[30] RP4-Based Plasmids for Conjugation between *Escherichia coli* and Members of the Vibrionaceae

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Introduction

The Vibrionaceae family includes both important pathogens and useful model organisms in the study of processes ranging from the regulation of light production by quorum sensing to the establishment of mutualistic animal–bacteria associations. Genetics has proven a powerful discipline in the study of *Vibrio* species, and the refinement of genetic tools for use with these important and interesting bacteria will facilitate future studies of their biology.

The self-transmissible broad host-range IncPα plasmid RP4[1] provides the means for genetic manipulation of many diverse bacteria, including members of the Vibrionaceae. Derivatives of RP4 facilitate the conjugal transfer of vectors engineered using *Escherichia coli* as a host into other bacterial species of interest. As a general strategy, the *cis*-acting origin of transfer, *oriT* or "mob," is cloned into vectors, which can then be mobilized if *trans*-acting *tra*-and *trb*-encoded RP4 transfer functions are provided. The *trans*-acting transfer functions are typically provided by an RP4-derived helper plasmid such as pRK2013[2,3] or by strains such as β2155, S17-1, or SM10, which have chromosomally integrated helper plasmids.[4,5] The RP4-based helpers pRK2013, S17-1, and SM10, together with the pSUP series of *oriT*-containing plasmids,[5,6] have been the foundation for genetic manipulations in many gram-negative bacteria including the Vibrionaceae. We have improved the utility of RP4-based conjugation for use with the Vibrionaceae (and other gram-negative bacteria) by modifying conjugal tools to meet three criteria.

Criterion 1: An ideal conjugal helper should not introduce insertion elements into recipient cells, except where that function is intended. If this criterion is not met, transposition of insertion elements from the donor into recipient DNA could complicate interpretation of experimental data. Helper plasmids and the chromosomes of helper strains contain *oriT* and are themselves self-transmissible; their use can result in unintended transfer of transposable elements into recipients.

[1] W. Pansegrau, E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, and C. M. Thomas, *J. Mol. Biol.* **239,** 623 (1994).
[2] D. H. Figurski and D. R. Helinski, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1648 (1979).
[3] G. Ditta, S. Stanfield, D. Corbin, and D. R. Helinski, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 7347 (1980).
[4] C. Dehio and M. Meyer, *J. Bacteriol.* **179,** 538 (1997).
[5] R. Simon, U. Priefer, and A. Pühler, *Biotechnology* **1,** 784 (1983).
[6] R. Simon, M. O'Connell, M. Labes, and A. Pühler, *Methods Enzymol.* **118,** 640 (1986).

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For example, SM10, S17-1, and β2155 can transfer dozens of insertion elements native to *E. coli* (e.g., IS*1*, IS2, IS*3*, IS*4*, IS*5*, IS*30*, IS*150*, and IS*186*) into recipients. Each of these strains also carries transposable elements on their integrated helper plasmids. For example S17-1 contains a chromosomal copy of RP4-2-Tc::Mu-Km::Tn*7* harboring Tn*7* and Mu, both of which have been shown to transpose in *Vibrio* species. Furthermore, we found that S17-1 can transfer Tn*7* to *Vibrio fischeri* recipients. Helper plasmid pRK2013 lacks the transposable elements IS*21* and Tn*1* of its parent vector, RP4; however, an examination of pRK2013's construction,[7–11] and Southern blotting (unpublished data) revealed that pRK2013 carries Tn*903*. Although unintended transposition of Tn*903* or Tn*7* into recipients can often be readily detected by screening for drug resistances encoded by these transposons, the presence of other elements (e.g., Mu, IS*1*, IS903) can only be screened by laborious PCR- or hybridization-based methods. Accidental incorporation of foreign insertion elements into recipient strains can be virtually eliminated by the use of a conjugal helper plasmid lacking insertion elements. We therefore constructed three such helper plasmids (pEVS101, pEVS103, and pEVS104) that lack insertion elements.

Criterion 2: For many purposes, a conjugal helper that replicates in the donor, but not in the recipient, is desirable. pRK2013 contains the ColE1 plasmid origin which allows its replication in certain recipients, particularly members of the Enterobacteriaceae and Vibrionaceae. Although pRK2013 can be readily lost from *Vibrio* strains during nonselective growth, its retention under selective pressure can be problematic in two ways. First, using pRK2013 we could not select for mobilization of constructs conferring kanamycin resistance (KnR), because this marker is found on pRK2013 and kanamycin (Kn) selection identifies, with high frequency, *Vibrio* transconjugants retaining pRK2013. Second, when the goal is to mobilize suicide plasmids for marker exchange or transposon mutagenesis, the homologous *oriT* regions of the helper and the suicide plasmid it is mobilizing can recombine, and the resulting product can be replicated by the helper's ColE1 origin. Thus, plasmid–plasmid recombinants may be the unintentional products of a mating intended to produce chromosomal insertions. We therefore constructed helper plasmid pEVS104 such that it contains only the R6Kγ origin of replication,[12] which is not maintained in *Vibrio* recipients.

Criterion 3: Optimally, mobilizable vectors should contain only a defined, minimal *oriT*. The *cis*-acting *oriT* region in many mobilizable (e.g., pSUP) plasmids

[7] S. N. Cohen, A. C. Y. Chang, H. W. Boyer, and R. B. Helling, *Proc. Natl. Acad. Sci. U.S.A.* **70,** 3240 (1973).
[8] M. A. Lovett and D. R. Helinski, *J. Bacteriol.* **127,** 982 (1976).
[9] A. Oka, H. Sugisaki, and M. Takanami, *J. Mol. Biol.* **147,** 217 (1981).
[10] R. P. Silver and S. N. Cohen, *J. Bacteriol.* **110,** 1082 (1972).
[11] T. Watanabe, C. Ogata, and S. Sato, *J. Bacteriol.* **88,** 922 (1964).
[12] R. Kolter, M. Inuzuka, and D. R. Helinski, *Cell* **15,** 1199 (1978).

is undefined[5,6] and includes *tra* genes flanking the *oriT* (i.e., *traJ* and *traK*) that are nonessential for *cis*-acting transfer function. The expression of these genes by mobilized plasmids is unnecessary and could present a burden on recipient cells making plasmid carriage unfavorable. Furthermore, the presence of this extraneous DNA can unnecessarily complicate cloning strategies. Plasmids with minimal *oriT* regions have been generated and were conjugally functional.[13,14] We have extended this approach by constructing a series of minimal *oriT* cassettes flanked by convenient restriction sites, facilitating the subcloning of *oriT* into other vectors. Using these cassettes we constructed plasmids with utility for allelic exchange or complementation in *Vibrio* species.

The new RP4-based constructs described here are at least as efficient as existing tools for mediating conjugal transfer between *E. coli* and members of the Vibrionaceae. Helper plasmid pEVS104 mediated conjugation into seven Vibrionaceae species as efficiently as did pRK2013, but, unlike pRK2013, did not result in recipients bearing helper-plasmid-encoded KnR. Shuttle vector pEVS78 was transferred to *V. fischeri* 100-fold more efficiently than pSUP102, and CC118λpir pEVS104 transferred pEVS78 to most *Vibrio* recipients more efficiently than did strain S17-1. Advantages of these new tools include: (i) conjugal helpers that do not introduce transposable elements into recipients, (ii) a conjugal helper, pEVS104, that does not replicate in *Vibrio* recipients, (iii) convenient *oriT* cassettes that are defined and free of extraneous gene sequences, and (iv) mobilizable cloning, marker exchange, and shuttle vectors incorporating this *oriT* cassette. The tools and methods described here should also be useful for mediating conjugation between *E. coli* and many other gram-negative bacteria. Helper plasmid pEVS104 will be particularly advantageous for recipients, such as the Enterobacteriaceae, that replicate ColE1-based vectors or support transposition of IS*903*, Mu, Tn*7*, or *E. coli* IS elements.

General Methods for Cloning and Conjugation

Bacterial strains used in conjugation experiments, and their origins, are listed in Table I. *E. coli* strains S17-1, DH5α, or DH5α-32 are used as the host for plasmids with either ColE1 or p15A origins of replication. Strain BW23474 serves as the host for plasmids with the R6Kγ origin of replication, with the exception of plasmid pEVS104, which is maintained in strain CC118λ*pir*. When added to LB medium[15] for selection of *E. coli,* tetracycline (Tc), ampicillin (Ap), chloramphenicol (Cm), and Kn are used at concentrations of 10, 100, 20, and 40 μg ml^{-1}, respectively. For selection of *E. coli* with erythromycin (Em), 150 μg ml^{-1} is added

[13] M. F. Alexeyev and I. N. Shokolenko, *Gene* **160,** 59 (1995).

[14] A. Schafer, A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Pühler, *Gene* **145,** 69 (1994).

[15] J. H. Miller, "A Short Course in Bacterial Genetics." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992.

TABLE I
BACTERIAL STRAINS, PLASMIDS, AND OLIGONUCLEOTIDES

Strain, plasmid, or oligonucleotide	Relevant characteristics[a]	Source or reference[b]
Bacterial strains		
E. coli		
DH5α	*F⁻ Φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(1)
DH5α-32	DH5α, demi-Tn5EmR::*trpA*	(2)
BW23474	*Δlac-169 robA1 creC510 hsdR514 uidA (ΔMluI)::pir-116 endA (BT333) recA1*	(3, 4)
S17-1	*thi pro hsdR hsdM recA* RP4 2-Tc::Mu-KnR::Tn7 (TpR, SpR, SmR)	(5)
CC118λ*pir*	*Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1,* lysogenized with λ*pir*	(6)
TH1191	*dam⁻ dcm⁻*	K. Visick
Vibrionaceae		
ES114	*V. fischeri;* wild-type *Euprymna scolopes* light organ isolate	(7)
ESR1	Spontaneous RfR derivative of ES114	(8)
ATCC33934	*V. orientalis*	ATCC
ATCC33869	*V. splendidus*	ATCC
KNH1	*V. parahaemolyticus*	(9)
BB120	*V. harveyi*	B. Bassler
KNH6	*P. leiognathi*	This study
HM21R	*A. veronii;* spontaneous RfR derivative of wild-type isolate HM21	(10)
Plasmids		
pACYC184	p15A ori; CmR, TcR	(11)
pSUP102	RP4 *oriT*-containing, ~2 kb, *Sau*3AI (partial digest) fragment in *BclI* site pACYC184; CmR, TcR	(12)
pSUP202	RP4 *oriT*-containing, ~2 kb, *Sau*3AI (partial digest) fragment in *Sau*3AI (partial digest) site pBR325; ColE1 ori, ApR, CmR, TcR	(5)
pBluescriptKS+	ColE1 ori, MCS::*lacZα*; ApR	Stratagene
pBCSK+	pBluescriptSK+, *cat* replaces *bla;* CmR	Stratagene
pCR2.1	ColE1 ori, MCS::*lacZα*, PCR-product cloning vector; ApR KnR	Invitrogen
pUC4K	ColE1 ori, KnR cassette; ApR KnR	(12)
pRK2013	ColE1 ori, RP4 *oriT, trb* and *tra,* R6-5 Tn*903* fragment; KnR	(13)
pKV38	oriR6K, EmR	K. Visick
pEVS70	*oriT* PCR product (from primers EVS49 and EVS50) in pCR2.1	This study
pEVS71	pEVS70 *oriT Xba*I fragment, and *Xba*I-*Xba*I MCS fragments, in pBCSK+ *Spe*I site, *lacZα*$^+$	This study
pEVS72	pEVS70 *oriT Xba*I fragment in pBCSK+ *Spe*I site, *lacZα*$^-$	This study
pEVS73	pEVS70 *oriT Xba*I fragment, and *Xba*I-*Xba*I MCS fragments, in pBluescriptKS+ *Spe*I site, *lacZα*$^+$	This study
pEVS75	*oriT* PCR product (from primers EVS58 and EVS59) in pCR2.1	This study
pEVS76	pEVS75*Alu*I *oriT* fragment in pUC4K *Stu*I site	This study
pEVS77	pEVS71 *Bam*HI *oriT* fragment in pACYC184 *BclI* site	This study

TABLE I (*continued*)

pEVS78	pEVS77 Δ*Eco*RV (ΔTcR)	This study
pEVS79	pEVS75 *Alu*I *oriT* fragment in pBCSK+ *Xmn*I site	This study
pEVS94	pEVS72 *Xba*I-*Bam*HI *oriT* fragment in *Xba*I-*Bam*HI digested pKV38; artificial MCS in *Xba*I site	This study
pEVS94S	5′-AATTGGTCGACC-3′ self-annealed and ligated into *Eco*RI site of pEVS94	This study
pEVS99	pKV38 *Xba*I digested, in pRK2013 *Avr*II site	This study
pEVS101	pEVS99 Δ*Stu*I (Δ Tn903)	This study
pEVS103	pEVS76 KnR *oriT Sal*I cassette in pEVS101 *Xho*I site	This study
pEVS104	pEVS103 Δ*Eco*RI (Δ ColE1)	This study
pEVS113	pUC4K KnR *Pst*I fragment in pEVS94S *Pst*I site, Δ*Eco*RV (Δ EmR)	This study
pEVS114	CmR *Pst*I fragment in pEVS94S *Pst*I site, Δ*Eco*RV (Δ EmR)	This study
Oligonucleotides		
EVS49	5′GGA TCC TCT AGA CTG GAA GGC AGT ACA CCT TGA TAG 3′	This study
EVS50	5′GGA TCC TCT AGA TTC CTG CAT TTG CCT GTT TCC AG 3′	This study
EVS58	5′ CAT GAT CGA GCT TAA TTC TGG AAG GCA GTA CAC CTT GAT AG 3′	This study
EVS59	5′ CAT GAT CGA GCT TAA TTC CTG CAT TTG CCT GTT TCC AG 3′	This study

[a] ATCC, American Type Culture Collection (Manassus, VA); MCS, multiple cloning site; ApR, ampicillin resistance; CmR, chloramphenicol resistance; EmR, erythromycin resistance; KnR, kanamycin resistance; RfR, rifampicin resistance; SpR, spectinomycin resistance; SmR, streptomycin resistance; TcR, tetracycline resistance; TpR, trimethoprim resistance.

[b] Key to references: (1) D. Hanahan, *J. Mol. Biol.* **166**, 557 (1983); (2) E. V. Stabb, E. G. Ruby, "American Society for Microbiology, 98th General Meeting," p. 375. American Society for Microbiology, Washington, D.C., 1998; (3) A. Haldimann, M. K. Prahalad, S. L. Fisher, S. K. Kim, C. T. Walsh, and B. L. Wanner, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14361 (1996); (4) W. W. Metcalf, W. Jiang, and B. L. Wanner, *Gene* **138**, 1 (1994); (5) R. Simon, U. Priefer, and A. Pühler, *Biotechnology* **1**, 784 (1983); (6) M. Herrero, V. De Lorenzo, and K. N. Timmis, *J. Bacteriol.* **172**, 6557 (1990); (7) K. J. Boettcher and E. G. Ruby, *J. Bacteriol.* **172**, 3701 (1990); (8) J. Graf, P. V. Dunlap, and E. G. Ruby, *J. Bacteriol.* **176**, 6986 (1994); (9) S. V. Nyholm, E. V. Stabb, E. G. Ruby, and M. J. McFall-Ngai, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10231 (2000); (10) J. Graf, *Infect. Immun.* **67**, 1 (1999); (11) A. C. Y. Chang and S. N. Cohen, *J. Bacteriol.* **134**, 1141 (1978); (12) J. Vieira and J. Messing, *Gene* **19**, 259 (1982); (13) D. H. Figurski and D. R. Helinski, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1648 (1979).

to BHI medium (Difco, Sparks, MD). With the exception of *Aeromonas veronii,* which is grown in LB, Vibrionaceae strains are grown in LBS medium.[16] When added to LBS or LB medium for selection of Vibrionaceae strains, rifampicin (Rf), trimethoprim (Tp), Cm, streptomycin (St), spectinomycin (Sp), and Kn are used

[16] E. V. Stabb, K. A. Reich, and E. G. Ruby, *J. Bacteriol.* **183,** 309 (2001).

at concentrations of 100, 5, 2, 200, and 100 μg ml^{-1}, respectively. Strain KNH6 was isolated from near-shore seawater in Kaneohe Bay, Oahu, Hawaii, and is designated *Photobacterium leiognathi* based on: (i) luciferase enzyme kinetics; (ii) galactose utilization; (iii) nonutilization of maltose, trehalose, and sucrose; and (iv) growth at 37°.[17,18]

Except in conjugations with *A. veronii,* donor and recipient cells are grown to stationary phase in LB or LBS, respectively, with appropriate antibiotics, and 100 μl of each culture is combined in a microfuge tube. The cells are pelleted by centrifugation, washed in fresh antibiotic-free LBS, repelleted, suspended in 10 μl of fresh LBS, dropped onto the surface of fresh LBS agar medium, incubated for 16 hr at 28°, resuspended in 750 μl of LBS, serially diluted, and plated. Matings with *A. veronii* are performed similarly, except that the recipient culture is grown in LB to an OD_{600} between 0.2 to 0.3, 800 μl of this *A. veronii* culture is combined with donor cells, and matings are incubated on LB agar medium at 37°. Selection, or strong enrichment, against *E. coli* donor strains is accomplished using Rf for matings with *A. veronii,* or by plating at 22° for *V. orientalis, V. splendidus, V. parahaemolyticus, V. fischeri,* and *P. leiognathi.* When *V. harveyi* BB120 is the recipient, plated mating mixtures are incubated at 28°, and recipient colonies are easily distinguished by their bioluminescence.

Construction of New Conjugal Helper Plasmids

Plasmids pEVS101, pEVS103, and pEVS104 are derivatives of pRK2013 that retain the *tra* and *trb trans*-acting functions necessary for conjugal transfer, but lack Tn*903* (Fig. 1 and Table I). Plasmids pEVS101, pEVS103, and pEVS104 each function as a conjugal helper plasmid. Helpers pEVS101 and pEVS103 contain both a ColE1 plasmid origin and an R6Kγ origin of replication, whereas pEVS104 contains only the R6Kγ origin and therefore requires the presence of the R6K *pir* gene in host strains for plasmid maintenance.[12]

Construction of Minimal oriT Cassettes

We use PCR (polymerase chain reaction) to amplify a 400-bp region of RP4 surrounding *oriT* (bp 51033 to 51432), and through cloning and subcloning of these amplification products we have generated small cassettes containing the *oriT* region flanked by convenient restriction sites in different orientations (Fig. 2). These cassettes contain the recognition, binding, and *nic* sites for TraI and TraJ, as well as the nearby bent DNA region that serves as the target for TraK. However, the

[17] J. J. Farmer III and F. W. Hickman-Brenner, *in* "The Prokaryotes," 2nd Ed. (A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer, eds.), Vol. 3, p. 2952. Springer-Verlag, New York, 1992.

[18] K. H. Nealson, *Methods Enzymol.* **57,** 153 (1978).

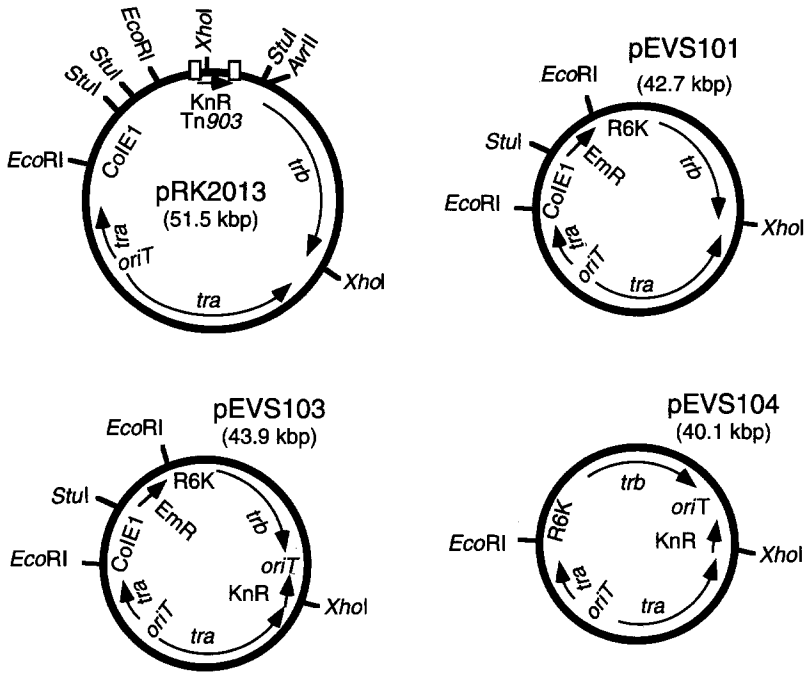

FIG. 1. New conjugal helper plasmids. Positions of plasmid origins, transfer origins, drug resistance genes, and selected restriction sites and transcripts (e.g., *tra* and *trb*) are indicated. Not all genes are shown (e.g., *kor* genes). *Stu*I sites blocked by *dcm* methylation are not shown. Plasmid construction is outlined in Table I.

constructs lack the *traJ* and *traK* genes flanking *oriT*.[1] We select for the presence of *oriT* cassettes in vectors based on conferred mobilizability from strain DH5α to DH5α-32. Oligonucleotide primer sequences and plasmids used to generate and isolate the *oriT* cassettes are listed in Table I. The sequence of each *oriT*-cassette-bearing plasmid is known and available on request.

The *oriT* cassettes in pEVS70, pEVS71, pEVS72, pEVS73, pEVS75, and pEVS76 are flanked by recognition sequences for common restriction enzymes, facilitating the subcloning of *oriT* into other vectors. The *oriT* cassette in pEVS75 is flanked on either side by the 4-bp recognition sequences for *Nla*III, *Sau*3AI, *Taq*I, *Alu*I, *Mse*I, and *Tsp*509I, and digestion with these enzymes results in DNA ends that are compatible with those generated by a number of 6-bp recognizing enzymes (e.g., *Eco*RI, *Cla*I, *Sph*I, *Bam*HI, and *Ase*I). This allows the pEVS75 *oriT* cassette to be cloned into a variety of sites without the introduction of additional 6-bp enzyme recognition sites that might otherwise complicate further cloning strategies (e.g., see the generation of pEVS79, below). The minimal *oriT* present on an *Alu*I fragment of pEVS75 is subcloned into the *Stu*I site in pUC4K (purified from *dcm*$^-$

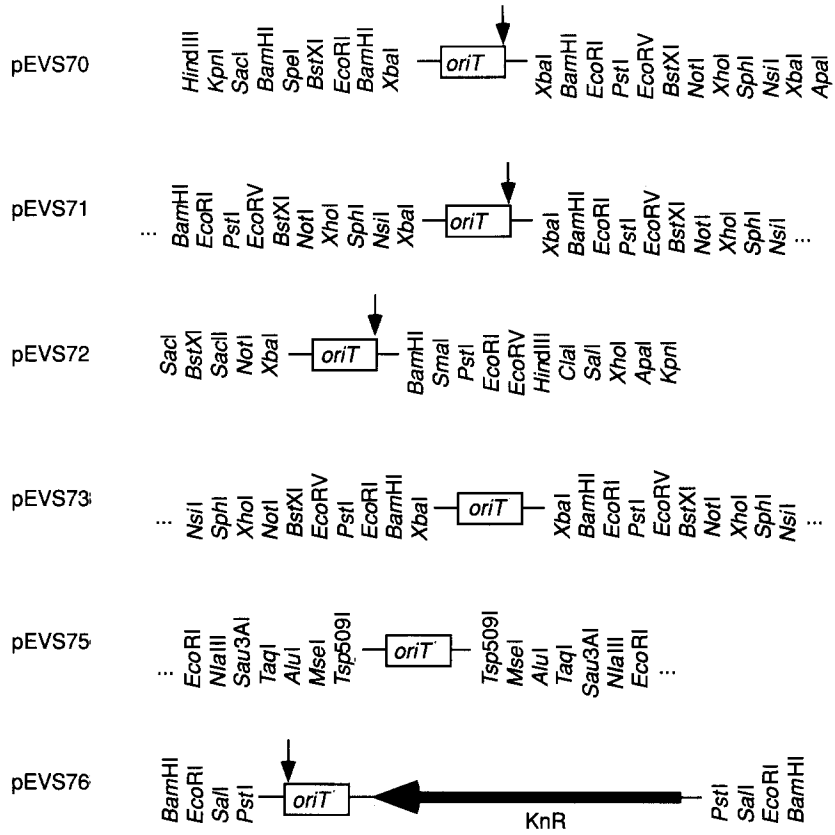

FIG. 2. Restriction maps of *oriT* cassettes. "..." indicates that the cassette shown is embedded within a multiple cloning site that extends beyond the restriction sites shown. Enzymes that cut more than once, and on both sides of the *oriT* cassette, are highlighted with bold type. During conjugation, the site of nicking occurs near the center of *oriT* and, in asymmetric constructs, an arrow indicates the end of *oriT* first transferred to a recipient. The construction of these cassettes is described in Table I, and plasmid sequences are available on request.

strain TH1191 to allow *Stu*I digestion), generating pEVS76. pEVS76 contains a KnR/*oriT* cassette flanked on either end by *Pst*I, *Sal*I, *Bam*HI, *Hin*cII, and *Eco*RI. KnR provides an antibiotic selection to facilitate cloning this cassette into other vectors making them both KnR and mobilizable. The cassette in pEVS71 contains a direct repeat of several restriction enzyme recognition sequences flanking *oriT*. Digestion of this plasmid with either *Bam*HI or *Nsi*I releases a cassette with *oriT* adjacent to a multiple cloning site, and incorporating this cassette into vectors can simultaneously confer mobilizability and a convenient multiple cloning site (e.g., see the generation of pEVS77 and pEVS78 below).

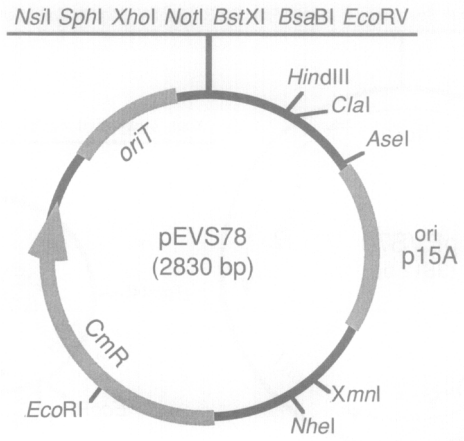

FIG. 3. Map of shuttle vector pEVS78. The *oriT*-containing *Bam*HI fragment of pEVS71 was cloned into the *Bcl*I site of pACYC184 generating pEVS77, and pEVS78 was generated by digestion of pEVS77 with *Eco*RV followed by self-ligation.

Construction of Shuttle Vectors Stable in Vibrio Recipients

We use the *oriT*-MCS cassette from pEVS71 to generate shuttle vector pEVS77 and its Δ*tetA* derivative pEVS78 (Table I). These vectors contain the p15A plasmid origin of replication, which is stably maintained in *V. fischeri*.[19,20] In triparental conjugation from *E. coli* to *V. fischeri,* we have found that pEVS78 (Fig. 3) is stably transferred 100-fold more efficiently than pEVS77 (see below), making it a more promising shuttle vector for use with *Vibrio* recipients. pEVS78 is small and contains several unique restriction sites, which should facilitate the cloning of genes of interest into this shuttle vector. To demonstrate the broader utility of pEVS78, we have conjugally transferred this plasmid to six other members of the Vibrionaceae: *V. orientalis, V. splendidus, V. harveyi, V. parahaemolyticus, A. veronii,* and *P. leiognathi*. The complete sequences of pEVS77 and pEVS78 are available on request.

Construction of Vectors for Allelic Exchange

We have also used the minimal *oriT* cassettes described above to generate mobilizable vectors for cloning and allelic exchange. The *oriT*-containing *Alu*I fragment of pEVS75 is cloned into the *Xmn*I site of pBCSK+ (Stratagene, La Jolla, CA) generating pEVS79 (Table I, Fig. 4). The introduction of the *oriT* fragment introduces only one restriction site (*Acc*I) also present in the multiple cloning site. Thus,

[19] K. L. Visick and E. G. Ruby, *in* "Bioluminescence and Chemiluminescence" (J. W. Hastings, L. J. Kricka, and P. E. Stanley, eds.), p. 119. John Wiley and Sons, New York, 1997.
[20] K. M. Gray and E. P. Greenberg, *J. Bacteriol.* **174,** 4384 (1992).

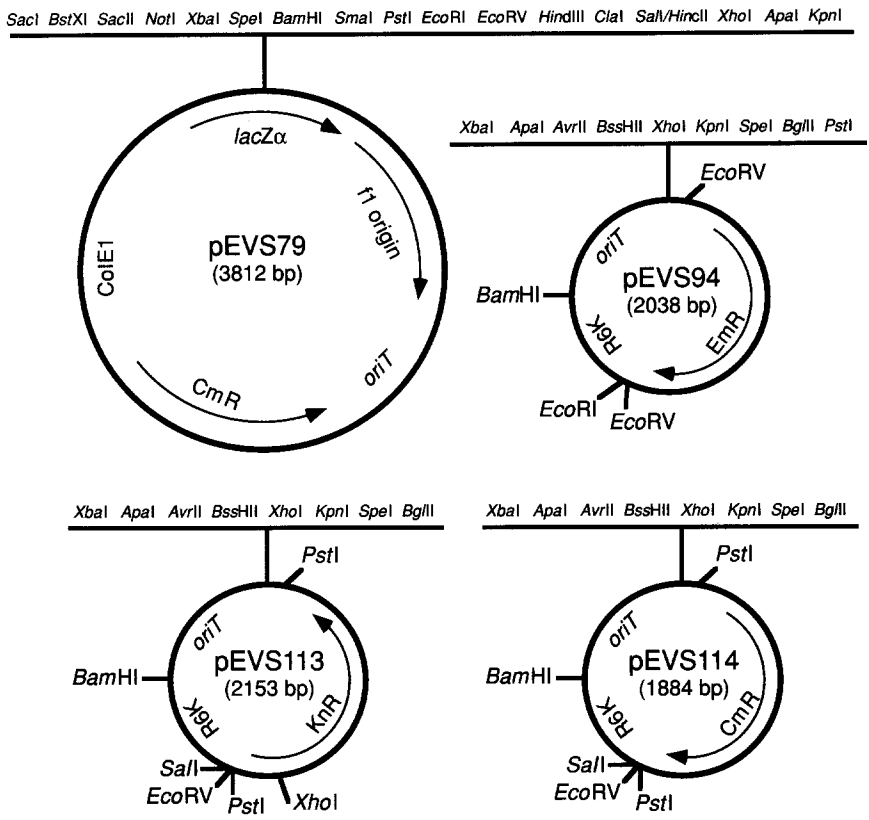

FIG. 4. Maps of cloning and allelic exchange vectors pEVS79, pEVS94, pEVS113, and pEVS114. The *oriT*-containing *AluI* fragment of pEVS75 was cloned into the *XmnI* site of pBCSK+ (Stratagene) generating pEVS79. The *oriT*-containing *XbaI–BamHI* fragment of pEVS72 and an artificial MCS were cloned into pKV38 to generate pEVS94. The unique *EcoRI* site in pEVS94 was replaced with a *SalI* site, the EmR-containing *EcoRV* fragment deleted, and KnR or CmR cloned into the *PstI* site to generate pEVS113 and pEVS114, respectively.

pEVS79 retains the utility of pBCSK+ as a cloning vector (e.g., blue/white screening, universal primer sites), while also being conjugally mobilizable. We and others (K. Visick, P. Fidopiastis, C. Lupp, D. Millikan; personal communications, 2002) have used pEVS79 to clone *V. fischeri* DNA, engineer mutations in it, and mobilize these mutant alleles back into the *V. fischeri* chromosome. Although pEVS79 contains the ColE1 origin and replicates as a plasmid in *V. fischeri* (and presumably in other *Vibrio* species), it is sufficiently unstable that recombinants, which are more stable than free plasmid, can be identified following nonselective growth. Interestingly, such single recombinants appear to resolve as double recombinants at an unusually high frequency, possibly because leaky replication of the plasmid

origin amplifies the duplicated region surrounding the site of the initial recombination event, providing additional homologous targets for double recombination. Replication of pEVS79 as a plasmid seems to be disfavored at lower temperature, and plating the results of matings selectively at 22°, rather than 28°, enriches for recombinants.

Another mobilizable vector suitable for cloning and marker exchange is pEVS94 (Fig. 4), which contains the minimal *oriT* cassette from pEVS72, a gene encoding EmR, and the R6Kγ origin of replication. Several unique restriction sites in the MCS of pEVS94, together with the small size of this vector (2038 bp), facilitate the cloning of fragments into pEVS94. Because the R6Kγ origin of replication does not replicate in *Vibrio* species, plasmid maintenance following selection for the vector-encoded resistance marker is not a concern, as it is for pEVS79 above. To obtain high yields of pEVS94 DNA, we used host strain BW23474,[21] which contains a chromosomal integrant of the *pir-116* allele and thereby maintains plasmids with the R6Kγ origin of replication in high copy.[22] The unique *Eco*RI site in pEVS94 was replaced with a *Sal*I generating pEVS94S. KnR and CmR determinants were cloned into the *Pst*I site of pEVS94S, and the EmR *Eco*RV fragment deleted in each case, generating plasmids pEVS113 and pEVS114 (Fig. 4). The complete sequences of pEVS79, pEVS94, pEVS94S, pEVS113, and pEVS114 are available on request.

Mobilization and Reconstruction of Transposon Insertions

The KnR/*oriT* cassette from pEVS76 can be used in conjunction with Tn*10* derivatives that contain the R6Kγ origin of replication to quickly reconstruct transposon-insertion mutations in different *V. fischeri* genetic backgrounds (Fig. 5) as follows: (i) mini-Tn*10*Cm transposons containing the R6Kγ origin of replication and conferring CmR are used to mutagenize the *V. fischeri* chromosome; (ii) in a mutant of interest, the Tn insertion is cloned directly, by digesting chromosomal DNA with an enzyme that does not cut in the transposon, self-ligating this CmR- and ori R6Kγ-containing fragment, and transforming an *E. coli* strain that contains the *pir* gene; (iii) the resulting plasmid is relinearized with the same enzyme used to excise it from the *V. fischeri* chromosome and ligated to the KnR/*oriT* cassette from pEVS76; (iv) the resulting construct is mobilized into another *V. fischeri* strain, selecting for KnR and CmR single recombinants; (v) screening for CmR, Kn-sensitive double recombinants allows the regeneration of the Tn::chromosome mutation in a different strain. This technique has been used to move a mini-Tn*10*Cm::*hadA* allele, initially isolated in *V. fischeri* strain KV150

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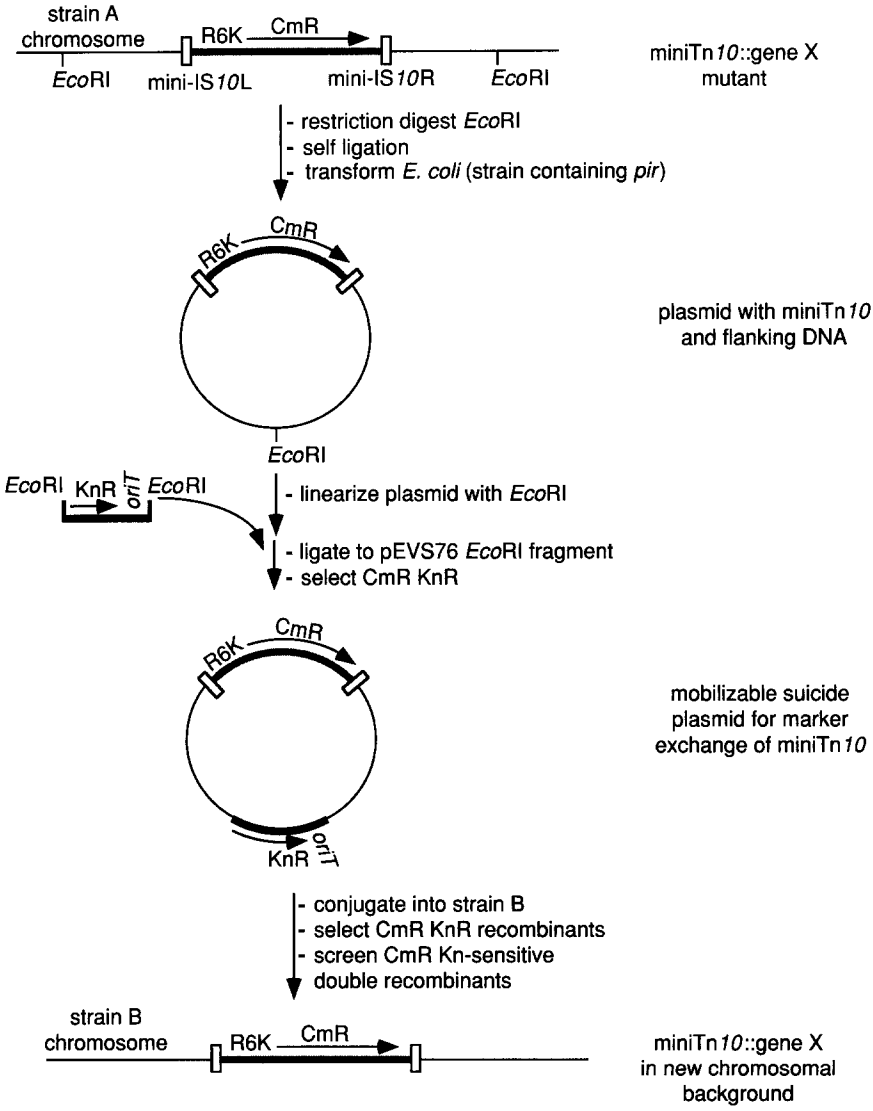

FIG. 5. Schematic representation of method for reconstruction of transposon insertions in new strain background.

(RfR, *lux*$^-$), into wild-type strain ES114 (B. Feliciano, personal communication, 2000). In this scheme for mobilization and reconstruction of transposon insertions, linearized suicide vectors such as pEVS94 could be used in much the same way as the KnR/*oriT* cassette from pEVS76.

TABLE II
CONJUGAL TRANSFER FROM *E. coli* TO *V. fischeri* STRAIN ES114

Mating	Donor strain(s)	Frequency of recipients	
		CmR	KnR
A	DH5α pEVS77	$<10^{-8}$	$<10^{-8}$[a]
B	CC118λ*pir* pRK2013 and DH5α pEVS77	2×10^{-5}	2×10^{-2}
C	CC118λ*pir* pEVS103 and DH5α pEVS77	3×10^{-5}	2×10^{-2}
D	CC118λ*pir* pEVS104 and DH5α pEVS77	4×10^{-5}	$<10^{-8}$[a]
E	CC118λ*pir* pEVS104 and DH5α pSUP102	3×10^{-5}	nd[b]
F	CC118λ*pir* pEVS104 and DH5α pACYC184	$<10^{-8}$	nd
G	CC118λ*pir* pEVS104	$<10^{-8}$	nd
H	CC118λ*pir* pEVS104 and DH5α pEVS78	7×10^{-3}	nd
I	CC118λ*pir* pEVS104 pEVS78	3×10^{-2}	nd
J	S17-1 pEVS78	4×10^{-3}	nd

[a] Very small KnR colonies arose at a frequency between 10^{-5} and 10^{-6}.
[b] nd, Not determined.

Comparison of Conjugation Methods

In many studies, spontaneous antibiotic-resistant mutant strains are used as conjugal recipients, providing a counterselection against donor *E. coli* cells. However, such marked strains are not truly wild type and may display unexpected mutant phenotypes. For example, RfR and naladixic acid-resistant mutants display pleiotropic patterns of gene expression,[23–26] including a reduced ability to compete with wild-type cells for colonization of host tissue.[27] Because we and others are interested in studying the interactions between *Vibrio* strains and their animal hosts, or the regulatory patterns of *Vibrio* strains, we have experimented, successfully, with the use of low growth temperature (22°) as an enrichment against *E. coli* donor cells following conjugal transfer to true wild-type *Vibrio* isolates. We have found that low-temperature incubation is an effective enrichment against *E. coli,* indistinguishable from rifampicin counterselection, and have used this approach to isolate transconjugants of wild-type strains of *V. fischeri* (Table II), *V. parahaemolyticus, V. splendidus, V. orientalis,* and *P. leiognathi.*

In triparental matings between *E. coli* and *V. fischeri* strain ES114, we have found that pEVS103 and pEVS104 are as efficient as pRK2013 at mediating

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the stable transfer of pEVS77, as measured by the frequency of CmR recipients (Table II; matings B, C, and D). Control experiments (Table II; matings A and G) have demonstrated that spontaneous CmR is absent (or rare) in *V. fischeri,* indicating that the frequency of CmR is a good measure of conjugal transfer. Although each helper plasmid confers KnR (Fig. 1), only the use of pEVS101 (data not shown), pEVS103, or pRK2013 gives rise to KnR recipients through plasmid maintenance or possibly, in the case of pRK2013, transposition of Tn*903* (Table II; matings B and C). In contrast, use of pEVS104, which contains only the R6Kγ plasmid origin of replication, does not result in levels of KnR recipients that are above background (Table II; matings A and D). The minimal *oriT* cassette present in pEVS77 mediates conjugal transfer as efficiently as the larger undefined *oriT*-containing fragment present in the otherwise isogenic pSUP102, while the *oriT*-lacking parent plasmid, pACYC184, is not transferred (Table II; matings D, E, and F). Interestingly, pEVS78, a Δ*tetA* derivative of pEVS77, is stably transferred 100-fold more efficiently than pEVS77 (Table II; matings D and H), and may therefore represent a more generally useful shuttle vector. We also have found that CC118γ*pir* pEVS104/pEVS78 transfers this shuttle vector 10-fold more efficiently than does S17-1 pEVS78 (Table II; matings I and J). Triparental mating using the combination of CC118λ*pir* pEVS104 and DH5α pEVS78 as donors is as efficient as biparental mating with donor S17-1 pEVS78 at transferring shuttle vector pEVS78 (Table II; matings H and J).

Similar results have been obtained in matings between *E. coli* and six other members of the Vibrionaceae: *V. orientalis, V. splendidus, V. harveyi, V. parahaemolyticus, A. veronii,* and *P. leiognathi* (data not shown). We have found that pEVS103 and pEVS104 are as efficient as pRK2013 at mediating the stable transfer of pEVS78. Also, for three species tested (*V. orientalis, V. harveyi,* and *V. parahaemolyticus*), the use of pRK2013 gives rise to KnR recipients through plasmid maintenance or transposition of Tn*903,* whereas use of pEVS104 does not result in KnR above background levels. In general, biparental matings result in more efficient transfer of pEVS78 than triparental matings, although for the recipients *A. veronii* and *P. leiognathi* triparental mating is similar to biparental mating using strain S17-1. In biparental matings we have found that CC118λ*pir* pEVS104/pEVS78 transfers the shuttle vector roughly 10-fold more efficiently than does S17-1 pEVS78, although these donors are roughly equivalent when *P. leiognathi* or *V. splendidus* serve as the recipient.

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