was denatured; thus, three distinct bands corresponding to the expected sizes based on nicking at the predicted *oriT* site were detected (Fig. 4C, lanes B, C, and D). In all three cases, the largest fragment corresponded to full-length single-stranded plasmid DNA and the different sizes of the other two bands equaled full-length single-stranded linear plasmid. The predicted sizes of the non-unit-length strand indicated on the right side of Fig.

```
    AATTCTACTAAGGGCGCACTTATACGCAGTAACTTCGTTACTTCGTATTTATGCG  BamHI
EcoRI  GATGATTCCCGCGTGAATATGCGTCATTGAAGCAATGAAGCATAAATACGCCTAG
       ************------ ------>   <----- -----
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FIG. 3. Synthetic 49-bp *oriT* fragment. This fragment was inserted into the *Eco*RI and *Bam*HI sites of pDL277 to create pVA2261. The conserved region that was aligned with other *oriT* sites in gram-negative bacteria is marked with asterisks. Palindromic sequences are marked with dashed lines and arrows. The design of the components of this fragment generated 4-bp, single-stranded *Bam*HI- and *Eco*RI-compatible termini.

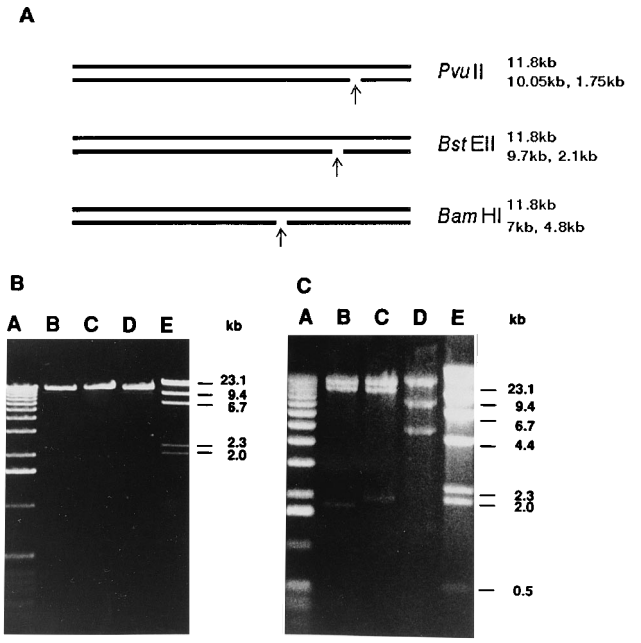

FIG. 4. Detection of specific nicking of pVA1857. (A) Schematic representation of single-stranded species generated by a site-specific nick (arrow) on endonuclease-digested pVA1857 DNA. The sizes of each single-stranded DNA fragment are listed on the right. (B and C) Products were analyzed by standard agarose gel electrophoresis (B) or alkaline agarose gel electrophoresis (C). Lanes: A, 1-kb ladder; B to D, pVA1857 DNA digested, respectively, with *Pvu*II, *Bst*EII, or *Bam*HI; E, λ *Hin*dIII fragments.

4A were observed and corresponded to the products of *oriT* site cleavage.

To localize the nick site more accurately, nucleotide sequence determinations were performed. The results revealed a break in the template DNA within the predicted *oriT* sequence. With such a template, the DNA polymerase reactions were expected to cause the reaction products to accumulate (or "pile up") in each of the four sequencing reaction lanes on the 3′ side of the *oriT* nick. These pile-up products are seen in the right-hand panel of the autoradiographs in Fig. 5, with the appropriate DNA sequence pattern leading up to the end of

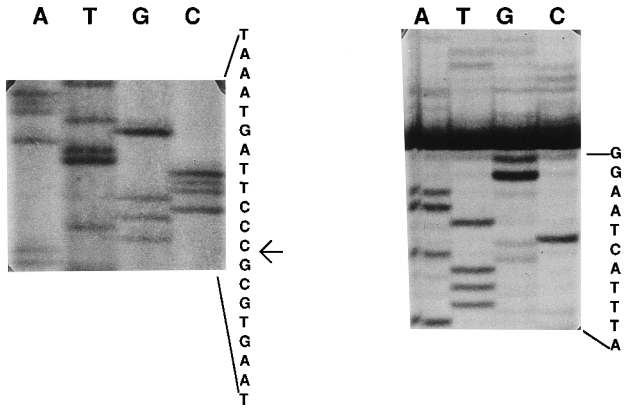

FIG. 5. Localization of the specific nicked site in the *oriT* region of pVA1857. The sequences of the two strands of the presumptive *oriT* are shown. The order of sequencing reactions is A, T, G, and C from left to right in each panel. The sequences, as written, read upward in the 5′-to-3′ direction. The arrow indicates the specific nick site.

the template. The left panel of Fig. 5 shows the DNA sequence of the complementary strand of *oriT* region. Analysis of the break in the DNA sequence by this strategy allowed us to define the precise nick site, as illustrated by the arrow in Fig. 5.

Analysis of ORF1 function. The nicking of the *oriT* site of pVA1702 was demonstrated in the *E. coli* plasmid, pVA1857, which contained the entire A region of pIP501. This suggested that endonuclease activity (nickase) of pVA1702 was encoded by the A region DNA sequence. To determine the DNA coding region of the nickase, plasmids containing the *oriT* sequence along with various segments of the A region were tested for their ability to generate *oriT*-nicked DNA. Four plasmids, pVA1857, pVA1860, pVA1861, and pVA2241, were used to evaluate the A region sequence needed for nicking in *E. coli* (Fig. 6A). pVA1857 contained a DNA fragment which included the entire A region of pVA1702; pVA1860 contained a DNA fragment which included the *oriT* site and the entire *orf1* gene; and pVA1861 contained a DNA fragment which included the *oriT* site and part of the *orf1* gene, which encoded 212 N-terminal amino acids of ORF1. pVA2241 contained the *oriT* site with no *orf1* gene sequences. Each of these plasmid DNA were prepared by the Clewell and Helinski (6) cleared-lysate technique and subjected to ethidium bromide-CsCl ultracentrifugation as described in Materials and Methods. All of these plasmids displayed two components (Fig. 6B) following CsCl-ethidium bromide equilibrium centrifugation. The lower components of these four plasmids, previously shown to be covalently closed circular DNA, had similar intensities of fluorescence. The upper components, previously shown to be open circular plasmid DNA, displayed differing fluorescence intensities. The fluorescence intensities of the upper components of pVA1857 and pVA1860 were similar. However, the upper component of pVA1861 was less intense than those of pVA1857 and pVA1860. Moreover, the intensity of the upper component of pVA2241 was lower than that of pVA1861. Thus, pVA1860, carrying only the *orf1* gene, gave rise to similar amounts of open circular plasmid DNA to those of pVA1857 containing the entire A region DNA fragment. Deletion of the gene encoding 442 C-terminal amino acids of ORF1 dramatically decreased the yield of open circular plasmid (Fig. 6A, pVA1861).

Next, we looked at the open circular products recovered in these experiments to evaluate whether nicking had occurred at the *oriT* site. To do this, the open circular plasmid DNAs of pVA1857, pVA1860, pVA1861, and pVA2241 were examined by alkaline agarose gel electrophoresis (Fig. 6C). *Bam*HI-digested pVA1857 and pVA1860, *Pvu*II-digested pVA1861, and *Xba*I-digested pVA2241 all were subjected to alkaline gel electrophoresis. pVA1857, pVA1860, and pVA1861 all revealed three bands on alkaline gels. The observed sizes were consistent with products resulting from nicking at the predicted *oriT* site. However, *Xba*I digests of pVA2241 gave rise to a single band on alkaline gel analysis, indicating it consisted of randomly nicked open circular plasmid molecules. The open circular form of plasmid pVA2241 observed following CsCl-ethidium bromide equilibrium centrifugation is presumably from nonspecific nicking of plasmid DNA during preparation. Taken together, these results indicated that the *orf1* gene product participated in the endonuclease cleavage at the *oriT* site. Additionally, some activity of the ORF1 protein remained after deletion of up to 442 amino acids from the C terminus of the protein as indicated by a small amount of *oriT*-nicked open circular plasmid DNA recovered from CsCl-ethidium bromide gradients.

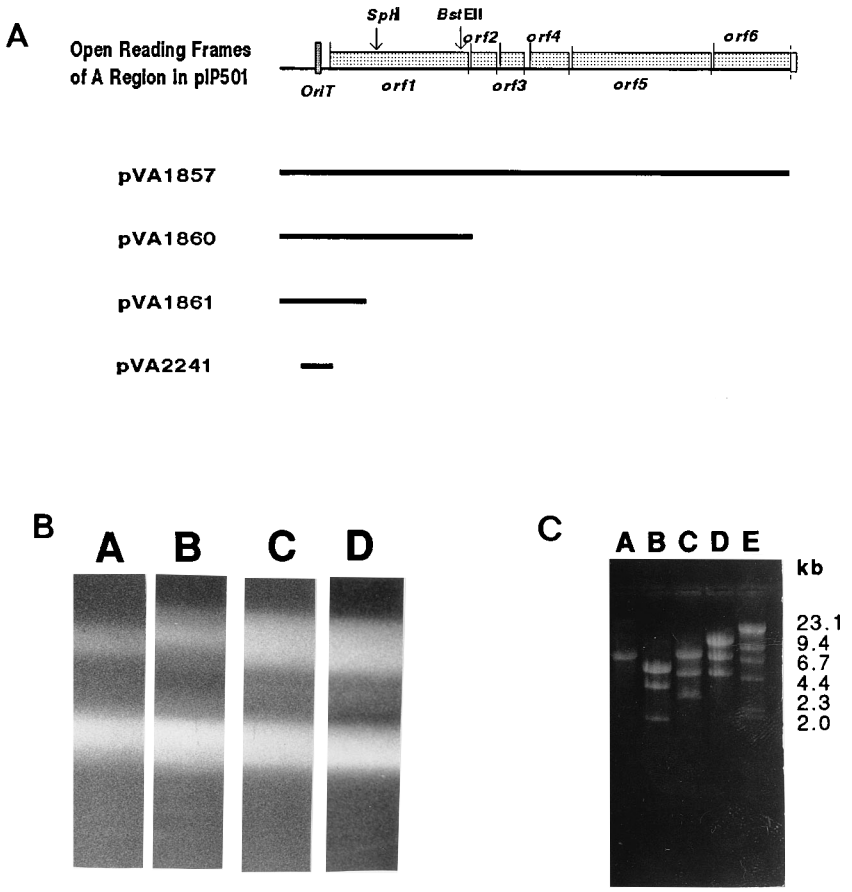

FIG. 6. Evaluation of ORF1 function. (A) Schematic presentation of the pVA1702 A region and plasmids used in *orf1* function analysis. pVA1857 contains the *oriT* region and the entire A region sequence of pVA1702. pVA1860 contains the *oriT* region and the *orf1* sequence of pVA1702. pVA1861 contains the *oriT* region and part of the *orf1* sequence of pVA1702. pVA2241 contains the *oriT* region of pVA1702. (B) Photograph of ethidium bromide-cesium chloride gradients displaying DNA recovered from *E. coli* lysate. A, B, C, and D indicate the gradient of pVA2241, pVA1861, pVA1860, and pVA1857, respectively. The upper components are open circular plasmids, and the lower components are closed circular plasmids. (C) Alkaline agarose gel electrophoresis of various open circular plasmid DNAs. Lanes: A, pVA2241 digested with *Xba*I; B, pVA1861 digested with *Pvu*II; C, pVA1860 digested with *Bam*HI; D, pVA1857 digested with *Bam*HI; E, λ *Hin*dIII ladder.

DISCUSSION

pIP501 is a paradigm for studying broad-host-range, gram-positive conjugal transfer. Our DNA sequence analysis revealed a putative *oriT* site and a nickase-like gene product in the pIP501 A region. We demonstrate here that mobilization of plasmids by pVA1702 is dependent on the presence of the *oriT* sequence. Analysis of plasmid DNA carrying this *oriT* sequence revealed that precise nicking of the sequence is reminiscent of that in gram-negative systems. Finally, our data implicated one of the genes (*orf1*) downstream of the *oriT* sequence in this nicking process, most probably by serving as the site-specific single-stranded endonuclease (nickase).

Gram-negative *oriT* sequences characteristically are 50 bp or less and are composed of a small sequence into which the nick site is embedded along with adjacent inverted repeat sequences. We discovered an *oriT*-like sequence in the pVA1702 A region which had striking similarities to the *oriT* sequences of RSF1010 and pTF1 (Fig. 1). *oriT* sites function in *cis* by interacting with the proteins of the so-called relaxosome to initiate transfer. We were able to construct various plasmids that could be assayed for pVA1702-promoted mobilization. pDL277, a nonconjugative plasmid containing a multiple cloning site, was used to create four plasmids that could be tested for pVA1702-promoted conjugative mobilization (Fig. 2). pDL277 was mobilized at barely detectable frequencies (10^{-7} to 10^{-8}) by pVA1702, and the linkage (83% coinheritance) of the kanamycin and spectinomycin resistance markers in the progeny suggested that this transfer was due to cointegrate formation (Table 3, donor strain V2213). However, addition of a synthetic *oriT* (Table 3, donor V2262) or 309-bp fragment carrying the *oriT* sequence resulted in pDL277 mobilization at frequencies of 10^{-4} to 10^{-5}. A 479-bp fragment from the A region not carrying the *oriT* sequence was mobilized at frequencies around 10^{-7}. These data support the role of the *oriT* sequence in transfer. A 3,799-bp fragment (V2246 donor) without the *oriT* was mobilized at frequencies similar to those seen for *oriT*-containing fragments in this system (10^{-4} to 10^{-5}). Analysis of transconjugants from matings with the V2246 donor indicated a tight linkage (99.3%) between the pDL277 spectinomycin resistance marker and the kanamycin resistance marker of pVA1702. This result is consistent with mobilization via cointegrate formation. However, in matings involving constructs with an intact *oriT*, the linkages of these two markers were 28.7 to 43.7%, suggesting mobilization in *trans* as being the predominant mode.

Postulated mobilization of pDL277 derivatives by cointegration can be ascribed to homologous sequences present on both pVA1702 and the pDL277 derivatives. Interestingly, Langella

et al. (21) have described a region on pIP501 which, when cloned in nonconjugative plasmids, confers high-frequency mobilization via cointegrate formation. This DNA fragment did not originate from the A region but, rather, was cloned from a region of the pIP501 plasmid designated C (20). The C region was characterized by Krah and Macrina (20) and was thought to be involved in the stable establishment of pIP501 in various host strains. Langella et al. (21) postulated that this *mob* site contained within the C region is able to serve as a hot spot for cointegrate formation. They reconciled their data with those of Krah and Macrina (20) by suggesting that their *mob* region is a site for plasmid multimer formation and resolution. This would account for a possible dual role of this region in plasmid replication (establishment) and intermolecular interactions. pVA1702 does not carry the C region, which contains the proposed cointegrate hot spot (20, 21), so this sequence had no influence on our mobilization studies.

To demonstrate that the putative *oriT* site was nicked in vivo, we used an *E. coli* host cell system. The low copy number of pVA1702 and difficulties in gently lysing *E. faecalis* precluded the isolation of protein-plasmid DNA complexes from this organism. The nicked site in the *oriT* region could be measured by cleaving relaxed DNA with a single-cleaving restriction enzyme and then analyzing the sizes of the single-stranded products on alkaline agarose gels. Results of such experiments clearly indicated that *oriT*-specific nicking occurred in *E. coli* (Fig. 4). DNA sequencing reactions further enabled us to explore the precise position of the nick created in the *oriT* sequence (Fig. 5). That nicking at *oriT* actually occurs in *E. faecalis* is strongly suggested by our plasmid mobilization studies with pVA2261 (Table 3, matings with V2262 donor). The addition of a 49-bp sequence representing the palindromic region and the 12-bp conserved region was enough to restore the ability of pDL277 to be mobilized by pVA1702.

The ability of pVA1860 to generate specifically *oriT*-nicked plasmid DNA in *E. coli* suggests that the *orf1* gene product is necessary and sufficient to achieve this cleavage (Fig. 6). This is an interesting observation in light of the usual need for accessory proteins to effect such cleavage in gram-negative systems (18). However, we cannot rule out that *E. coli* specified proteins are working in concert with the *orf1* gene product in these experiments. The genetic and biochemical conservation of the *orf1* gene product is further suggested in the behavior of the pVA1861 plasmid (Fig. 6). Even though this plasmid contains *oriT* and the coding region for only the first 242 N-terminal amino acids of ORF1, this truncated protein is still able to function. Although a relatively small amount of nicked plasmid DNA is recovered from cleared lysate, the fragments demonstrated on alkaline gels are consistent with the plasmid DNA specifically nicked at the *oriT* site (Fig. 6B and C). Other workers have shown that the homologous proteins encoded by *E. coli* plasmids contain endonuclease activities in their N-terminal regions, usually in the first one-third of the gene (2, 28). This also appears to be the case for the *orf1* gene product.

We assume from our observations that the role of the *oriT*-ORF1 protein demonstrated in our mobilization studies is similar to that associated with the self-transfer of pVA1702. That is, the *oriT* site is nicked as part of the initiating events that accompany the transfer of the conjugative plasmid itself. The means to demonstrate this directly with *E. faecalis* carrying pVA1702 are not available at present. Thus far, we have been unable to isolate pVA1702 mutants containing a defective *oriT* site or a defective *orf1* gene.

Although work with other gram-positive conjugal transfer systems provided evidence that plasmid DNA was nicked and that single strands of the molecule were transferred from donor to recipient, direct demonstration of a functional *oriT* site has not been previously made (23). Staphylococcal conjugative plasmid transfer is sensitive to novobiocin, suggesting the need for enzymatic unwinding of DNA in the donor cell which would be consistent with single-strand transfer (1). In addition, physical evidence that nonconjugative staphylococcal plasmids require nicking to be mobilized has been reported (26). Our conjugal mobilization data, taken together with the demonstrated in vivo nicking seen in *E. coli*, argue for the role of specific, single-strand cleavage in plasmid mobilization. Thus, plasmid mobilization promoted by pVA1702 (pIP501) works in a fashion similar to that seen in gram-negative bacteria. The striking homology of the *oriT* site of pVA1702 with that of the gram-negative plasmids RSF1010 and pTF1 suggests that this aspect of the conjugal transfer apparatus has shared a common molecular ancestor. Beyond the *orf1* gene and the *oriT* site, no other pVA1702 (pIP501) sequences characterized in our work or in other studies appear to be related to other known prokaryotic conjugal transfer systems. We are presently investigating the roles of the gene products encoded by *orf2* to *orf6* in pVA1702-promoted conjugal transfer.

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