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Characterization and DNA Sequence of the Mobilization Region of pLV22a from *Bacteroides fragilis*

THOMAS J. NOVICKI[1] AND DAVID W. HECHT[1,2]*

Department of Medicine, VA Hospital, Hines, Illinois 60141,[1] and Department of Medicine, Microbiology, and Immunology, Program in Molecular Biology, Loyola University Chicago, Maywood, Illinois 60153[2]

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A 4.2-kb plasmid (pLV22a) native to *Bacteroides fragilis* LV22 became fused to a transfer-deficient *Bacteroides* spp.-*Escherichia coli* shuttle vector by an inverse transposition event, resulting in a transferrable phenotype. The transfer phenotype was attributable to pLV22a, which was also capable of mobilization within *E. coli* when coresident with the IncPβ R751 plasmid. Transposon mutagenesis with Tn*1000* localized the mobilization region to a 1.5-kb DNA segment in pLV22a. The mobilization region has been sequenced, and five open reading frames have been identified. Mutants carrying disruptions in any of the three genes designated *mbpA*, *mbpB*, and *mbpC* and coding for deduced products of 11.3, 30.4, and 17.1 kDa, respectively, cannot be mobilized when coresident with R751. Mutations in all three genes can be complemented in the presence of the respective wild-type genes, indicating that the products of *mbpA*, *mbpB*, and *mbpC* have roles in the mobilization process and function in *trans*. The deduced 30.4-kDa MbpB protein contains a 14-amino-acid conserved motif that is also found in the DNA relaxases of a variety of conjugal and mobilizable plasmids and the conjugative transposon Tn*4399*. Deletion analysis and complementation experiments have localized a *cis*-acting region of pLV22a within *mbpA*.

Bacterial conjugation is a process in which plasmid or chromosomal DNA is transferred from a bacterial donor cell to a suitable recipient cell. As a result, genetic information, including antibiotic resistance determinants, can be rapidly disseminated to a variety of bacterial species (4, 5). A conjugative plasmid has two sets of requirements for self-transfer: (i) a *cis*-acting origin of transfer, *oriT*, and (ii) numerous *trans*-acting functions that initiate DNA transfer and replication and establish cell-cell contacts. In contrast, mobilizable plasmids typically contain an *oriT* and encode one or two cognate proteins necessary for transfer initiation processes. Coresident conjugative plasmids and the chromosome are thought to subsequently provide the other necessary *trans*-acting proteins for conjugation (1, 9).

A general model for the processing of DNA to initiate transfer, based primarily on experiments with the F factor of *Escherichia coli*, has been described elsewhere. Initial events in transfer include the formation of a relaxation complex at $oriT_F$, with subsequent site- and strand-specific nicking at that site (21). Following nicking, a single strand is transferred to the recipient cell in a 5′-to-3′ direction with complementary strand synthesis in the new host. For detailed reviews of conjugative transfer involving the F factor and selected R factors, see articles by Willetts and Wilkins (21) and Guiney and Lanka (5).

The transfer regions of two *Bacteroides fragilis* transfer factors have been described elsewhere. Hecht et al. have previously described one conjugative plasmid, pBFTM10, that is self-transferrable (Tra^+) in *B. fragilis* (7). In addition, when fused to the *E. coli* replicon pDG5 to give pGAT400, pBFTM10 can also transfer from *B. fragilis* to *E. coli* (7). In *E. coli*, pGAT400 can be mobilized (Mob^+) in the presence of the IncPβ plasmid R751 (7, 19). pBFTM10 contains two genes, *btgA* and *btgB*, and a *cis*-acting *oriT* region that are required for self-transfer in *Bacteroides* spp. and mobilization in *E. coli* when coresident with R751 (7). The mobilization cassette of a second *B. fragilis* transfer factor, Tn*4399*, has recently been described by Murphy and Malamy (8, 12). Tn*4399* also contains two genes, *mocA* and *mocB*, and a *cis*-acting *oriT* region that are required for mobilization in the presence of R751, although these sequences are not homologous with pBFTM10. We now describe the mobilization region of a second *B. fragilis* plasmid, pLV22a. pLV22a was discovered by its ability to mobilize a transfer-deficient (Tra^-) deletion derivative of pGAT400 in *cis*. The mobilization region of pLV22a has been localized and characterized by insertion mutations, deletions, and DNA sequence analysis. pLV22a contains three genes, encoding deduced products of 11.3, 30.4, and 17.1 kDa, that are required for mobilization in *E. coli*. Deletion and mutation experiments have also identified a *cis*-acting region of this plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Media, antibiotic levels, and growth conditions for *Bacteroides* spp. and *E. coli* have been previously described (7). Antibiotics and concentrations used for the selection of strains and plasmids included the following: ampicillin, 200 μg/ml; chloramphenicol, 40 μg/ml; streptomycin, 50 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 10 μg/ml (for *E. coli*) or 5 μg/ml (for *Bacteroides* spp.). Strains containing R751 were grown on Mueller-Hinton medium containing 10 μg of trimethoprim per ml.

Recombinant DNA techniques. Chromosomal DNA was prepared essentially as described by Saito and Miura (17). Plasmid DNA was prepared by (i) miniprep alkaline lysis (18), (ii) CsCl equilibrium gradient separation (18), and (iii) affinity column (Qiagen Corp., Chatsworth, Calif.) purification. All restriction endonucleases, DNA ligase, and S1 nuclease were purchased from Promega (Madison, Wis.).

Construction of plasmids pBF328Δ, pTJ5a, pTJ8-WT, pTJ6, pTJ6XB, and pΔLVO1. To develop a non-tetracycline-resistant cloning vector compatible with pACYC184 for use in the complementation assays, the tetracycline resistance gene of pBR328 was deleted by digestion with *Msc*I-*Eco*RV followed by self-ligation of the resulting 3.8-kb fragment to give pBR328Δ (3). pBR328Δ contains the pBR328 replication region and ampicillin resistance gene and is mobilization deficient (Mob^-). pTJ5a and pTJ8-WT were created by ligation of the 5.3-kb *Eco*RI fragment of pTJ47-1 containing pLV22a and $IS4400_L$ into the unique

* Corresponding author. Mailing address: Loyola University Chicago, Bldg. 54, Room 101, 2160 S. First Ave., Maywood, IL 60153. Phone: (708) 216-2792. Fax: (708) 216-2269. Electronic mail address: dhecht@wpo.it.luc.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) and/or characteristic(s)[a]	Source or reference
Strains		
B. fragilis LV22	Tc^r	This study
B. thetaiotaomicron BT4001	Rif^r	Shoemaker and Salyers (20)
B. thetaiotaomicron BT22.47	Tc^r, Rif^r	This study
E. coli HB101	Sm^r	Sambrook et al. (18)
E. coli DW1030	Sp^r	Robillard et al. (16)
E. coli DH5α	Nal^r	Gibco BRL (Gaithersburg, Md.)
Plasmids		
R751	IncPβ, Tra^+, Tmp^r	Meyer and Shapiro (13)
F′*lac*	IncFl, Tra^+, lac^+	Pasteur Institute
pACYC184	Mob^-, Cm^r, Tc^r	Sambrook et al. (18)
pUC19	Ap^r	Sambrook et al. (18)
pBR322	Mob^-, Tc^r, Ap^r	Sambrook et al. (18)
pBR328	Mob^-, Cm^r, Tc^r, Ap^r	Covarrubias et al. (3)
pBR328Δ	Mob^-, Ap^r, deletion derivative of pBR328	This study
pGAT400	(Tra^+), (Cc^r), Ap^r	Hecht and Malamy (8)
pGAT400ΔBglII	(Tra^-), (Cc^r), Ap^r, deletion derivative of pGAT400	Hecht and Malamy (8)
pLV22a	LV22 native plasmid	This study
pTJ47-1	(Tra^+), Mob^+, Ap^r, pLV22a::pGAT400ΔBglII fusion	This study
pTJ47-3	(Tra^+), Mob^+, Ap^r, pLV22a::pGAT400ΔBglII fusion	This study
pTJ5a	Mob^+, Tc^r, pLV22a inserted into *Eco*RI site of pACYC184	This study
pTJ6	Mob^+, Cm^r, pLV22a ligated to 3.8-kb pACYC184 *Xba*I-*Bam*HI fragment	This study
pTJ6XB	Mob^+, Cm^r, deletion derivative of pTJ6	This study
pTJ8-WT	Mob^+, Ap^r, pLV22a ligated to *Eco*RI site of pBR328Δ	This study
pΔLVO1	Mob^+, Cm^r, deletion derivative of pTJ6XB	This study
pB22a	Mob^+, Ap^r, pLV22a ligated to the 3.9-kb *Aat*II-*Bam*HI fragment of pBR322	This study
pTJ7	Mob^-, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pTJ18	Mob^-, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pTJ38	Mob^-, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pTJ39	Mob^-, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pTJ49	Mob^-, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pTJ67	Mob^-, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pTJ28	Mob^+, Tc^r, Tn*1000* insertion mutant of pTJ5a	This study
pBTJ7	Mob^-, Ap^r, Tn*1000* insertion mutant of pLV22a ligated to *Eco*RI site of pBR328Δ	This study
pBTJ18	Mob^-, Ap^r, Tn*1000* insertion mutant of pLV22a ligated to *Eco*RI site of pBR328Δ	This study
pBTJ38	Mob^-, Ap^r, Tn*1000* insertion mutant of pLV22a ligated to *Eco*RI site of pBR328Δ	This study
pBTJ39	Mob^-, Ap^r, Tn*1000* insertion mutant of pLV22a ligated to *Eco*RI site of pBR328Δ	This study
pBTJ49	Mob^-, Ap^r, Tn*1000* insertion mutant of pLV22a ligated to *Eco*RI site of pBR328Δ	This study
pBTJ67	Mob^-, Ap^r, Tn*1000* insertion mutant of pLV22a ligated to *Eco*RI site of pBR328Δ	This study

[a] Ap^r, Cm^r, Nal^r, Rif^r, Sp^r, Sm^r, Tc^r, and Tmp^r indicate resistance to ampicillin, chloramphenicol, naladixic acid, rifampin, spectinomycin, streptomycin, tetracycline, and trimethoprim, respectively. Tra refers to the self-transfer ability of a plasmid, while Mob refers to the phenotype of a Tra^- plasmid mobilized when coresident with R751. Phenotypes in parentheses are expressed only in *Bacteroides* spp., while phenotypes without parentheses are expressed only in *E. coli.*

*Eco*RI sites of pACYC184 and pBR328Δ, respectively. pB22a was created by ligation of the 5.3-kb *Eco*RI fragment of pTJ47-1 that contains pLV22a and $IS4400_L$ into the *Eco*RI site of the pGEM7Zf(+) polylinker. The fragment was then excised by digestion with *Aat*II-*Bam*HI and ligated to the 3.9-kb *Aat*II-*Bam*HI fragment of pBR322. pTJ6 was created by ligation of the 5.3-kb *Eco*RI fragment of pTJ47-3 that contains pLV22a and $IS4400_L$ into the *Eco*RI site of the pUC19 polylinker. The fragment was then excised by digestion with *Xba*I-*Bam*HI and ligated to the 3.8-kb *Xba*I-*Bam*HI fragment of pACYC184. To delete $IS4400_L$ and regions of pLV22a not required for mobilization, pTJ6 was digested with *Xba*I-*Bst*XI and treated with S1 nuclease to generate blunt ends. The resulting 5.5-kb fragment that contains the pLV22a Mob region was self-ligated to give pTJ6XB (see Fig. 2C). To delete the first 31 bp of the *mbpA* coding region and upstream sequences, pTJ6XB was digested with *Ase*I-*Bsa*WI and treated with S1 nuclease to generate blunt ends. The resulting 5.3-kb fragment was self-ligated to give pΔLVO1 (see Fig. 2C).

Transposon mutagenesis. Tn*1000* insertion mutations in the pLV22a portion of pTJ5a were generated according to the F′*lac* protocol as previously described (6). Each mutant that was determined to contain an insertion in pLV22a had all internal Tn*1000 Bgl*II fragments deleted to prevent further transposition events mediated by the transposon. In order to perform the complementation assays, a set of plasmids containing insertion mutations identical to those of pTJ7, pTJ18, pTJ38, pTJ39, pTJ49, and pTJ67 was constructed. *Bsa*WI fragments internal to Tn*1000* were deleted from pTJ7, pTJ18, pTJ38, pTJ39, pTJ49, and pTJ67, leaving 65 bp of Tn*1000* DNA at the sites of insertion. A 5.4-kb *Eco*RI fragment from each insertion mutation, containing pLV22a and the 65 bp of Tn*1000*, was then cloned into the unique *Eco*RI site of pBR328Δ. The resulting plasmids were designated pBTJ7, pBTJ18, pBTJ38, pBTJ39, pBTJ49, and pBTJ67, respectively.

Plasmid mobilization experiments. Quantitative *Bacteroides* spp.-*E. coli* filter matings were performed as previously described (7). Mobilization of plasmids in *E. coli* in the presence of R751 was determined by mixing mid-log-phase cultures of *E. coli* HB101, containing R751 and the plasmid to be assayed for mobilization, with *E. coli* DW1030 (donor-to-recipient ratio, 1:9; total volume of cells, 1.5 ml). After pelleting, the cells were suspended in 100 μl of phosphate buffered saline (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 145 mM NaCl; pH 6.9) and plated onto a 25-mm-diameter Nalgene GN-6 filter (Nalge Co., Rochester, N.Y.) supported on Luria agar plates. The filters were incubated for only 3 h at 37°C to limit secondary mobilization events (7). The cells were then suspended and serially diluted in phosphate buffered saline and plated onto the appropriate antibiotic media. The mobilization frequencies in the presence of R751 were

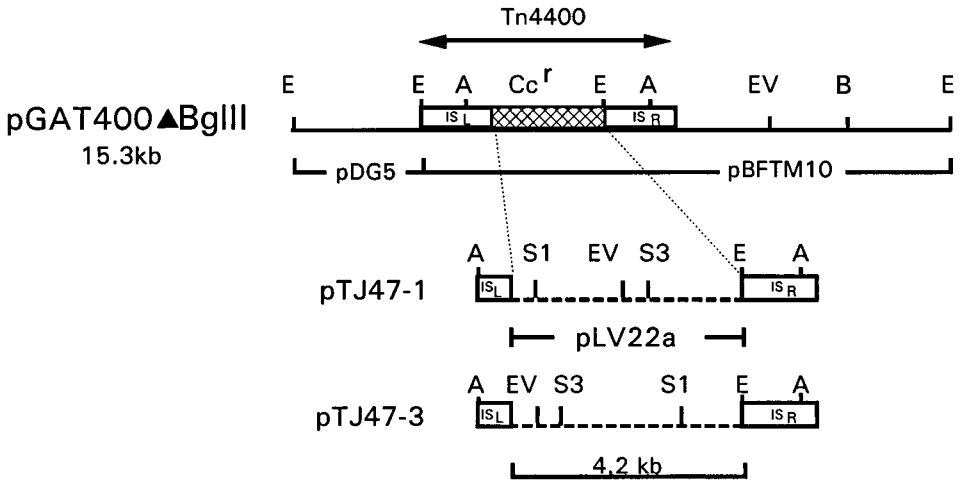

FIG. 1. Restriction map of pGAT400ΔBglII (solid line). The locations of Tn*4400* and the clindamycin resistance determinant (Cc^r) are shown. pTJ47-1 and pTJ47-3 illustrate the location and two orientations (16) of pLV22a (dashed line) between IS*4400*$_L$ (IS$_L$) and IS*4400*$_R$ (IS$_R$) following fusion. Tn*4400* DNA (hatched area in pGAT400ΔBglII) between IS*4400*$_L$ and IS*4400*$_R$ was missing following fusion with pLV22a. E, *Eco*RI; A, *Ava*I; EV, *Eco*RV; B, *Bgl*II; S1, *Sac*I; S3, *Sau*3AI.

calculated by dividing the number of Mob$^+$ plasmid transconjugants by the number of R751 transconjugants in the same experiment.

DNA sequencing. Nucleotide sequences were determined from double-stranded DNA templates with the Sequenase system (U.S. Biochemical Corp., Cleveland, Ohio). The outward-facing primers specific to the gamma and delta ends of Tn*1000* were 5′-TCAATAAGTTATACCAT-3′ and 5′-GAATTATCTC-CTTAACG-3′, respectively. Other primers, 17 to 20 bases in size, were synthesized as needed to fill gaps and obtain the complementary DNA sequence.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank under accession no. U25716.

RESULTS

Mobilization of pGAT400ΔBglII by pLV22a. pLV22a, a plasmid native to *B. fragilis* LV22, was originally transferred to *Bacteroides thetaiotaomicron* BT4001 in a mating experiment designed to separate two putatively conjugative plasmids present in LV22. One transconjugant that contained only pLV22a, BT22.47 (data not shown), was tested for its ability to transfer the Tra$^-$ pGAT400ΔBglII deletion derivative of pGAT400 to *E. coli* (7). A conjugation experiment between the donor BT22.47, containing pGAT400ΔBglII, and *E. coli* HB101 produced 25 transconjugants when selecting for pGAT400ΔBglII. Restriction analysis of plasmid DNA from five transconjugants revealed two plasmids that appeared unaltered, one plasmid that was associated with a 4-kb deletion internal to Tn*4400*, and two plasmids, pTJ47-1 and pTJ47-3, that contained new DNA (data not shown). Restriction analyses of both pTJ47-1 and pTJ47-3 demonstrated that the new DNA was 4.2 kb in size and was located between the two insertion sequences of Tn*4400* (Fig. 1), while the internal portion of Tn*4400* between IS*4400*$_L$ and IS*4400*$_R$ had been deleted. The new DNA contained within pTJ47-1 and pTJ47-3 had identical endonuclease restriction sites, although at different locations between the IS*4400* sequences. This suggested that the newly acquired DNA fragment was a plasmid in two different orientations. DNA sequence analysis of the junctions of both pTJ47-1 and pTJ47-3 indicated that the new DNA was acquired as a result of an inverse transposition by Tn*4400* (16). A Southern hybridization with pTJ47-1 and pTJ47-3 as probes against LV22 and BT4001 demonstrated homology with only the 4.2-kb plasmid, pLV22a, native to LV22 (data not shown).

Mobilization properties of pLV22a in *E. coli*. pBFTM10 (in pGAT400) and Tn*4399* are mobilizable within *E. coli* when coresident with the IncPβ plasmid R751 (7, 12, 19). We suspected that pLV22a may also have contained transfer genes allowing the mobilization of pTJ47-1 and pTJ47-3 in *E. coli*. To test pTJ47-1 and pTJ47-3 for mobilization in *E. coli*, HB101 donors containing R751 and either pTJ47-1 or pTJ47-3 were mated with DW1030. As shown in Table 2, pTJ47-1, pTJ47-3, and pGAT400 were mobilized at similar frequencies when coresident with R751, while pGAT400ΔBglII was mobilization deficient (Mob$^-$). Thus, mobilization of pTJ47-1 and pTJ47-3 could be attributed to the presence of pLV22a in *cis* in these plasmids.

Localization and subcloning of the mobilization region of pLV22a. To determine if the Mob$^+$ phenotype was attributable to only pLV22a, fragments from both pTJ47-1 and pTJ47-3 were subcloned into pACYC184 in two different cloning sites to give pTJ5a and pTJ6, respectively (Fig. 2A and B). Frequencies of mobilization for both pTJ5a and pTJ6 from HB101 donors in the presence of R751 were similar to those for pTJ47-1 and pTJ47-3 (Table 2), indicating that mobilization in *E. coli* was attributable to pLV22a alone.

As a first step in defining the regions of pLV22a required for plasmid mobilization in *E. coli*, insertion mutagenesis of pTJ5a with Tn*1000* was performed (6). A total of 26 insertion mutations were mapped within the pLV22a segment of pTJ5a (Fig. 2A). Each mutant was then separately tested for mobilization from HB101 donors containing R751 (Table 3). Six mutations

TABLE 2. R751 mobilization of pGAT400 and Tra$^-$ plasmids[a]

Plasmid	Presence of pLV22a in *cis*	Frequency of mobilization (mean ± SE)[b]
pGAT400	−	$(2.9 \pm 1.2) \times 10^{-2}$
pGAT400ΔBglII	−	$<10^{-5}$[c]
pTJ47-1	+	$(3.7 \pm 1.9) \times 10^{-3}$
pTJ47-3	+	$(3.9 \pm 0.6) \times 10^{-3}$
pTJ6	+	$(1.0 \pm 0.5) \times 10^{-2}$
pTJ6XB	+	$(7.5 \pm 2.8) \times 10^{-3}$

[a] Plasmids were mobilized from *E. coli* HB101 containing R751 to *E. coli* DW1030.

[b] The mobilization frequency is defined as the number of mobilized plasmid transconjugants divided by the number of R751 transconjugants in the same experiment.

[c] $<10^{-5}$ indicates a frequency of mobilization below the limit of detectability.

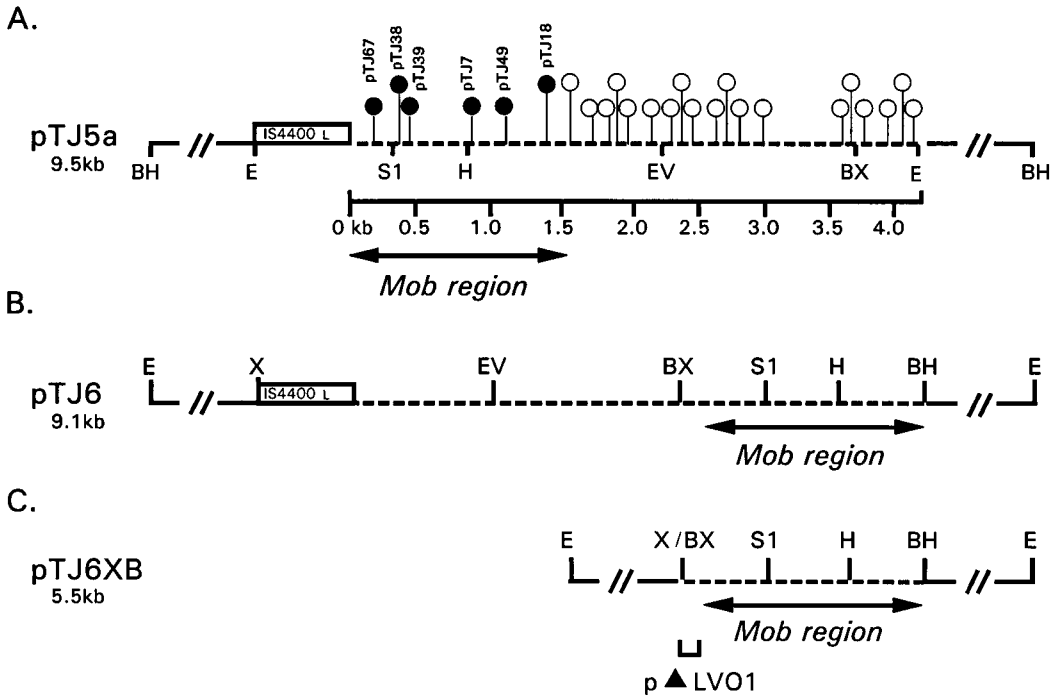

FIG. 2. (A) pTJ5a. pLV22a from pTJ47-1 ligated into the *Eco*RI site of pACYC184 is shown. Solid circles, Mob$^-$ Tn*1000* insertion mutations; open circles, Mob$^+$ insertions. (B) pTJ6. pLV22a from pTJ47-3 ligated into the 3.8-kb *Xba*I-*Bam*HI pACYC184 fragment is shown. (C) pTJ6XB. The deletion of the 3.6-kb *Xba*I-*Bst*XI fragment of pTJ6 upstream of the pLV22a mobilization region is shown. p▲LVO1 represents a 0.3-kb deletion of pTJ6XB DNA, including the amino terminus of *mbpA* (see text for details). Solid lines with hash marks indicate pACYC184. Dashed lines denote pLV22a. Restriction enzyme abbreviations are the same as those in the legend to Fig. 1 with the addition of the following: BH, *Bam*HI; BX, *Bst*XI; H, *Hin*dIII; X, *Xba*I.

located within a 1.5-kb region of pLV22a resulted in a Mob$^-$ phenotype. The other 20 insertion mutations remained Mob$^+$ and were located throughout the remaining 2.7 kb of pLV22a (Fig. 2A). To determine if the 1.5-kb region of pLV22a defined by Tn*1000* mutagenesis was sufficient for mobilization when coresident with R751, the upstream 3.6-kb *Xba*I-*Bst*XI fragment including IS*4400*$_L$ of pTJ6 was deleted to give pTJ6XB (Fig. 2C). pTJ6XB, containing only 1.7 kb of pLV22a DNA, was mobilized efficiently when R751 was present in donor cells (Table 2).

TABLE 3. R751 mobilization of pTJ5a derivatives with Tn*1000* insertions[a]

Mobilization gene	Plasmid	Presence of wild-type pLV22a in *trans*	Frequency of mobilization (mean ± SE)[b]
mbpC	pTJ49	−	$<10^{-5}$[c]
	pTJ49	+	$(1.0 \pm 0.8) \times 10^{-2}$
	pTJ18	−	$<10^{-5}$
	pTJ18	+	$(6.6 \pm 2.9) \times 10^{-3}$
mbpB	pTJ38	−	$<10^{-5}$
	pTJ38	+	$(3.8 \pm 1.4) \times 10^{-3}$
	pTJ39	−	$<10^{-5}$
	pTJ39	+	$(9.0 \pm 6.5) \times 10^{-3}$
	pTJ7	−	$<10^{-5}$
	pTJ7	+	$(6.2 \pm 3.0) \times 10^{-3}$
mbpA	pTJ67	−	$<10^{-5}$
	pTJ67	+	$(2.7 \pm 1.1) \times 10^{-3}$

[a] Plasmids were mobilized from *E. coli* HB101 containing R751 to *E. coli* DW1030.
[b] The mobilization frequency is defined as the number of mobilized plasmid transconjugants divided by the number of R751 transconjugants in the same experiment.
[c] $<10^{-5}$ indicates a frequency of mobilization below the limit of detectability.

DNA and protein sequence analysis of the mobilization region of pLV22a. Both DNA strands of the 1.5-kb mobilization region of pLV22a were sequenced (Fig. 3). Five open reading frames (ORFs), capable of encoding proteins ranging in size from 11.1 to 30.4 kDa, were identified on the two strands, as illustrated in Fig. 4. *orf* 1, *orf* 2, and *orf* 3, found in the top strand, code for potential protein products of 99, 264, and 150 amino acids, respectively, based upon methionine start codons. *orf* 4 and *orf* 5, found in the bottom strand, encode potential protein products of 118 and 169 amino acids, respectively. All Tn*1000* insertions with Mob$^-$ phenotypes were located within these five ORFs. The insertion mutation of pTJ67 was located within *orf* 1, the mutations of pTJ38 and pTJ39 were within *orf* 2, the mutation of pTJ7 was within *orf* 2, *orf* 4, or *orf* 5, and the mutations of pTJ49 and pTJ18 were within *orf* 3. *orf* 3 was also altered by the deletion of the two terminal amino acid residues in plasmid pTJ47-3 as a result of the original fusion of pLV22a with pGAT400ΔBglII.

The gene product of *orf* 3, MbpC, functions in *trans* and is required for mobilization. The region between nucleotides 1155 and 1615 (Fig. 3) contains only one potential ORF, *orf* 3, which could code for a 17.1-kDa protein. Insertion mutations of pTJ49 and pTJ18 disrupted *orf* 3 and could not be mobilized from HB101 donors, even when coresident with R751 in *trans* (Table 3). When wild-type pLV22a was provided in *trans* on a compatible plasmid (pB22a), both pTJ49 and pTJ18 could be mobilized at frequencies similar to that of wild-type pTJ5a (Table 3). These data suggest that the DNA sequence disrupted by the insertions of pTJ49 and pTJ18 encodes a critical

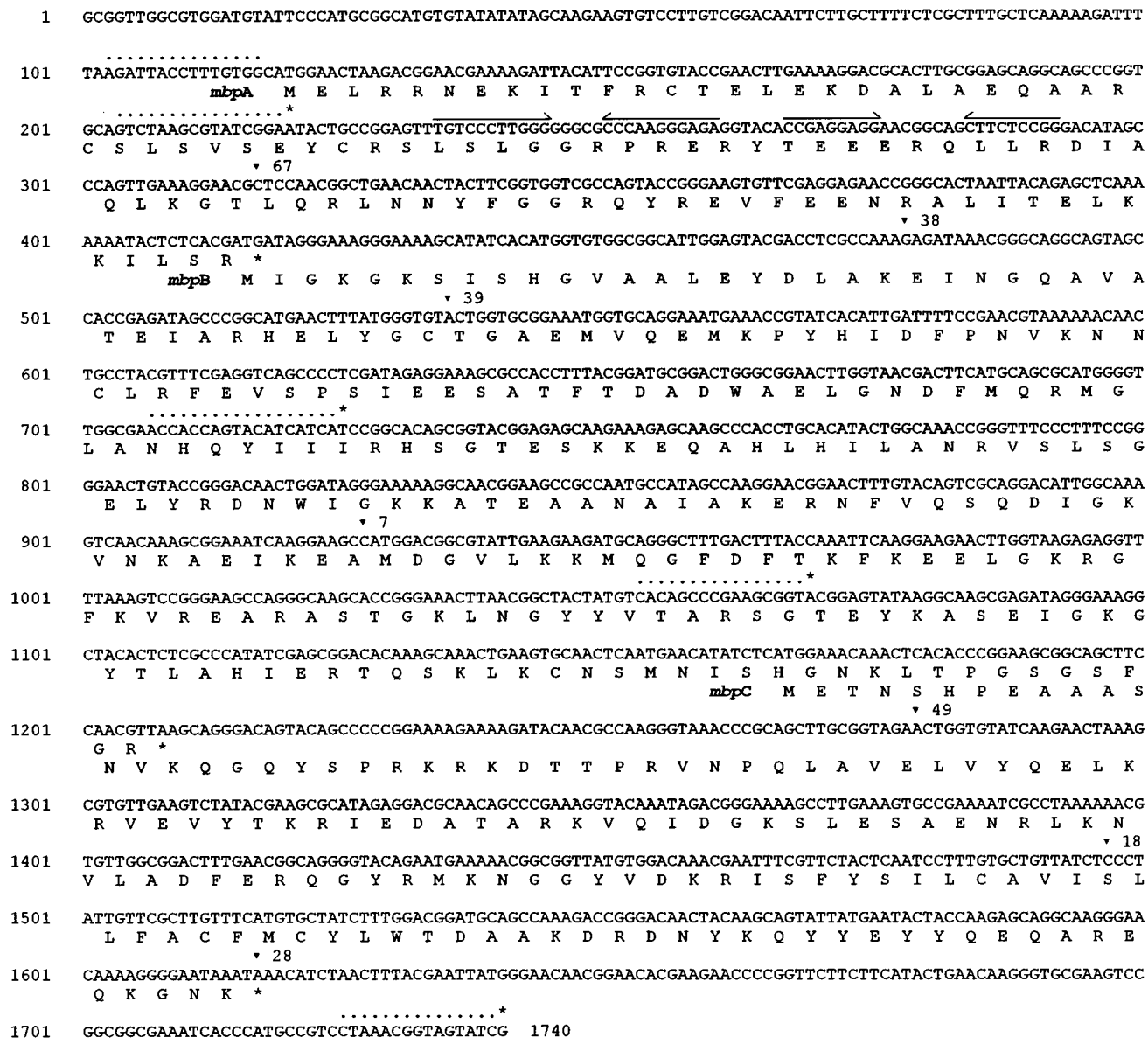

FIG. 3. Experimentally determined double-stranded sequence of a 1.7-kb region of pLV22a, including the mobilization region. Base 100 corresponds to 0 kb in Fig. 2. The sites of Tn*1000* insertions used for sequence determination (numbered inverted triangles), the synthetic oligonucleotides used for sequencing (dots), the synthetic oligonucleotides on the opposite strand used for sequencing (dots followed by an asterisk), and the inverted repeat sequences (solid lines with arrowheads) are indicated above the nucleotide sequence. The deduced amino acid sequences of MbpA, MbpB, and MbpC are given below the nucleotide sequence.

protein that is a *trans*-acting factor in the mobilization process. However, as demonstrated by the Mob$^+$ pTJ47-3, the two terminal amino acids of *orf* 3 are not required for mobilization (Table 2). To confirm that the mutations of pTJ49 and pTJ18 are in the same functional ORF, pBTJ49 and pBTJ18 were used to complement pTJ18 and pTJ49, respectively, in *trans*. pBTJ49 did not complement pTJ18, nor did pBTJ18 complement pTJ49, supporting the hypothesis that both mutations are in *orf* 3. Thus, *orf* 3 is required for mobilization and is designated *mbpC* (mobilization of *Bacteroides* plasmids).

The DNA and protein sequence of *mbpC* were compared with those of the IncP plasmids, pBFTM10, Tn*4399*, and the NBU1 mobilization regions (7, 10, 12). No significant homology or similarity between MbpC and the transfer proteins of these plasmids, conjugal transposons, or other proteins in GenBank could be detected by the Bestfit or FASTA programs of the Genetics Computer Group DNA analysis software.

The gene product of *orf* 2, MbpB, functions in *trans* and is required for mobilization. As shown in Table 3, pTJ38, pTJ39, and pTJ18, containing Tn*1000* insertion mutations in *orf* 2, could not be mobilized. pTJ38, pTJ39, and pTJ7 were mobilized in *trans* at frequencies similar to that of wild-type pTJ5a when coresident with wild-type pLV22a (pB22a). This suggests that all three insertion mutations disrupt a gene required for mobilization and that the product of *orf* 2, designated MbpB, functions in *trans* in the mobilization process. Whether the mutation contained in pTJ7 might also disrupt a product(s) potentially encoded by *orf* 4 or *orf* 5 is not known.

To determine if the mutations contained in pTJ38, pTJ39, and pTJ7 were functionally affecting the same ORF, pBTJ38,

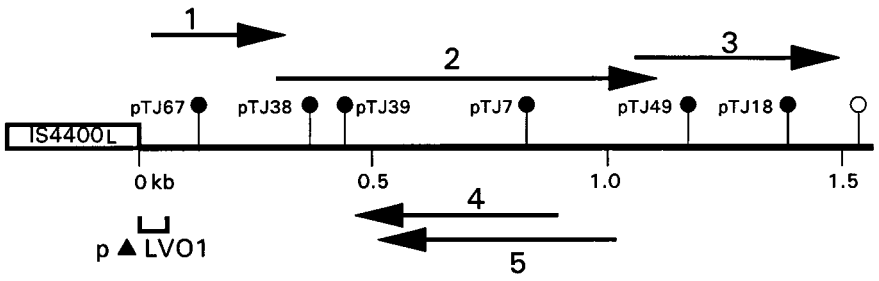

FIG. 4. ORFs and corresponding insertion mutations. The region shown is derived from the translation of the DNA sequence of the pLV22a mobilization region. The numbers above the arrows identify corresponding ORFs (see the text for details). Solid circles, Mob$^-$ Tn*1000* insertion mutations; open circles, Mob$^+$ insertions; p▲LVO1, deletion of *mbpA* (see the legend to Fig. 2 for a description).

pBTJ39, and pBTJ7 were used to complement pTJ38, pTJ39, and pTJ7. No complementation between any combination of these plasmids was found, indicating that pTJ38, pTJ39, and pTJ7 disrupt potential coding sequences in *mbpB* (Table 4). However, insertion mutations that disrupt *mbpB* could also be polar on *mbpC*, if the two genes are in an operon (Fig. 4). Experiments pairing pBTJ39 and pTJ18 as well as pTJ39 and pBTJ18 in HB101 donors containing R751 demonstrated near-wild-type mobilization for both complemented plasmids (Table 4). This indicates that insertion mutations in *mbpB* are not polar on *mbpC* and that *mbpB* is required for mobilization.

MbpB shares homology with DNA relaxases. The derived protein sequence of MbpB was compared with those of other plasmid mobilization proteins and was found to share similarities with DNA-relaxing enzymes encoded by a wide variety of conjugal elements and a conjugal transposon (Fig. 4A). These include *Agrobacterium* Ti and Ri plasmids, a mobilizable plasmid from *Thiobacillus ferrooxidans*, broad-host-range IncPα and IncPβ plasmids, mobilizable plasmids from *Staphylococcus aureus*, and the *B. fragilis* conjugal transposon Tn*4399*. Alignment of mobilization proteins by the method of Pansegrau and Lanka (14) reveals a 14-amino-acid sequence motif that is highly conserved among DNA relaxases. Comparing the amino acid sequence of MbpB directly with that of Tn*4399* by Bestfit analysis revealed 38% identity over a 72-amino-acid region, which includes the relaxase motif (Fig. 5B). No homology with the other two characterized *B. fragilis* transfer factors, pBFTM10, or NBU1 mobilization proteins was detected.

TABLE 4. Cross-complementation between pLV22a mutants in the presence of IncPβ plasmid R751[a]

Mobilization gene or region	Complementing plasmid	Mobilized plasmid	Frequency of mobilization[b] (mean ± SE)
Wild type	NA[c]	pTJ5a	$(3.2 \pm 0.4) \times 10^{-3}$
	NA	pTJ8-WT	$(1.1 \pm 0.1) \times 10^{-2}$
mbpC	pBTJ49	pTJ18	$<10^{-5}$[d]
	pBTJ18	pTJ49	$<10^{-5}$
mbpB	pBTJ7	pTJ38	$<10^{-5}$
	pBTJ38	pTJ7	$<10^{-5}$
	pBTJ7	pTJ39	$<10^{-5}$
	pBTJ39	pTJ7	$<10^{-5}$
	pBTJ38	pTJ39	$<10^{-5}$
	pBTJ39	pTJ38	$<10^{-5}$
	pBTJ18	pTJ39	$(2.5 \pm 1.4) \times 10^{-3}$
	pBTJ39	pTJ18	$(4.7 \pm 2.0) \times 10^{-3}$
mbpA	pBTJ67	pTJ39	$(1.1 \pm 0.4) \times 10^{-3}$
	pBTJ39	pTJ67	$(6.3 \pm 5.8) \times 10^{-5}$
	pBTJ67	pTJ18	$(1.9 \pm 0.8) \times 10^{-3}$
	pBTJ18	pTJ67	$(6.2 \pm 1.3) \times 10^{-4}$
cis acting	pTJ8-WT	pΔLVO1	$<10^{-5}$
	pΔLVO1	pTJ67	$<10^{-5}$
	pΔLVO1	pTJ39	$(1.0 \pm 0.3) \times 10^{-2}$
	pΔLVO1	pTJ18	$(7.5 \pm 0.4) \times 10^{-3}$

[a] Plasmids were mobilized from *E. coli* HB101 containing R751 to *E. coli* DW1030.

[b] The mobilization frequency is defined as the number of mobilized plasmid transconjugants divided by the number of R751 transconjugants in the same experiment.

[c] NA, not applicable.

[d] $<10^{-5}$ indicates a frequency of mobilization below the limit of detectability.

The gene product of *orf* 1, MbpA, functions in *trans* and is required for mobilization. The region between nucleotides 119 and 416 (Fig. 3) contains only one potential ORF, *orf* 1, which is disrupted by the insertion mutation of pTJ67. As shown in Table 3, pTJ67 could not be mobilized when coresident with R751 in *trans*, suggesting that Tn*1000* disrupts a critical mobilization gene. When wild-type pLV22a was provided in *trans*, pTJ67 mobilized at wild-type frequencies. Thus, the product of *orf* 1 appears to function in *trans*, is required for mobilization, and is designated *mbpA*.

Insertion mutations in *mbpA* could also have been polar on *mbpB*, if the two genes were in an operon (Fig. 4). Experiments pairing pBTJ67 and pTJ39 as well as pBTJ39 and pTJ67 were performed in HB101 donors containing R751 (Table 4). pBTJ67 complemented pTJ39 efficiently, while pBTJ39 demonstrated only partial complementation of pTJ67. To ensure that mutations in *mbpA* were not polar on *mbpC*, complementation experiments pairing pBTJ67 and pTJ18 (*mbpC*) as well as pBTJ18 and pTJ67 were also performed (Table 4). Again, pBTJ67 complemented pTJ18 efficiently, confirming that the mutation contained in pTJ67 is not polar on *mbpC*. However, only partial complementation of pTJ67 occurred when pBTJ18 was provided in *trans*, similar to the results with pBTJ39.

Comparing the DNA and protein sequences of *mbpA* and its deduced protein sequence with sequences contained within GenBank by using Bestfit or FASTA programs (Genetics Computer Group DNA analysis software) did not reveal any homology.

Localization of a *cis*-acting region within *mbpA*. A deletion mutation, pΔLVO1, was generated by a deletion of 50 bp from the 5′ end of the mobilization region contained in pTJ6, including the first 31 bp of *mbpA* (Fig. 2B and C). As expected, pΔLVO1 could not be mobilized, even when R751 was present in donor cells. Additional studies also demonstrated that pΔLVO1 could complement insertion mutations in both *mbpB* (pBTJ39) and *mbpC* (pBTJ18) but not in *mbpA* (pBTJ67)

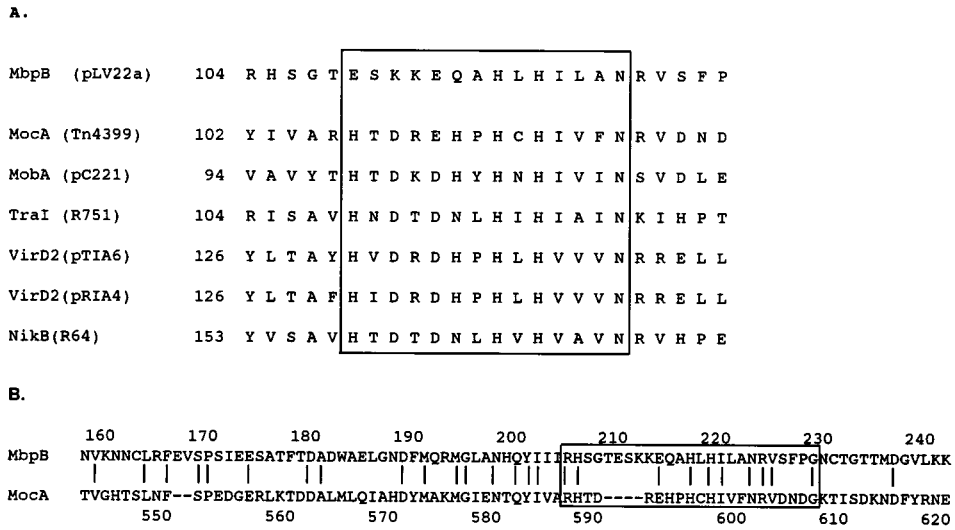

FIG. 5. (A) Alignment of MbpB relaxase sequence and other putative and known relaxase sequences. The boxed sequences are the conserved regions, according to Pansegrau and Lanka (14). The number to the left of each plasmid sequence denotes the position of the first illustrated amino acid. (B) Alignment of amino acid sequences from MbpB (pLV22a) and MocA (Tn*4399*) (12), overlapping the putative relaxase region (boxed area). Vertical lines between the sequences denote amino acid identities.

(Table 4), confirming that the deletion in this plasmid did not affect the expression of downstream genes.

Although pΔLVO1 and pTJ67 both disrupt *mbpA*, pΔLVO1 could not be complemented by wild-type pLV22a (pJT8-WT) (Table 4). This suggested that in addition to a *trans*-acting function of MbpA, a *cis*-acting function was also located within *mbpA* and/or its immediate upstream sequences. Additional DNA sequence analysis of the 1.5-kb mobilization region revealed two adjacent sets of imperfect inverted repeats (14 and 9 bp long, respectively), located in *mbpA* (Fig. 3). These findings support the argument for the location of a *cis*-acting region, possibly the pLV22 *oriT*, on the basis of similar findings with both pBFTM10 (7) and Tn*4399* (12).

DISCUSSION

We have characterized the mobilization region of a new cryptic *B. fragilis* plasmid, pLV22a, that can be transferred from *Bacteroides* spp. to *E. coli* and mobilized in the presence of an IncP plasmid in *E. coli*. pLV22a was discovered by its fusion, following an inverse transposition event, to the Tra^- pGAT400ΔBglII, resulting in a Tra^+ phenotype and a deletion of a portion of Tn*4400*. In addition, however, transfer of pGAT400ΔBglII from *Bacteroides* spp. to *E. coli* also occurred without apparent fusion to pLV22a. Instead, some transconjugant plasmids were found to have either a deletion of Tn*4400* without the addition of new DNA or no apparent alteration. The reason for these other plasmid products is unknown, but we speculate that those associated with deletions may be the result of an initial fusion of pGAT400ΔBglII with pLV22a, followed by a loss of pLV22a and a portion of Tn*4400*. The unaltered plasmids may also be the result of a fusion with pLV22a that was later excised precisely or nearly precisely. Alternatively, it is possible that an unknown transfer factor in the donor strain may have mobilized pGAT400ΔBglII in *trans*.

pLV22a also has mobilization properties in *E. coli* when coresident with the IncP plasmid R751, similar to those observed for shuttle plasmids containing pBFTM10, NBU1, or the Tn*4399* mobilization cassette (7, 10, 12). On the basis of genetic analysis, it was determined that the region of pLV22a required for mobilization in *E. coli* is small (1.5 kb) and contains three genes encoding products that all function in *trans*. The three genes are either contiguous (*mbpA* and *mbpB*) or overlapping (*mbpB* and *mbpC*) and appear likely to be expressed by independent promoters that have not yet been identified (Table 4). However, in some constructs $IS4400_L$ may be responsible for promoting the expression of *mbpA*. Two additional ORFs identified in the bottom strand, *orf* 4 and *orf* 5, were also disrupted by the mutation contained in pTJ7. Their potential roles, if any, in the mobilization of pLV22a are unknown and are currently under investigation.

The mobilization region of pLV22a is different from those of the other published *Bacteroides* spp. transfer factors, pBFTM10, Tn*4399*, and NBU1. The mobilization of these transfer factors in *E. coli* in the presence of R751 requires two genes for pBFTM10 and Tn*4399* and one gene for NBU1 (7, 10, 12). The mobilization of pLV22a, in contrast, requires three genes. One of the mobilization proteins (BtgA) from pBFTM10 is known to bind to its cognate *oriT* and is thought to be involved in relaxosome formation (2). The functions of BtgB (pBFTM10), MocA, and MocB (Tn*4399*) or Mob (NBU1) in plasmid mobilization have not been demonstrated, but they may also be involved in DNA processing. Whether all three pLV22a genes are required for DNA processing is not known, but other plasmids, such as ColE1, also require three genes for relaxosome formation (11). In addition to requiring DNA processing functions, pBFTM10, Tn*4399*, NBU1, and pLV22a all require additional mating functions for mobilization in *E. coli*. These likely include establishing and regulating effective mating contacts, which are apparently provided in *trans* by R751, although a possible role of the chromosome is unknown.

Of the three deduced pLV22a mobilization proteins, only MbpB was found to have homology with other sequences deposited in GenBank. MbpB shares a common 14-amino-acid sequence motif with nine other confirmed and potential relaxases encoded by a variety of conjugal or mobilizable plasmids from both gram-negative and gram-positive hosts (14). However, because mutations in *mbpB* could not be complemented by the DNA-processing proteins of R751 (Table 3) or the IncPα plasmid pRK231 (data not shown), it appears that

MbpB is pLV22a specific. This is not surprising, since DNA relaxases function by interacting specifically with their respective target *oriT* regions to create a nicked open circular form of plasmid DNA prior to transfer (15). MbpB has an even greater degree of homology with MocA of the *B. fragilis* conjugal transposon Tn*4399* in the region encompassing the conserved relaxase motif (12). It is interesting that although pLV22a and Tn*4399* appear otherwise unrelated, it is possible that Tn*4399* and pLV22a acquired *mocA* and *mbpB*, respectively, from a common ancestor. In contrast, MbpB was not homologous with BtgA or BtgB (pBFTM10) or with Mob (NBU1), and thus a common ancestor for these proteins is less likely.

The *oriT* of pLV22a appears to be located, at least in part, within *mbpA*. Support for this conclusion is given by the inability of wild-type pLV22a to complement the deletion derivative pΔLVO1. In addition, two sets of inverted repeats were identified near the amino terminus of *mbpA*, which is similar to observations for the *oriT* in Tn*4399* but contrasts with those for pBFTM10 and NBU1, in which the *oriT* sequences are upstream of *btgA* and *mob*, respectively (2, 10, 12). Whether the inverted repeats in the pLV22a *oriT* are required for *oriT* function is unclear, as they are not disrupted in pΔLVO1. However, the insertion mutation pTJ67, located 100 bp downstream of the inverted repeats, also affected the *cis*-acting function and was only partially complemented by both the *mbpB* mutant pBTJ39 and the *mbpC* mutant pBTJ18. We speculate that the location of the pTJ67 mutation may partially disrupt the *cis*-acting function by its close proximity. The reason that wild-type pLV22a (pB22a) fully complemented pTJ67 is unclear, but this complementation may be due to higher levels of expression of mobilization proteins by the high-copy-number plasmid pB22a. Studies are in progress to clone and characterize the minimal pLV22a *oriT*.

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