Genetic Analysis of the Conjugal Transfer Determinants Encoded by the Streptococcal Broad-Host-Range Plasmid pIP501

E. REGIS KRAH III AND FRANCIS L. MACRINA*

Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298-0678

Received 23 May 1989/Accepted 10 August 1989

pIP501 is a 30.2-kilobase (kb) broad-host-range conjugative streptococcal plasmid which encodes chloramphenicol and erythromycin resistance. A smaller conjugation-proficient derivative of pIP501 has been constructed and designated pVA1702. pVA1702 is 25.2 kb, encodes only kanamycin resistance, and confers conjugative ability in an *Enterococcus faecalis* mating system. The temperature-sensitive transposon delivery vectors pTV1ts and pTV32 carrying Tn917 and Tn917lac, respectively, were used to create insertion mutants of pVA1702 in *E. faecalis*. These insertions enabled us to localize the determinants conferring conjugation to two separate regions of pVA1702: the 7.5-kb region A and the 8.8-kb region B. A nested set of deletions of the 7.5-kb region was subcloned in *Escherichia coli*, and the expression of these deletions was examined in an *E. coli* minicell system. The genes for three conjugal proteins have been located and designated *cnjA*, *cnjB*, and *cnjC* (for conjugation). Two other peptides that are transcribed in a divergent direction from the *cnj* genes were encoded in the A region, but it is not known whether they are involved in conjugation. These studies indicate that the conjugation genes of pIP501 are arranged in multiple transcriptional units.

Conjugative plasmids encoding macrolide, lincosamide, and streptogramin B resistance (MLS^r) have been found in a wide variety of clinically important streptococci (2-4, 13, 14). Besides sharing a common MLS^r determinant, members of this group range from 26 to 33 kilobases (kb) in size (13), have similar restriction enzyme digest patterns (7), and have considerable DNA sequence similarities as determined by hybridization studies (13). Hence, these plasmids are considered evolutionarily related. Unlike the pheromone-responding plasmids of *Enterococcus* (*Streptococcus*) *faecalis* that have a limited range of transfer to other members of the genus *Enterococcus* (4), these plasmids have a broad host range. In filter matings, these plasmids transfer to a wide variety of streptococci (2, 3, 7, 13, 14, 22) and to other gram-positive bacteria such as staphylococci (23), bacilli (20), lactobacilli (10), pediococci (11), *Listeria* spp. (3), and clostridia (20). These plasmids must encode a conjugal transfer system which is transcribed, translated, and correctly processed so as to function in a wide variety of hosts. Two of these plasmids, pIP501 (1, 8) and pAMβ1 (16), have been characterized at the molecular level and have been used to develop several useful gene cloning and transposon delivery systems (1, 22, 24). Despite this, there is very little known about the physiological genetic basis of conjugal transfer conferred by these plasmids.

We have begun to characterize the organization of the genes conferring conjugation on pIP501 so as to gain a greater understanding of the mechanism of transfer specified by these plasmids. pIP501 is 30.2 kb in size and confers chloramphenicol resistance and MLS^r. Removal of the MLS^r gene and insertion of the pVA380-1 replicon to create pVA797 do not affect conjugation frequency (8) or host range (22). Deletions of 8 kb or more, created in vivo during transformation of *Streptococcus sanguis* Challis, results in a transfer-deficient phenotype (9), but such plasmids are not helpful in locating specific genes required for conjugation.

In this study, we report the construction of a smaller conjugation-proficient derivative of pVA797 designated

pVA1702. pVA1702 is 25.2 kb and encodes only kanamycin resistance (Km^r). pTV1ts and pTV32 were used to deliver Tn917 and Tn917lac, respectively, in an *E. faecalis* background to insertionally mutagenize pVA1702. Genes governing conjugation have been found on two separate regions of pVA1702 spanning 16 kb. By using deletion analysis, the conjugation genes in one of these regions have been mapped and their protein products have been identified.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Tables 1 and 2 and in Fig. 1. *S. sanguis* and *E. faecalis* strains were grown in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broth, and *Escherichia coli* strains were grown in Luria broth (GIBCO Diagnostics, Madison, Wis.). Agar (Difco) was added to 1.5% to make solid media. *S. sanguis* cultures and mating mixtures were grown in GasPak jars (Becton Dickinson and Co., Cockeysville, Md.) in an atmosphere of 10% H₂, 10% CO₂, and 80% N₂. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following concentrations: carbenicillin, 50 µg/ml; chloramphenicol, 5 µg/ml for *S. sanguis* and 10 µg/ml for *E. faecalis*; kanamycin, 1,000 µg/ml for transconjugant selection and plasmid maintenance and 800 µg/ml for selection of protoplast transformants; spectinomycin, 200 µg/ml; streptomycin, 200 µg/ml; rifampin, 20 µg/ml; fusidic acid, 20 µg/ml; erythromycin, 5 µg/ml for *S. sanguis*, 20 µg/ml for *E. faecalis*, and 8 µg/ml for selection of protoplast transformants. Media and solutions used in protoplast transformation experiments were employed as described by Wirth et al. (26).

Nucleic acid methodology. Restriction endonucleases and T4 ligase were purchased from New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as described by the manufacturers. Agarose gel electrophoresis was performed as described elsewhere (19). Large-scale isolation of plasmid DNA from *E. faecalis* (5) and transformation of protoplasts were performed as described by Wirth et al. (26). To avoid

* Corresponding author.

TABLE 1. Plasmids

Plasmid	Size (kb)	Replicon	Drug resistance ^a	Comments	Source or reference
pIP501	30.1	pIP501	Cm, Em	Parental plasmid	14
pVA797	31.1	pIP501, pVA380-1	Cm	pIP501 derivative	8
pVA1229	36.5	pIP501, pVA380-1	Cm, Em	pVA797::Tn917 derivative	G. Dunny
pVA1700	26.6	pIP501, pVA380-1	Cm	pVA1229 derivative	This study
pVA1701	29.7	pIP501	Km	pVA797 derivative	This study
pVA1702	25.2	pIP501	Km	pVA1700 derivative	This study
pVA1702B	25.2	pIP501	Km	pVA1702 derivative	This study
pStr4A	10.4	pVA380-1	Sp, Km	Source of Km gene	D. Le Blanc
pTV1ts	12.4	pE194	Cm, Em	Tn917 vehicle	27
pTV32	15.6	pE194	Cm, Em	Tn917lac vehicle	27
pVA838	9.2	pVA380-1, pACYC184	Cm, Em	<i>E. coli</i> -streptococcus shuttle plasmid	17
pVA1856	16.0	pVA380-1, pACYC184	Em	pVA1702::Tn917lac derivative	This study
pGEM3	2.87	ColE1	Cb	<i>E. coli</i> cloning vector	Promega
pVA1857	11.8	ColE1	Cb	pVA1702::Tn917lac derivative	This study
pVA1858	10.0	ColE1	Cb	pVA1702::Tn917lac derivative	This study
pVA1859	9.2	ColE1	Cb	pVA1702::Tn917lac derivative	This study
pVA1860	7.5	ColE1	Cb	pVA1702::Tn917lac derivative	This study
pVA1861	6.2	ColE1	Cb	pVA1860 derivative	This study
pVA1862	7.0	ColE1	Cb	pVA1858 derivative	This study

^a Cb, Carbenicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Sp, spectinomycin. Concentrations used are described in Materials and Methods.

problems associated with host restriction systems, all conjugative plasmids used in the construction of pVA797 derivatives were moved into an *E. faecalis* OG1-SS host background by conjugation or protoplast transformation before isolation. Large-scale plasmid isolation from *S. sanguis* was done as previously described (18). *E. coli* plasmid isolation and transformation were performed as described previously (19).

Generation of pVA1702::Tn917 and pVA1702::Tn917lac derivatives. The temperature-sensitive plasmids pTV1ts and pTV32 (27) were used as Tn917 delivery vehicles to insert transposons into pVA1702 in an *E. faecalis* host background (6, 25). In addition to the Em^r markers on Tn917 and Tn917lac, carried by pTV1ts and pTV32, respectively, these plasmids also contained a Cm^r determinant. Both plasmids can replicate at 30°C but not at 42°C (27). pVA1702 was introduced into *E. faecalis* strains carrying pTV1ts or pTV32 by conjugation. The strain carrying both plasmids was maintained on plates containing kanamycin and chloramphenicol. Overnight cultures were grown at 30°C in broth containing chloramphenicol and kanamycin, diluted 1:1,000 into drug-free BHI broth, grown for 12 h at 42°C, similarly diluted, grown for another 12 h, and placed onto media containing erythromycin. To isolate plasmids carrying inserts, colonies were scraped from the agar surface, lysed using the sarcosyl lysis protocol (5), and subjected to CsCl-ethidium bromide ultracentrifugation (19). Purified plasmids were used to transform *E. faecalis* protoplasts, which were selected on erythromycin and then screened for Km^r. Such transformants were assumed to contain transposed derivatives of pVA1702. Colonies were maintained on plates containing kanamycin and were tested for conjugative ability. The locations of the transposon insertions were determined by restriction endonuclease mapping.

Conjugation transfer experiments. The conjugation frequency of pVA797 in isogenic *E. faecalis* matings is referred to as the wild-type frequency (approximately 10⁻³). The transfer frequency was expressed as the number of transconjugants observed divided by the number of input donor cells at the initiation of the mating. For screening conjugative ability of Tn917 and Tn917lac inserts in pVA1702, a

0.01-ml sample of an overnight donor culture was mixed with 0.1 ml of an overnight culture of recipient in 1 ml of fresh BHI broth in an Eppendorf tube. The cells were pelleted, and the supernatant was discarded. The cells were suspended in the remaining broth (approximately 50 µl), transferred to a nitrocellulose filter on a BHI agar plate, and incubated at 37°C for 2 h. Cells were recovered by vortexing filters in 2 ml of BHI broth and grown on selective media. Strains carrying pVA1702 insertion derivatives which did not yield transconjugants in the assay described above were tested for a lower conjugation frequency by increasing the number of donor and recipient cells in filter matings. Two milliliters of the donor culture was mixed with 2 ml of the recipient culture, pelleted, and washed in 5 ml of fresh medium. Cells were pelleted, suspended in 100 µl of fresh BHI broth, transferred to a nitrocellulose filter, and incubated at 37°C overnight on solid media. Cells were recovered as described above. Overnight intragenic and intergenic matings between streptococci and enterococci were performed as described above, except that 1 ml of donor and recipient cultures was used.

Mapping of transposon insertions in pVA1702. Plasmid DNA for mapping transposon insertions was isolated from *E. faecalis* by the procedure of Ehrenfeld and Clewell (6). The location of the Tn917 or Tn917lac insertion site was found by digestion of the plasmid by the restriction enzymes *Bst*EII (three sites in pVA1702 and none in Tn917 or Tn917lac) and *Sal*I (no sites in pVA1702, one at the midpoint of Tn917, and two in Tn917lac). Digestion with *Bam*HI (no sites in pVA1702 and one near the terminus of Tn917lac) was used to determine the orientation and placement of the transposon.

Detection of expression of lacZ. Tn917lac carries a promoterless *lacZ* gene near its terminus (Fig. 2). Insertion of the transposon into an operon in the proper orientation generates a transcriptional fusion, with the *lacZ* gene using a ribosome binding site provided in the transposon sequences to ensure proper translation (27). *E. faecalis* cells carrying pVA1702::Tn917lac plasmids were picked onto L broth plates with kanamycin onto which 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 10 mg/ml of *N,N*-

TABLE 2. Bacterial strains

Species and strain	Description ^a	Source or reference
<i>S. sanguis</i>		
V288	Challis strain, naturally competent	18
V797	V288 carrying pVA797	8
V1349	Carries pStr4A	D. Le Blanc
V1828	Spontaneous Fs ^r variant of V481	This study
V1829	V1828 carrying pVA1702B	This study
<i>E. faecalis</i>		
OG1-SS	St ^r Sp ^r , host strain	6
OG1-RF	Rf ^r Fs ^r conjugal recipient	6
V1501	OG1-SS carrying pVA797	This study
V1507	OG1-SS carrying pVA1229	This study
V1827	OG1-SS carrying pStr4A	This study
V1700	OG1-SS carrying pVA1700	This study
V1701	OG1-SS carrying pVA1701	This study
V1702	OG1-SS carrying pVA1702	This study
V1830	OG1-SS carrying pVA1701B	This study
V1831	OG1-SS carrying pVA1702B	This study
V1500	OG1-SS carrying pTV1	D. Clewell
V1740	OG1-SS carrying pTV32	D. Clewell
<i>E. coli</i>		
HB101	<i>hsdS20 recA13 proA2 endA leu-6 thi-1 supE44 rpsL20 ara-14 galK2 lacY1 xyl-5 mtl-1</i>	B. Stocker
V838	pVA838; HB101 background	17
V1852	pGEM3; HB101 background	This study
V1856	<i>EcoRI-BamHI</i> fragment of pVA1702::Tn917lac insert no. 104 cloned into pVA838; HB101 background	This study
V1857	<i>EcoRI-BamHI</i> fragment of pVA1702::Tn917lac insert no. 104 cloned into pGEM3; HB101 background	This study
V1858	<i>EcoRI-BamHI</i> fragment of pVA1702::Tn917lac insert no. 64 cloned into pGEM3; HB101 background	This study
V1859	<i>EcoRI-BamHI</i> fragment of pVA1702::Tn917lac insert no. 9 cloned into pGEM3; HB101 background	This study
V1860	<i>EcoRI-BamHI</i> fragment of pVA1702::Tn917lac insert no. 33 cloned into pGEM3; HB101 background	This study
V1861	<i>EcoRI-SphHI</i> fragment of pVA1860 cloned into pGEM3; HB101 background	This study
V1862	<i>SphI-BamHI</i> fragment of p1858 cloned into pGEM3; HB101 background	This study
M2141	$\Delta(\text{pro-lac})$ Str ^r <i>minA minB</i>	21
V1863	M2141(pVA838)	This study
V1864	M2141(pVA1856)	This study
V1865	M2141(pGEM3)	This study
V1866	M2141(pVA1857)	This study
V1867	M2141(pVA1858)	This study
V1868	M2141(pVA1859)	This study
V1869	M2141(pVA1860)	This study
V1870	M2141(pVA1861)	This study
V1871	M2141(pVA1862)	This study

^a Drug resistance phenotypes: Fs, fusidic acid; St, streptomycin; Sp, spectinomycin; Rf, rifampin.

FIG. 1. Construction of pVA1701 and pVA1702. A conjugation-proficient pVA797::Tn917 insertion (pVA1229) was digested with *AvaI* and self-ligated, deleting 4.5 kb of plasmid DNA and all but 5 base pairs of the transposon. The *Cm*^r gene and pVA380-1 origin of pVA1700 were removed as a 3.8-kb *BclI-EcoRI* fragment, and a *Km*^r gene was inserted in their place, creating pVA1702. pVA1701 was derived directly from pVA797 by using the identical restriction enzyme strategy to replace the *Km*^r gene. ori, Origin of replication.

dimethylformamide; Sigma) had been spread. The cells were incubated overnight at 37°C. Expression of the *lacZ* gene was detected visually by the appearance of blue colonies (27). Colonies not expressing *lacZ* were white.

Expression of pVA1702 sequences in *E. coli* minicells. At the *BamHI* site provided by Tn917lac insertions, a series of nested *EcoRI-BamHI* fragments was cloned into pGEM3 (Promega Biotec, Madison, Wis.) (see Fig. 4) by using HB101 as a host and then transformed into an *E. coli* minicell-producing strain. Isolation and [³⁵S]methionine (Dupont, NEN Research Products, Boston, Mass.) labeling of *E. coli* minicells were done as previously described (15). Radiolabeled ³⁵S-plasmid-encoded proteins were electrophoresed in sodium dodecyl sulfate-10% polyacrylamide gels with a 5% stacking gel (12). After electrophoresis, gels were stained, destained, and dried (12). Molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.) were used as standards to determine the molecular weight of labeled peptides. Autoradiography was carried out for 48 to 104 h with Kodak X-Omat R film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Construction of pVA1700, pVA1701, and pVA1702. A plasmid derived from pVA797 was constructed by using a restriction site provided by a Tn917 insertion at 7.9 kb (Fig.

FIG. 2. Transposon insertion map of pVA1702. A linearized restriction map of the plasmid is shown with 1-kb increments indicated below the map. Positions of transposon insertions are indicated by vertical lines along the graph below the restriction map. Insertions which do not affect conjugation are below the graph, whereas insertions which abolish detectable transfer are above the graph. The A and B transfer regions are indicated below the insertion map. Tn917lac insertions are followed by an L. The proposed orientations of transcription of the A and B regions are represented by arrows. The insert shows the Tn917lac element in the rightward configuration with respect to the restriction map.

1, pVA1229) which did not affect conjugative transfer. Digestion with *Ava*I and self-ligation deleted 4.5 kb of plasmid sequences and all but 5 base pairs of Tn917 sequences, creating pVA1700. pVA1700 was 26.5 kb and was transferred at wild-type frequencies in isogenic *E. faecalis* filter matings.

To use the pTV1ts and pTV32 delivery vehicles for Tn917 and Tn917lac, respectively, a resistance gene other than that for *Cm*^r had to be placed on pVA1700. The *Cm*^r gene of pVA1700 was removed as a 3.8-kb *Bcl*I-*Eco*RI restriction fragment, and the *Km*^r gene of pStr4A was ligated in its place as a 2.5-kb *Bcl*I-*Eco*RI fragment, resulting in the construction of pVA1702 (Fig. 1). The *Bcl*I-*Eco*RI deletion of pVA1700 also removed the pVA380-1 origin of replication which had been added during the construction of pVA797 (8). pVA1701 was derived directly from pVA797. The *Cm*^r gene and pVA380-1 origin of replication were removed and exchanged for the pStr4A *Km*^r gene by using the same restriction strategy used in the construction of pVA1702 (Fig. 1). pVA1229, pVA1700, and pVA1701 all transferred at the wild-type frequency in isogenic *E. faecalis* filter matings (data not shown). pVA1702 reproducibly transferred at a slightly higher frequency than that of the wild type (3.08×10^{-3} versus 1.30×10^{-3}). Although pVA1701 and pVA1702 transferred at wild-type frequencies in isogenic and nonisogenic *E. faecalis* matings (data not shown), the transfer frequency of pVA1702 to *S. sanguis* decreased 10^6 -fold. pVA1701 transfer frequency decreased only 100-fold (1.21×10^{-9} for pVA1702 versus 1.73×10^{-5} for pVA1701).

Transposon mutagenesis of pVA1702. pVA1702 is a pIP501 derivative which maintained conjugative proficiency. Because of its reduced size, it was judged the best candidate for transposon insertional mutagenesis. An analysis of the conjugation functions of pVA1702 was initiated by using the

transposons Tn917 and Tn917lac delivered by the temperature-sensitive vectors pTV1ts and pTV32, respectively (27), in an *E. faecalis* OG1-SS system (6, 25). Plasmids carrying transposon insertions were isolated as described in Material and Methods. From 12 separate temperature-curing experiments (8 with pTV1ts and 4 with pTV32), approximately 300 *Km*^r *Em*^r *Cm*^s colonies were isolated and tested for conjugative ability in filter matings. The plasmid DNA was isolated, and the site and orientation of the insertion were determined by restriction endonuclease analysis. Tn917lac inserts were screened for β -galactosidase production by testing for the ability of colonies to hydrolyze X-Gal. Approximately 60% of the insertions mapped between positions 2.75 and 3.25 of pVA1702, with Tn917 and Tn917lac having a strong preference for insertion into this region (Fig. 2). Tn917 had a second preferred insertion site at 14.0 kb, where 30% of the insertions occurred. Of the remaining inserts, 29 were considered unique (12 were Tn917, and 17 were Tn917lac).

Transposon insertion into two separate regions of pVA1702 (Fig. 2, A and B) resulted in an undetectable transfer phenotype ($<10^{-9}$ transconjugants per donor cell). None of the inserts that were isolated resulted in a reduced transfer phenotype. Region A was 7.5 kb, flanked by inserts 7A and 104, and defined by 17 unique insertions. All Tn917lac insertions within region A except 103 were oriented with the promoterless *lacZ* gene reading rightward on the linear plasmid map (see transposon orientation insert in Fig. 2). Insert 103 was located at the extreme right of the A region and was orientated with the promoterless *lacZ* gene reading leftward. All Tn917lac inserts in region A except 103 resulted in X-Gal hydrolysis. Colonies containing plasmids with inserts in different regions of the plasmid gave various intensities of blue on X-Gal plates (Table 3). Region B was

TABLE 3. Summary of pVA1702::Tn917 and pVA1702::Tn917lac insertions^a

Plasmid and insertion no.	Strain no.	Position (base pair)	Conjugation frequency	X-Gal hydrolysis	Tn917 Orientation
pVA1702::Tn917					
97	V1721	3200	2.49×10^{-4}	NA	NA
2	V1720	6990	$<4.45 \times 10^{-9}$	NA	NA
72	V1732	7570	$<8.00 \times 10^{-9}$	NA	NA
85	V1734	7850	$<2.00 \times 10^{-9}$	NA	NA
59	V1716	8470	$<1.38 \times 10^{-9}$	NA	NA
56	V1735	8800	$<8.70 \times 10^{-9}$	NA	NA
1	V1723	8940	$<6.25 \times 10^{-9}$	NA	NA
91	V1731	9600	$<9.09 \times 10^{-9}$	NA	NA
120	V1717	9760	$<8.03 \times 10^{-9}$	NA	NA
115	V1718	12980	$<6.75 \times 10^{-9}$	NA	NA
17	V1711	13970	$<1.72 \times 10^{-9}$	NA	NA
92	V1713	20200	8.93×10^{-4}	NA	NA
pVA1702::Tn917lac					
7A	V1832	3700	1.30×10^{-4}	++++	LW
1A	V1833	6300	$<2.67 \times 10^{-9}$	+++	RW
33	V1834	6800	$<2.40 \times 10^{-9}$	+++	RW
12	V1835	7100	$<3.17 \times 10^{-9}$	+++	RW
10	V1836	7600	$<3.45 \times 10^{-9}$	+++	RW
11	V1837	8300	$<1.50 \times 10^{-9}$	+++	RW
9	V1838	8450	$<3.50 \times 10^{-9}$	+++	RW
87	V1839	9200	$<4.26 \times 10^{-9}$	+++	RW
64	V1840	9600	$<3.77 \times 10^{-9}$	+++	RW
103	V1841	11000	$<2.60 \times 10^{-9}$	—	LW
104	V1842	11300	1.87×10^{-4}	+	RW
95	V1844	12400	$<3.00 \times 10^{-9}$	—	RW
81	V1845	13970	$<1.83 \times 10^{-9}$	—	RW
82	V1846	17200	$<2.30 \times 10^{-9}$	—	RW
6	V1847	20500	2.76×10^{-4}	+++	RW
31	V1848	24000	4.38×10^{-4}	+++	RW
93	V1849	25100	3.56×10^{-3}	—	LW

^a NA, Not applicable; + and —, degree of hydrolysis. Orientation of transcription of the *lacZ* gene on the linear map of pVA1702 was rightward (RW) or leftward (LW), as shown in Fig. 2 (insert).

8.8 kb and was flanked by inserts 104 and 92. It was defined by five unique transfer-deficient insertions. All of the B-region insertions were oriented so that transcription of the *lacZ* gene would be rightward, but none of them resulted in detectable X-Gal hydrolysis.

Subcloning and analysis of the A region in *E. coli*. In focusing our efforts on the A transfer region, a series of nested deletions was constructed by using a unique *EcoRI* site on pVA1702 and an internal *BamHI* site located at 276 bp on Tn917lac (Fig. 3A). The *EcoRI*-*BamHI* fragments from four separate insertions were subcloned onto the *E. coli* pGEM3 vector and introduced into the *E. coli* minicell-producing strain M2141 for analysis of plasmid-encoded peptides. Plasmid pVA1857 encompassed the entire A region and encoded five peptides in minicells with apparent molecular weights of 79, 75, 67, 52, and 27 kilodaltons (kDa) (Fig. 3B, lane 4). These proteins were designated p79, p75, p67, p52, and p27, respectively. When this same *EcoRI*-*BamHI* fragment was ligated into the *E. coli*-streptococcal shuttle vector pVA838, creating pVA1856, the same five peptides were produced by minicells containing this plasmid (Fig. 3B, lane 2). pVA1858, which contains a deletion of 1.7 kb into the A region, no longer produced p75 or p52, but a new peptide of 55 kDa was detected. The new peptide was presumed to be a 3' truncation of p75. This suggested that

the start of the p75 gene was located at 8.2 kb. pVA1859, which has a deletion 1.1 kb further into the A region than that of pVA1858, encoded a peptide of 25 kDa in minicells. This peptide was presumed to be a second truncation of p75, and placement and transcription orientation of the p75 gene were confirmed. pVA1860, with a deletion 1.7 kb further into the transfer region than that of pVA1859, no longer specified the production of p27. pVA1861, with a deletion 0.9 kb further into the A region than that of pVA1860, resulted in the loss of both p79 and p67. The amount of DNA deleted was not large enough to encode for either peptide. pVA1862, with a 3.1-kb deletion of pVA1859 which removed 2.6 kb from the left side of the transfer region (Fig. 3A and B, lane 9), resulted in the loss of p79 and p67, and a smaller peptide of 33 kDa was observed. This smaller peptide was presumed to be the product of a 3' deletion of the gene encoding p79 or p67.

DISCUSSION

We have described the construction and analysis of pVA1702, a conjugation-proficient derivative of pIP501. This plasmid carried a contiguous 22.2-kb region of pIP501 and was conjugally transferred at a slightly higher frequency than pIP501 in an isogenic *E. faecalis* mating system. In intergenic matings, pVA1702 failed to transfer to *S. sanguis*, whereas pVA1701 did so at near to wild-type frequencies. The change in host range ability was accompanied by the deletion of a DNA fragment (4.5 kb; Fig. 1). By increasing the number of donor bacteria in a filter mating by 100-fold and extending the mating time from 2 to 24 h, it was possible to isolate *S. sanguis* transconjugants when *E. faecalis* carrying pVA1702 was used as the donor. The loss of host range ability appeared to be due to the disruption of a function(s) which is required for stable maintenance in different hosts, and we are continuing our investigation of this phenomenon.

The regions involved in conjugal transfer of pVA1702 were located by transposon mutagenesis. The technique used to generate insertions prevented the isolation of insertions in the *Km^r* gene or in the plasmid origin of replication. Both Tn917 and Tn917lac were used with the hope that the modifications of Tn917 in the construction of Tn917lac would alter the regional insertion specificity exhibited by this transposon (27). Approximately 60% of all insertions for both transposons were isolated between 2.75 and 3.25 kb on the plasmid map. In this same region on pVA1229 there was a Tn917 insertion that provided the restriction endonuclease site allowing the genesis of pVA1702 (Fig. 1 and 2). Tn917 had a second preferred insertion site at 14.0 kb, where 30% of the insertions occurred. Insertion mutagenesis revealed that the conjugation genes appeared to be in at least two separate regions of the plasmid, designated A and B (Fig. 2). Region A was 7.5 kb and was defined by 17 unique insertions between 3.7 kb and 11.2 kb. However, no insertions were isolated between 3.7 and 6.2 kb. Region B was 8.8 kb and was defined by seven unique insertions spanning 11.3 to 20.1 kb. Three of these insertions were clustered at the far left end of the region. The use of Tn917lac with its promoterless *lacZ* gene suggested a preliminary transcription map of the plasmid. All inserts in region A except 7A, which is at the extreme left end of the region, indicated that transcription was occurring in a rightward direction on the linear plasmid map (Table 3; Fig. 2), whereas inserts in the B region suggested that transcription was occurring in the leftward direction (Table 3; Fig. 2). It is possible, however, that transcription in the B region could be rightward but heavily

FIG. 3. Deletion analysis of the A transfer region. (A) Restriction map of the A region, with 1-kb increments indicated below the map. The restriction fragments cloned into the pGEM3 vector are indicated by horizontal lines. All subclones except pVA1861 end with a *Bam*HI site provided by different *Tn917lac* insertions. (B) Autoradiograph of the proteins encoded by these subclones labeled with [35 S]Met, as described in Materials and Methods, and separated on a polyacrylamide gel. Arrows indicate plasmid-encoded peptides of the A region with M_r s (from top to bottom) of 79, 75, 67, 52, and 27 kDa.

repressed. The insertion mutagenesis map of pVA1702 has several gaps (Fig. 2). This may reflect the nonrandom insertion of the transposon, and the isolation of insertions in these regions may simply require more screening. Alternatively, an insertion into these sites may be lethal to the cell or make the plasmid highly unstable. Because of the apparent strong regional specificity of the *Tn917* transposons and the present lack of another suitable transposon element, it was decided to forgo further mutant isolation.

When pVA797 (Fig. 1) was cloned in toto into *E. coli* and introduced into a minicell-producing strain, a number of plasmid-encoded peptides were observed (9). Following this strategy, efforts were focused on region A of pVA1702 to determine the number of proteins it encoded and to map the locations of their respective genes. A series of nested deletions of the A transfer region was subcloned onto the *E. coli* vector pGEM3 by using an *Eco*RI site at 2.5 kb on pVA1702 and a *Bam*HI site at 287 bp on *Tn917lac* (Fig. 3). pVA1857, which contains the entire A region, was found to encode five unique peptides of M_r s 79, 75, 67, 52, and 27 kDa (Fig. 3, lane 4). These same five peptides were observed when the identical DNA fragment was cloned into streptococcal shuttle vector pVA838 (Fig. 3, lane 2). On the basis of observations by Evans et al. (9) and the fact that an identical minicell protein profile was obtained when the A region was cloned into two separate vectors, we feel that these proteins were being transcribed from their native promoters in *E. coli*. Using a conversion factor of 37 kDa of protein equaling 1 kb of DNA, the total amount of plasmid-encoded protein observed in *E. coli* minicells (300 kDa) is in good agreement with the total potential coding capacity of the 8.3-kb clone encompassing the A region (306 kDa). Deletion analysis of this region showed that the genes encoding the peptides p75, p52, and p27 are located between 6.8 and 11.3 kb (Fig. 3). Insertional mutagenesis of this area indicated that all three proteins were involved in conjugal transfer, and *lacZ* insertions indicated that the proteins were transcribed from left to

right on the plasmid map (Fig. 2). Sequential deletions from the right end of the A region indicated that the order of the genes encoding these peptides is p27, p75, and p52, designated (for conjugation) *cnjA*, *cnjB*, and *cnjC*, respectively (Fig. 4). Since these proteins are involved in a similar function and appear to be transcribed in the same direction, it is possible that they are transcribed as a single polycistronic mRNA. We plan to test this hypothesis.

pVA1861, which contains a deletion 0.9 kb further into the A region than that of pVA1860, resulted in the loss of both p79 and p67 (Fig. 3B, lane 8). The amount of DNA deleted was not large enough to encode for either peptide. pVA1862, a 3.1-kb deletion derivative of pVA1859 which removed 2.6 kb from the left side of the transfer region, resulted in the loss of both p79 and p67 and the appearance of a novel 33-kDa peptide. This smaller peptide was presumed to be a truncated product of p79 or p67. The genes encoding p79 and p67 are apparently transcribed in opposite orientation to the

FIG. 4. Location of the conjugation genes of the A transfer region. A linear map of the A region is presented, with 1-kb increments indicated below the map. From 0 to 3 kb is the DNA fragment which carried the *Km^r* gene which was ligated to pIP501 sequences. The orientations of transcription of *cnjA*, *cnjB*, and *cnjC* are indicated by arrows. The orders of p79 and p67 are unknown. These proteins are presented solely to indicate that there is enough DNA present to encode for two peptides of this size.

FIG. 5. Composite map of pIP501. Symbols: ■, *Cm*^r gene, *Em*^r gene, *cop*, and origin of replication (*ori*) positioned as described elsewhere (1, 8); ▨, region which affects transfer to *S. sanguis*. Genes of the A region are positioned as described in the text; the B region extends from 17 to 21 kb. Arrows indicate the proposed orientations of transcription for both regions. Dashed line indicates the possible extent of the B region.

other genes in the A region. This was confirmed by the transcription of the *Tn917lac* insertion at 3.7 kb (Table 3; Fig. 2). Since there are no useful restriction sites between 3 and 6 kb, it has not been possible to map the order of the genes encoding p79 or p67. Additionally, since the only transposon isolated in this area was at the extreme end of the region, the insertion might not interrupt the coding sequence of a protein. It is presently unknown if these proteins are involved in conjugal transfer.

A composite map of pIP501 is presented in Fig. 5. This plasmid is a member of a group of conjugative plasmids which are distinct from other gram-positive conjugative elements (4). Using deletion analysis and transposon mutagenesis, we have demonstrated that the genes encoding the transfer apparatus of pIP501 are spread over two regions which encompass 16 kb of the plasmid. Since this 16-kb region can be split by an insertion which does not affect transfer, it is unlikely that all of the conjugation genes are transcribed as a single large transcript. A detailed investigation of the A region has located the genes encoding three transfer peptides of 25, 76, and 45 kDa. It is possible that these proteins are processed to a different form in streptococci. Regardless, this work has allowed the mapping of the genes encoding these proteins and has opened new avenues for investigation of the roles of these proteins.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant R37 DE04224 to F.L.M.

We sincerely thank D. Clewell for providing us with *E. faecalis* strains carrying pTV1ts and pTV32.

LITERATURE CITED

1. Behnke, D., and M. S. Gilmore. 1981. Location of antibiotic resistance determinants, copy control, and replication functions on the double-selective streptococcal cloning vector pGB301. *Mol. Gen. Genet.* **184**:115-120.

2. Bougueleret, L., G. Bieth, and T. Horodniceanu. 1981. Conjugative R plasmids in group C and G streptococci. *J. Bacteriol.* **145**:1102-1105.
3. Buu-Hoi, A., G. Beth, and T. Horaud. 1984. Broad host range of streptococcal macrolide resistance plasmids. *Antimicrob. Agents Chemother.* **25**:289-291.
4. Clewell, D. B. 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. *Microbiol. Rev.* **45**:409-436.
5. Clewell, D. B., Y. Yagi, G. M. Dunny, and S. K. Schultz. 1974. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. *J. Bacteriol.* **117**:283-289.
6. Ehrenfeld, E. E., and D. B. Clewell. 1987. Transfer functions of *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. *J. Bacteriol.* **168**:3473-3481.
7. Engel, H. W. B., N. Soedirman, J. A. Rost, W. J. van Leeuwen, and J. D. A. van Embden. 1980. Transferability of macrolide, lincomycin, and streptogramin resistances between group A, B, and D Streptococci, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. *J. Bacteriol.* **142**:407-413.
8. Evans, R. P., and F. L. Macrina. 1983. Streptococcal R plasmid pIP501: endonuclease site map, resistance determinant location, and construction of novel derivatives. *J. Bacteriol.* **154**:1347-1355.
9. Evans, R. P., R. B. Winter, and F. L. Macrina. 1985. Molecular cloning of a pIP501 derivative yields a model replicon for the study of streptococcal conjugation. *J. Gen. Microbiol.* **131**:145-153.
10. Gibson, E. M., N. M. Chace, S. B. Londen, and J. Londen. 1979. Transfer of plasmid-mediated antibiotic resistance from streptococci to lactobacilli. *J. Bacteriol.* **137**:614-619.
11. Gonzalez, C. F., and B. S. Kunka. 1983. Plasmid transfer in *Pediococcus* spp.: intergeneric and intrageneric transfer of pIP501. *Appl. Environ. Microbiol.* **46**:81-89.
12. Hames, B. D. 1981. An introduction to polyacrylamide gel electrophoresis, p. 1-91. In B. D. Hames and D. Rickwood (ed.), *Gel electrophoresis of proteins, a practical approach*. IRL Press, Washington, D.C.
13. Horaud, T., C. L. Bouguenec, and K. Pepper. 1985. Molecular genetics of resistance to macrolides, lincosamides, and streptogramin B (MLS) in streptococci. *J. Antimicrob. Chemother.* **16**(Suppl A):111-135.
14. Horodniceanu, T., D. H. Bouanchaud, G. Bieth, and Y. A. Chabbert. 1976. R plasmids in *Streptococcus agalactiae* (group B). *Antimicrob. Agents Chemother.* **10**:795-801.
15. Kennedy, N., L. Beutin, M. Achtman, R. Skurry, U. Rahmsdorf, and P. Herrlich. 1977. Conjugation proteins encoded by the F sex factor. *Nature (London)* **270**:580-585.
16. Le Blanc, D. L., and L. N. Lee. 1984. Physical and genetic analysis of streptococcal plasmid pAMB1 and cloning of its replication region. *J. Bacteriol.* **157**:445-453.
17. Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones. 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. *Gene* **25**:145-150.
18. Macrina, F. L., K. R. Jones, and P. H. Wood. 1980. Chimeric streptococcal plasmids and their use as molecular cloning vehicles in *Streptococcus sanguis* (Challis). *J. Bacteriol.* **143**:1425-1435.
19. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. Oultram, J. D., and M. Young. 1985. Conjugal transfer of plasmid pAMB1 from *Streptococcus lactis* and *Bacillus subtilis* to *Clostridium acetobutylicum*. *FEMS Microbiol. Lett.* **27**:129-134.
21. Reeve, J. 1979. Use of minicells for bacteriophage directed polypeptide synthesis. *Methods Enzymol.* **68**:493-503.
22. Romero, D. A., P. Slos, C. Robert, I. Castellino, and A. Mercenier. 1987. Conjugative mobilization as an alternate vector delivery system for lactic streptococci. *Appl. Environ.*

- Microbiol. **29**:807–813.
23. Schaberg, D. R., D. B. Clewell, and L. Glatzer. 1982. Conjugative transfer of R-plasmids from *Streptococcus faecalis* to *Staphylococcus aureus*. Antimicrob. Agents Chemother. **22**: 204–207.
 24. Trieu-Cuot, P., C. Carlier, and P. Courvalin. 1988. Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli*. J. Bacteriol. **170**:4388–4391.
 25. Weaver, K. E., and D. B. Clewell. 1988. Regulation of the pAD1 sex pheromone response in *Enterococcus faecalis*: construction and characterization of *lacZ* transcriptional fusions in a key control region of the plasmid. J. Bacteriol. **170**:4343–4352.
 26. Wirth, R., F. Y. An, and D. B. Clewell. 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli*-*S. faecalis* shuttle vector. J. Bacteriol. **165**:831–836.
 27. Youngman, P. 1987. Plasmid vectors for recovering and exploiting Tn917 transpositions in *Bacillus* and other gram-positive bacteria, p. 79–103. In K. G. Hardy (ed.), Plasmids, a practical approach. IRL Press, Washington, D.C.