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Characterization of pES213, a small mobilizable plasmid from *Vibrio fischeri*

Anne K. Dunn [a], Mark O. Martin [b], Eric V. Stabb [a,*]

[a] *Department of Microbiology, University of Georgia, Athens, GA 30602, USA*
[b] *Department of Biology, Occidental College, Los Angeles, CA 90041, USA*

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Abstract

Most *Vibrio fischeri* strains isolated from the *Euprymna scolopes* light organ carry plasmids, often including both a large (>40 kb) plasmid, and one or more small (<12 kb) plasmids. The large plasmids share homology with pES100, which is the lone plasmid in *V. fischeri* type strain ES114. pES100 appears to encode a conjugative system similar to that on plasmid R721. The small plasmids lack extensive similarity to pES100, but they almost always occur in cells that also harbor a large plasmid resembling pES100. We found that many or all of these small plasmids share homology with pES213, a plasmid in strain ES213. We determined the 5501-bp pES213 sequence and generated selectable antibiotic resistance encoding pES213 derivatives, which enabled us to examine replication, retention, and transfer in *V. fischeri*. An 863-bp fragment of pES213 with features characteristic of θ-type replicons conferred replication without requiring any pES213 open reading frame (ORF). We estimated that pES213 derivatives were maintained at 9.4 copies per genome, which corresponds well with a model of random plasmid segregation to daughter cells and the $\sim 10^{-4}$ per generation frequency of plasmid loss. pES213 derivatives mobilized between *V. fischeri* strains at frequencies up to $\sim 10^{-4}$ in culture and in the host, apparently by employing the pES100 conjugative apparatus. pES213 carries two homologs of the putative pES100 origin of transfer (*oriT*), and *V. fischeri* strains lacking the pES100 conjugative relaxase, including a relaxase mutant, failed to serve as donors for transmission of pES213 derivatives. In other systems, genes directing conjugative transfer can function in *trans* to *oriT*, so it was noteworthy that ORFs adjacent to *oriT*, VFB51 in pES100 and *traYZ* in pES213, enhanced transfer 100- to 1000-fold when provided in *cis*. We also identified and disrupted the *V. fischeri recA* gene. RecA was not required for stable pES213 replication but surprisingly was required in donors for efficient transfer of pES213 derivatives. These studies provide an explanation for the prevalence and co-occurrence of pES100- and pES213-type plasmids, illuminate novel elements of pES213 mobilization, and provide the foundation for new genetic tools in *V. fischeri*.

* Corresponding author. Fax: +1 706 542 2674.
E-mail address: estabb@uga.edu (E.V. Stabb).

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1. Introduction

The marine γ-proteobacterium *Vibrio fischeri* serves as an important model for studies of bioluminescence, quorum-sensing gene regulation, and beneficial animal–bacteria interactions. Each of these fields has enhanced our understanding of *V. fischeri* genetics and made use of genetic tools in *V. fischeri*. In particular, the recent development of the light organ symbiosis between *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes*, as a model animal–bacteria mutualism has driven interest in *V. fischeri* genetics, because unlike bioluminescence and quorum-sensing phenomena this complex symbiosis cannot be effectively studied using a subset of *V. fischeri* genes cloned in *Escherichia coli*. This interest in the genetics of symbiotic *V. fischeri* prompted the recent genome sequencing of *V. fischeri* strain ES114, a wild-type isolate from *E. scolopes*. ES114 was chosen as a type strain partly for its genetic simplicity, in that it lacks small plasmids and contains only a single large (45.8-kb) plasmid, pES100. As a result, the genome project did not encompass small *V. fischeri* plasmids.

Vibrio fischeri symbionts of *E. scolopes* often harbor plasmids. Boettcher and Ruby (1994) found that 56% of *V. fischeri* isolates from *E. scolopes* carried plasmids, and of these 15% carried a large plasmid with homology to pES100, 4% carried small (<12-kb) plasmids that lacked significant homology to pES100, and 81% harbored both a large plasmid similar to pES100 and one or more small plasmids. Although this frequent co-occurrence of distinct large and small plasmid types suggested a connection between them, no functional interrelationship was determined. However, conjugation may play an important role in plasmid distribution among *E. scolopes* isolates, and the light organ, which is densely colonized by closely related bacteria, appears especially conducive to plasmid transfer. Only *V. fischeri* colonizes this tissue, usually as a mix of strains, and hundreds of millions of cells are packed at densities of $10^{10}\,ml^{-1}$ (Ruby, 1996). Although plasmid exchange in this natural setting seems likely, it has not been documented.

pES100 is large enough to encode a complete conjugative apparatus, but the small *V. fischeri* plasmids must either utilize a remarkably compact conjugative system or, more likely, co-opt factors from pES100 or one of the two *V. fischeri* chromosomes to mobilize. In other described systems, small plasmids need only carry an origin of transfer (*oriT*) in *cis* if the cognate relaxase is provided in *trans* in combination with other DNA-processing proteins and a type-IV secretion system (Burns, 2003; Cascales and Christie, 2004; Francia et al., 2004; Frost et al., 1994; Pansegrau et al., 1994; Pansegrau and Lanka, 1996). However, even if these *V. fischeri* plasmids are transmissible it is not known whether the conjugative system belongs to a well-characterized family or one of the groups lacking a well-described representative (Francia et al., 2004). Therefore, the mechanisms of *V. fischeri* plasmid transfer could include novel elements.

Also unknown are the mechanisms by which these *V. fischeri* plasmids replicate. θ-type replicons are prevalent in the γ-proteobacteria, but among these plasmids there is considerable diversity in the specific composition of the replication origin, *oriV* (Del Solar et al., 1998). Delineating the requirements for replication of *V. fischeri* plasmids will add to our understanding of plasmid replication strategies, and it will constitute an important first step in developing useful new vectors. Native *V. fischeri* plasmids have not been exploited as genetic tools in this bacterium despite the growing interest in *V. fischeri* as a model system and the shortcomings of plasmids currently available.

In this study, we sequenced and characterized *V. fischeri* plasmid pES213. This plasmid was found in strain ES213, an isolate from *E. scolopes* that contains both a large plasmid with significant homology to pES100 and multiple smaller plasmids (Boettcher and Ruby, 1994). Our results illuminate the mechanisms by which pES213 is replicated, maintained, and mobilized in *V. fischeri*. Our data suggest that pES100 is a conjugative

plasmid capable of mobilizing pES213, with the unusual requirements of an ORF in *cis* to the origin of transfer and RecA in the donor. This study will underpin more in-depth analyses of these novel requirements for conjugation, and will provide the basis for new genetic approaches using *V. fischeri* as a model organism.

2. Materials and methods

2.1. Bacteria, plasmids, media, and reagents

Oligonucleotide primers and most bacterial strains used in this study are described in Tables 1 and 2, respectively. Additional *V. fischeri* strains ES12, ES66, ES79, ES191, ES209, ES213, ES235, ES240, ES334, ES566, ES595, ES602, ES620, and ES657 are wild-type *E. scolopes* isolates (Boettcher and Ruby, 1994).

Selected plasmids generated in this study are described in Table 3, and more detailed description of their construction follows. Plasmids pECBAC1 (Frijters et al., 1997), pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA), pEVS61 (Stabb et al., 2001), and pVO8 (Visick and Ruby, 1997), as well as pEVS92, pEVS94S, pEVS104, and pEVS114 (Stabb and Ruby, 2002) have been described elsewhere. We isolated a kanamycin-resistance encoding mini-Tn*5*-<*Not*I-Kan-3>-tagged derivative of pES213 and named this pES213A, as described below and in Table 3. The transposon in pES213A inserted upstream of bp 1 of the pES213 sequence (GenBank AY465897), yielding a duplication of bp 1–9. The mobilizable ($oriT_{RP4}$) suicide vector pEVS118 was derived from pEVS114 by deleting an *Eco*RV to *Hin*cII fragment. pEVS118 was digested with *Spe*I and *Xba*I and ligated to *Spe*I-digested pES213 to generate pES213C, which could be mobilized from *E. coli* to *V. fischeri* using RP4-based helpers. Deletion of a *Bam*HI fragment in pES213C removed portions of the transposon and pEVS118 along with intervening pES213 sequences yielding pES213D. Insertion of a small linker between the *Not*I–*Bsr*GI sites of pES213D yielded pES213Kn. H. Sørum kindly provided the *Vibrio salmonicida* trimethoprim-resistance gene *dfr* (GenBank AJ277063) cloned in pUC19. We PCR amplified *dfr* using primers JB1 and JB2, cloned the product in PCR-BluntII-TOPO, and used a *Not*I–*Bam*HI fragment from the resulting plasmid, pJLB1, as a source for *dfr* in pES213Tp. To generate pEVS122, we digested pEVS92 with *Bam*HI, generated blunt ends using Klenow fragment, ligated this into the *Srf*I site of pECBAC1, and then deleted an *Nco*I fragment. An internal ORF VFB50 (relaxase) fragment was initially cloned as a PCR product into pCR-BluntII-TOPO and was subsequently subcloned as an *Apa*I/*Spe*I fragment into *Apa*I/*Spe*I-digested pEVS118 to generate pEVS158 (Table 3).

Table 1
Oligonucleotide primers and probe used in this study[a]

Name	Sequence
AKD2a	CCCTTCATGACGTTGTGATTACCCGTTG
AKD2b	GGTTTCATGATGGGCGTACTGGAGACAA
AKD2c	TTTGTCATGAGTGCGGTCTTCTTGAGTA
AKD2d	GGGGTCATGATTAATACAAGGAGTTAGC
EVS86	CCTAGGTTCAATTAGTTAGTTTGTTGTGTC GTG
EVS87	ACTAGTAAGAGGGATTTGAAGAAACGTCT TAAG
EVS106	TGATATTAGGTGTAGTATTTGCGG
EVS109	GGAGGCGGCAACGCTGGAATTGGTGATTA TCT
EVS110	TATTCGGGAATAACAACATGGATATGTGG TTT
EVS113	ATGTGATATGATCTATCATGAAAGGACTTA
JB1	GCGCTTCGAACTCTGAGGAAGAATTGTG
JB2	GCGCCCTAGGTTAGTTAGTTAACCCTTTT GCCAGA
RTKnF	TGATGCGCTGGCAGTGTT
RTKnP	TGCGCCGGTTGCATTCGATTCCTGT
RTKnR	CTCGCATCAACCAAACCGTTA

[a] Oligonucleotide sequences are shown 5′–3′.

Plasmids were maintained in *E. coli* strain DH5α, except as noted. Plasmids containing the R6K γ origin of replication (R6Kγ *oriV*) were maintained in CC118λ*pir*, BW23473, BW23474, DH5α-λ*pir*, or GM2163-λ*pir*, with the latter two strains being used for blue/white screening and the generation of unmethylated DNA for *Cla*I digestion, respectively. pES213Kn was maintained in DH5α or JM107. *E. coli* was grown in LB medium (Miller, 1992) or brain heart infusion (BHI) and *V. fischeri* was grown in either a seawater-based complex medium (SWT) (Boettcher and Ruby, 1990) wherein seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, Ohio) or in a

Table 2
Bacterial strains used in this study[a]

Strain	Relevant characteristics[b]	Source or reference
E. coli		
BW23473	Δ*lac-169 robA1 creC510 hsdR514 uidA* (Δ*Mlu*I)::*pir endA* (*BT333*) *recA1*	Haldimann et al. (1996), Metcalf et al. (1994)
BW23474	Δ*lac-169 robA1 creC510 hsdR514 uidA* (Δ *Mlu*I)::*pir-116 endA* (*BT333*) *recA1*	Haldimann et al. (1996), Metcalf et al. (1994)
CC118λ*pir*	Δ(*ara-leu*)*araD*Δ*lacX*74*galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ *pir*	Herrero et al. (1990)
DH5α	F$^-$Φ80*dlacZ*Δ*M*15 Δ(*lacZYA-argF U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
DH5α-λ *pir*	DH5α lysogenized with λ *pir*	This study
GM2163	F$^-$ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam13*::Tn*9 xylA5 mtl-1 thi-1 mcrB1 hsdR2*	A. Karls
GM2163-λ *pir*	GM2163 lysogenized with λ *pir*	This study
JM107	F$^-$*traD*36 *lacI*^{q} Δ*lacZ*)M15 Δ(*lac-proAB*) *proA*$^+$*B*$^+$/*e*14$^-$ (McrA$^-$) *hsdR17 endA1 gyrA96 thi relA1 glnV44*	A. Karls
V. fischeri		
AKD512	ESR1 *recA*$^-$ (*recA*::pEVS140XNP) EmR	This study
DM135	ES114 miniTn*7-gfp* EmR	D. Millikan
ES114	Wild-type *E. scolopes* isolate, carries pES100	Boettcher and Ruby (1990)
ES560	Wild-type *E. scolopes* isolate, lacks endogenous plasmids	Boettcher and Ruby (1990)
ESR1	Spontaneous RfR derivative of ES114	Graf et al. (1994)
EVS135	ES560 miniTn*7-gfp* EmR	This study
EVS158	ES114 VFB50::pEVS158 (putative-relaxase mutant) CmR	This study
EVS401	ES114 Δ*pilA*::*aph*; KnR	Stabb and Ruby (2003)
EVS510	ES114 *recA*$^-$ (*recA*::pEVS140XNP) EmR	This study
JP100	ESR1 *recA*$^-$ (*recA*-Tn*5*-<Kan-2>) RfR KnR	This study

[a] All other *V. fischeri* strains are wild-type *E. scolopes* isolates (Boettcher and Ruby, 1994).
[b] *Abbreviations used:* CmR, chloramphenicol resistance; EmR, erythromycin resistance; KnR, kanamycin resistance; RfR, rifampicin resistance.

Tris-buffered, high-salt, rich, complex medium (LBS) (Stabb et al., 2001). Agar (15 mg ml^{-1}) was added to solidify media for plating experiments. Chemicals were obtained from Sigma Chemical (St. Louis, MO). Klenow fragment, DNA ligase, and restriction enzymes were obtained from New England Biolabs (Beverly, MA), except for *Srf*I, which was obtained from Stratagene (La Jolla, CA). KOD HiFi and *Pfu*-Turbo DNA polymerases were obtained from Novagen (Madison, WI) and Stratagene (La Jolla, CA), respectively. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). One kilobase-ladder DNA standard was obtained from Fisher Scientific (Pittsburgh, PA). When added to LB medium for selection of *E. coli*, trimethoprim, chloramphenicol, and kanamycin was used at concentrations of 10, 20, and 40 μg ml^{-1}, respectively. *E. coli* was grown on BHI medium for selection of resistance to 150 μg ml^{-1}erythromycin. When added to LBS medium for selection or screening in *V. fischeri*, chloramphenicol, erythromycin, trimethoprim, rifampicin, and kanamycin were used at concentrations of 2, 5, 10, 100, and 100 μg ml^{-1}, respectively.

2.2. Molecular genetic techniques and sequence analysis

Plasmids were purified using Qiagen Mini-, Midi-, or Maxi-prep kits (Qiagen, Valencia, CA). Plasmids that required the pES213 origin for replication were maintained in, and isolated from, *V. fischeri* strains when possible. Optional washes in the manufacturer's protocol improved the quality

Table 3
Selected plasmids used in this study[a]

Plasmid(s)	Relevant characteristics[b]
Generation of pES213 derivatives	
pES213	One plasmid endogenous to *V. fischeri* ES213
pES213A	pES213 with Tn*5*-<*Not*I-Kan-3> insertion upstream of bp 1[c]; pES213 *oriV*, KnR
pES213C	pES213A fused (at 2309 bp)[c] to pEVS118; pES213 *oriV*, R6Kγ *oriV*, $oriT_{RP4}$ CmR, KnR
pES213Kn	pES213 (2309–5501 bp)[c], pES213 *oriV*, KnR, $oriT_{RP4}$
pES213KnB	pES213Kn Δ*Bsa*BI (Δ *traYZ*)
pES213Tp	pES213Kn, KnR replaced by TpR; pES213 (2309–5501 bp)[c], pES213 *oriV*, TpR, $oriT_{RP4}$
pES213A library for sequencing and functional characterization	
pEVS122	R6Kγ *oriV*, $oriT_{RP4}$ EmR, *lacZα*, *cosN*, *loxP*, *incD*
pA2, pA11	pES213A *Alu*I fragments in pEVS122 *Sma*I site
pD2, pD4, pD8	pES213A *Dra*I fragments in pEVS122 *Sma*I site
pD3P, pD6P	pES213A *Dra*I-*Pst*I fragments in pEVS122 *Sma*I–*Pst*I sites
pS12, pS20, pS22, pS27, pS41, pS43, pS44, pS63	pES213A sheared fragments in pEVS122 *Sma*I site
pS12P	pS12 Δ*Pst*I-fragment
pS12PS, pS44S, pS63S	*Spe*I-, *Bgl*II-, and *Bst*BI-containing linker in *Kpn*I site of pS12P, pS44, and pS63, respectively, Δ*Spe*I fragment
pS12PB	*Spe*I-, *Bgl*II-, and *Bst*BI-containing linker in *Kpn*I site of pS12P, Δ*Bgl*II fragment
pS12PBB	*Spe*I-, *Bgl*II-, and *Bst*BI-containing linker in *Kpn*I site of pS12P, Δ*BstB*I fragment
pS12PSB	pS12PS digested *Bsp*HI, filled, self-ligated
pS12PSC	pS12PS digested *Cla*I, filled, self-ligated
pS12PSNH	pS12PS Δ *Nco*I–*Hpa*I (Δ*incD*)
pS12PSS	pS12PS Δ *Sac*I (Δ*cosN-loxP*)
pS12PSNHS	pS12PSNH (Δ *incD*) Δ *Sac*I (Δ*cosN-loxP*)
pS12PSEN	pES12PS Δ *Eco*RV–*Nco*I (ΔR6Kγ *oriV*)
pS43B	pS43 Δ*Bgl*II–*Bam*HI
pSS1	pS12PS *Sca*I–*Ssp*I fragment in pEVS122 *Sma*I site
Delineating in cis requirements for Vibrio–Vibrio transfer	
pEVS126	p15A *oriV*, $oriT_{RP4}$ KnR
pAKD160	PCR product (primers AKD1a and AKD1b, pES100 template) in pEVS126 *Bst*Z17 I site
pAKD161	PCR product, (primers AKD1a and AKD1c, pES100 template) in pEVS126 *Bst*Z17 I site
pAKD162	PCR product (primers AKD1a and AKD1d, pES100 template) in pEVS126 *Bst*Z17 I site
pAKD163	PCR product (primers AKD2a and AKD2b, pES213 template) in pEVS126 *Bst*Z17 I site
pAKD164	PCR product (primers AKD2c and AKD2d, pES213 template) in pEVS126 *Bst*Z17 I site
pAKD165	PCR product (primers AKD2b and AKD2c, pES213 template) in pEVS126 *Bst*Z17 I site
pAKD166	pAKD165 Δ *Bsa*BI (Δ *traYZ*)

Mutant analyses	
pVf100	ES114 *recA*-containing *Bgl*II fragment in pVO8 *Bam*HI site; CmR, EmR
pVf101	pVf100 Tn*5*-<Kan-2> insertion, *recA*$^-$ CmR, EmR, KnR
pEVS94S	R6Kγ *oriV*, *oriT*$_{RP4}$ EmR
pEVS140XNP	Internal *Xba*I–*Nsi*I *V. fischeri recA* fragment in *Xba*I and *Pst*I sites of pEVS94S
pDMA5	p15A *oriV*, *lacZ*α, CmR
pEVS131	*recA* PCR product (primers EVS86 and EVS87, ES114 template) *Spe*I/*Avr*II digested, in pDMA5B *Spe*I site; CmR
pEVS118	R6Kγ *oriV*, *oriT*$_{RP4}$, CmR
pEVS158	internal VFB50 (relaxase) fragment (PCR product, primers EVS109 and EVS110) in pEVS118; CmR

[a] Plasmids from other sources, additional plasmids generated in this study, and further details of construction are described in the text.
[b] *Abbreviations used:* CmR, chloramphenicol resistance; EmR, erythromycin resistance; KnR, kanamycin resistance; TpR, trimethoprim resistance.
[c] bp designations correspond to GenBank AY465897.

of plasmids isolated from *V. fischeri*. Plasmid DNA isolated from strain ES213 was subjected to in vitro Tn*5*-<*Not*I/Kan-3> mutagenesis (Epicentre, Madison, WI) and the resulting DNA was transformed into *E. coli* DH5α to isolate pES213A. Plasmid pVf100 was similarly mutated in vitro with Tn*5*-<Kan-2>, to generate pVf101. We used the ZeroBlunt-TOPO PCR Cloning Kit (Invitrogen) to clone PCR products into pCR-BluntII-TOPO. Between restriction and ligation reactions DNA was recovered with the Wizard DNA Cleanup kit (Promega, Madison, WI) or the DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, CA).

PCR amplification of the trimethoprim-resistance determinant *dfr1* was accomplished with *Pfu*-Turbo, and all other PCR-based cloning was performed using KOD HiFi DNA Polymerase (Novagen) following manufacturers' recommendations for cycle programs based on predicted DNA product size. Annealing temperature for each primer set was determined by subtracting 5 °C from the lowest primer melting temperature calculated using OligoAnalyzer 3.0 (http://biotools.idtdna.com/analyzer/). All reactions were performed using an iCycler (Bio-Rad Laboratories, Hercules, CA).

The number of pES213Kn copies per genome was determined using Real-Time PCR. Primers RTKnF and RTKnR (Table 1) amplified a 129-bp fragment of the *aph* (kanamycin resistance) gene present on pES213Kn and in single copy on the chromosome of strain EVS401. The probe, oligonucleotide RTKnP (Table 1), was 5′ labeled with 6-carboxyfluorescein (FAM) and 3′ labeled with Black Hole Quencher-1. Reactions were run with Platinum Quantitative PCR SuperMix-UDG (Invitrogen) in 50-μl final volumes with 8 μM of each primer and 4 μM of probe. Reactions were incubated at 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 45 s, and 72 °C for 30 s. Templates were prepared by boiling lysis of *V. fischeri* cells grown in LBS at 28 °C in the absence of antibiotic selection to an optical density at 595 nm (OD_{595}) of 1.0, pelleted and resuspended in an equal volume sterile distilled water. One microliter of each sample was used per reaction. Cultures were also plated to determine the CFU ml^{-1}, so

that minor differences in culture density were taken into account. Purified pES213Kn and/or deionized water were added to 1 μl of boiled ES114 lysate as control standards. Melt curves, using SYBER-Green to detect double stranded DNA, and gel electrophoresis did not detect any non-specific PCR products. Each sample was run in duplicate or triplicate, and reaction efficiency was between 92 and 97%, in three separate experiments.

DNA sequencing was conducted on an ABI automated DNA sequencer at the University of Georgia Molecular Genetics Instrumentation Facility or at the University of Michigan DNA Sequencing Core Facility. Deposited sequence data were generated by sequencing both DNA strands. Sequence analysis, e.g., the identification of open reading frames (ORFs), was performed using DNA Strider 1.2/1.3 and/or Sequencher 4.1.2 (Gene Codes, Ann Arbor, MI), and sequence comparisons were conducted with either CLUSTAL W (Thompson et al., 1994) or BLAST (Altschul et al., 1990) algorithms, using the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992) for protein alignment. Putative DnaA boxes were searched for using the consensus determined by Schaefer and Messer (1991), 5′-(T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C)-3′. We deposited the pES213 sequence in GenBank (Accession code AY465897).

Southern blotting was performed with the DIG DNA Labeling and Detection kit using the chemiluminescent substrate CDP-Star (Roche Biochemicals, Indianapolis, IN). Blots were hybridized with the probe overnight at 60 °C, and the nylon membranes were washed under high-stringency conditions, with two final 20-min washes in 1.5 mM sodium citrate, 15 mM NaCl, 0.1% SDS, pH 7.0, at 68 °C.

2.3. Selection and measurement of UV-resistance

We selected *E. coli* clones resistant to UV light by exposing plates to a UV light box, and we quantitatively measured UV-sensitivity in *E. coli* and *V. fischeri* using a UV-Stratalinker 1800 (Stratagene). To obtain quantitative UV-sensitivity data, *V. fischeri* strains were grown in LBS, pelleted by centrifugation, resuspended in Instant Ocean, placed in 500-μl aliquots in 24-well microtiter plates, exposed to either 3000 μJ/cm^2 UV light or no UV light, and dilution plated onto LBS agar to determine the UV-survival frequency.

2.4. Conjugation

Mobilizable vectors were transferred from *E. coli* to *V. fischeri* by triparental mating using conjugative helper plasmid pEVS104 as described (Stabb and Ruby, 2002). *Vibrio*-to-*Vibrio* conjugation was performed using a biparental mating method that was similar to the triparental method, but which excluded the RP4-based conjugative helper pEVS104. Mating mixtures were spotted on LBS agar medium at 28 °C for 8- to 12-h prior to selective plating. To assess conjugative transfer in the symbiosis, *E. scolopes* hatchlings were infected with *V. fischeri* as described previously (Ruby and Asato, 1993). Juvenile *E. scolopes* were exposed to a mixed inoculum for between 12- and 14-h before being rinsed in inoculum-free seawater. Inocula were pregrown unshaken in 5 ml of SWT in 50-ml conical tubes at 28 °C to an OD_{595} between 0.3 and 1.0. After 24 or 48 h, squid were homogenized and the homogenates plated to recover and enumerate bacteria. For both in culture and in host experiments, *Vibrio*-to-*Vibrio* transfer frequencies indicate the number of CFUs with the selectable markers of both the recipient and the plasmid divided by the number of CFUs with the recipient marker, i.e., the apparent frequency of transconjugants per recipient. Controls indicated that spontaneous antibiotic resistance was rare or absent and did not significantly skew the calculated transfer frequencies. Similarly, when strains DM135 and EVS135, which express green fluorescent protein, were used as recipients, we confirmed the green fluorescence of putative transconjugants thereby ruling out the possibility that these were spontaneous drug-resistant variants of the donors.

3. Results

3.1. Isolation of pES213-derivatives from V. fischeri

The goal of this study was to characterize one of the small plasmids found in *V. fischeri* strains

isolated from the *E. scolopes* light organ (Boettcher and Ruby, 1994). To accomplish this goal we used in vitro transposon (Tn*5*-<*Not*I/Kan-3>) mutagenesis to tag plasmid DNA from strain ES213 and isolate one of the plasmids from this strain. The kanamycin resistance determinant in the transposon allowed selection in *E. coli* of pES213A, presumably a transposon-carrying derivative of an endogenous *V. fischeri* plasmid. Restriction analyses of pES213A and the mixture of plasmids in strain ES213 were consistent with the interpretation that pES213A was formed by insertion of Tn*5*-<*Not*I/Kan-3> into one of the small plasmids native to ES213. We designated this native *V. fischeri* plasmid pES213. Thus, pES213 is the parent of pES213A and the subsequent derivatives of pES213A that were generated in this study.

To reintroduce pES213-derived plasmids back into *V. fischeri*, we generated derivatives of pES213A that contain an RP4 origin of transfer ($oriT_{RP4}$), including pES213C, pES213Kn, and pES213Tp (Table 3). We could then readily transfer these pES213 derivatives by conjugation from *E. coli* into *V. fischeri* in the presence of an RP4-based helper plasmid. Although pES213A, pES213C, pES213Kn, and pES213Tp replicate in *E. coli* they were relatively unstable in this heterologous host. For example, <50% of DH5α or JM107 cells retained pES213Kn after 10 generations of non-selective growth in culture. In contrast, pES213 derivatives were usually very stable in *V. fischeri*, and pES213Kn was retained by >99% of *V. fischeri* cells after 10 generations of non-selective growth.

3.2. Generating a pES213 library for sequence and functional analyses

To sequence pES213 and determine its functional domains, we cloned fragments of pES213A into pEVS122, which carries; (i) a multiple cloning site within *lacZ*α to allow blue/white screening for inserts and sequencing with standard primers, (ii) *ermR*, encoding erythromycin resistance, for selection in *E. coli* or *V. fischeri*, (iii) $oriT_{RP4}$, enabling conjugative transfer from *E. coli* to *V. fischeri* (Stabb and Ruby, 2002), and (iv) the R6Kγ *oriV* which permits the plasmid to replicate in strains carrying the *pir* gene (Kolter et al., 1978) but is non-functional in wild-type *V. fischeri*. By sequencing the pES213A fragments cloned in pEVS122, aligning overlapping data to assemble a single circular sequence, and deleting the Tn*5*-<*Not*I/Kan-3> nucleotides along with the 9-bp duplication generated by the Tn*5*-<*Not*I/Kan-3> insertion, we extrapolated the sequence of pES213 (GenBank AY465897), which is summarized in Fig. 1A and described in greater detail below.

3.3. Analyses of the pES213 sequence

Within the 5501 bp comprising pES213 we identified nine potential ORFs (Fig. 1A). As described below, two of these, designated TraY and TraZ, bear similarity to a single ORF in *V. fischeri* plasmid pES100 and appear to function in plasmid transfer. BLASTP and BLASTX searches revealed that ORF1 was similar to a group of ORFs found in bacterial genomes, often on phage or plasmids, that resemble the phage-resistance protein AbiF from *Lactococcus lactis* plasmid pNP40 (Garvey et al., 1995). ORF1 is 24% identical and 41% similar to AbiF over a stretch of 250 amino acids, although it is more similar to uncharacterized ORFs in this group. We also found that a 240-residue portion of ORF2 is 25% identical and 45% similar to a hypothetical protein from *Photorhabdus luminescens*, however, none of the other ORFs showed significant similarity to database entries or to ORFs in the ES114 genome.

BLASTN analyses of the pES213 DNA sequence revealed a 118-bp region with 90% identity to a plasmid isolated from the coral pathogen *Vibrio shiloi*. As described below, this homology falls within the minimal region required for pES213 replication. Although this region overlaps ORF5, this small putative ORF does not appear to be conserved in the *V. shiloi* plasmid. Two other regions were similar to non-coding sequence on pES100 and, as discussed below, were designated *oriT* and *oriT*′.

3.4. Localizing the pES213 replication origin

We mobilized the library of pES213-derived inserts cloned in pEVS122 from DH5α-λ*pir* into *V. fischeri* ES114, utilizing RP4-based conjugation

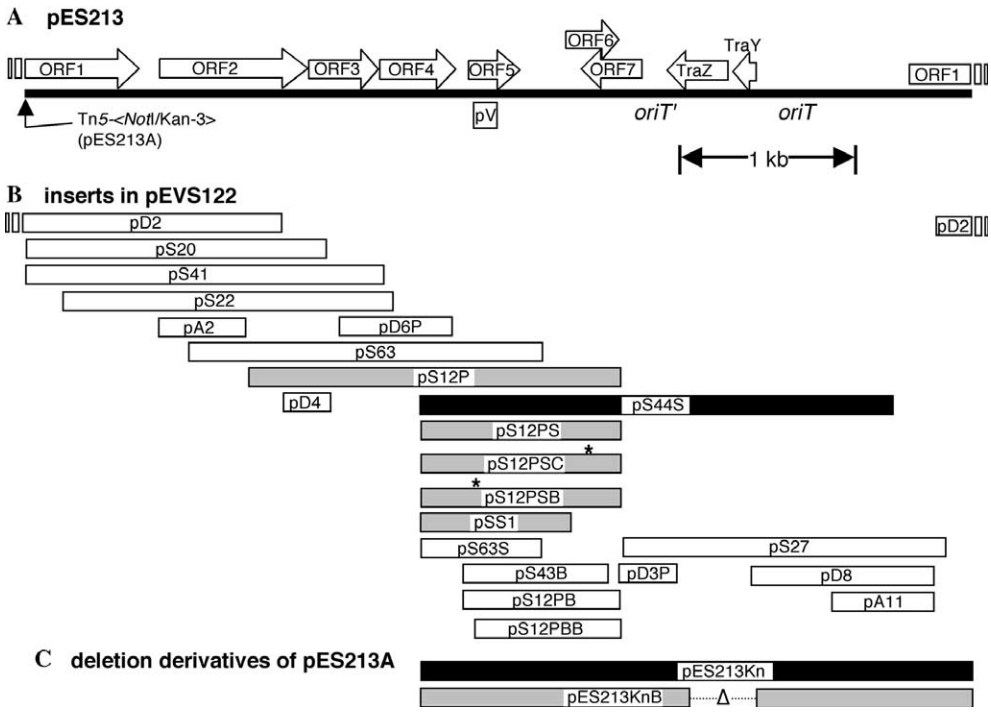


Fig. 1. Sequence and functional analysis of pES213. (A) Location and orientation of ORFs, insertion site of mini-Tn*5* in pES213A, and regions of sequence similarity to pES100, marked by *oriT* and *oriT'*, and to a *V. shiloi* plasmid (GenBank AF009904) labeled "pV." (B) Regions of pES213 (aligned beneath corresponding region in A) inserted in pEVS122 in each respective clone. Asterisks above pS12PSC and pS12PSB indicate the location of introduced frame-shifting mutations. (C) Regions of pES213 in deletion derivatives of pES213A. These clones, like those in pEVS122 (B), contain an $oriT_{RP4}$ to enable their introduction into *V. fischeri* from *E. coli*. (B and C) Black boxes indicate that the clone replicated in *V. fischeri* and was efficiently transferred from strain ES114 to strain ESR1 (without an RP4-based helper), gray boxes indicate the clone replicated in *V. fischeri* but was not transferred from ES114 to ESR1, and open boxes indicate the clone did not replicate in *V. fischeri*.

and selecting for erythromycin resistance. Because pEVS122 does not replicate in *V. fischeri*, and we have never observed spontaneous erythromycin resistance in *V. fischeri*, we were able to identify inserts that enabled plasmid replication (Fig. 1B). We also purified plasmids from erythromycin-resistant recipients, demonstrating that erythromycin resistance reflected autonomous plasmid replication rather than plasmid integration into the genome. We found that an 1155-bp insert in pS12PS conferred replication in *V. fischeri* and encompassed ORF5 and ORF6; however, these putative genes were disrupted by frame-shift mutations in pS12PSB and pS12PSC without a loss of replication proficiency (Fig. 1B). Thus, none of the open reading frames on pES213 is required for plasmid replication. We also generated deletion derivatives of pES213A carrying $oriT_{RP4}$, and mobilized them from *E. coli* to *V. fischeri* (Fig. 1C).

We considered the possibility that the plasmid- or phage-derived R6Kγ*oriV*, *cosN*, *incD* (*sopC*), or *loxP* sequences present on pEVS122 were contributing to the ability of pS12PS to replicate in *V. fischeri*. However, deletion derivatives of pS12PS, including pS12PSS (Δ*cosN-loxP*), pS12PSNH (Δ*incD*), pS12PSNHS (Δ*cosN-loxP* Δ*incD*), and pS12PSEN (ΔR6Kγ *oriV*), replicated in *V. fischeri*. Therefore, these pEVS122 vector sequences were not essential for replication.

The smallest subclone capable of replication in *V. fischeri* that we identified was pSS1 (Fig. 1B), which contained only 863 bp of pES213 DNA. We examined this sequence for characteristics of plasmid

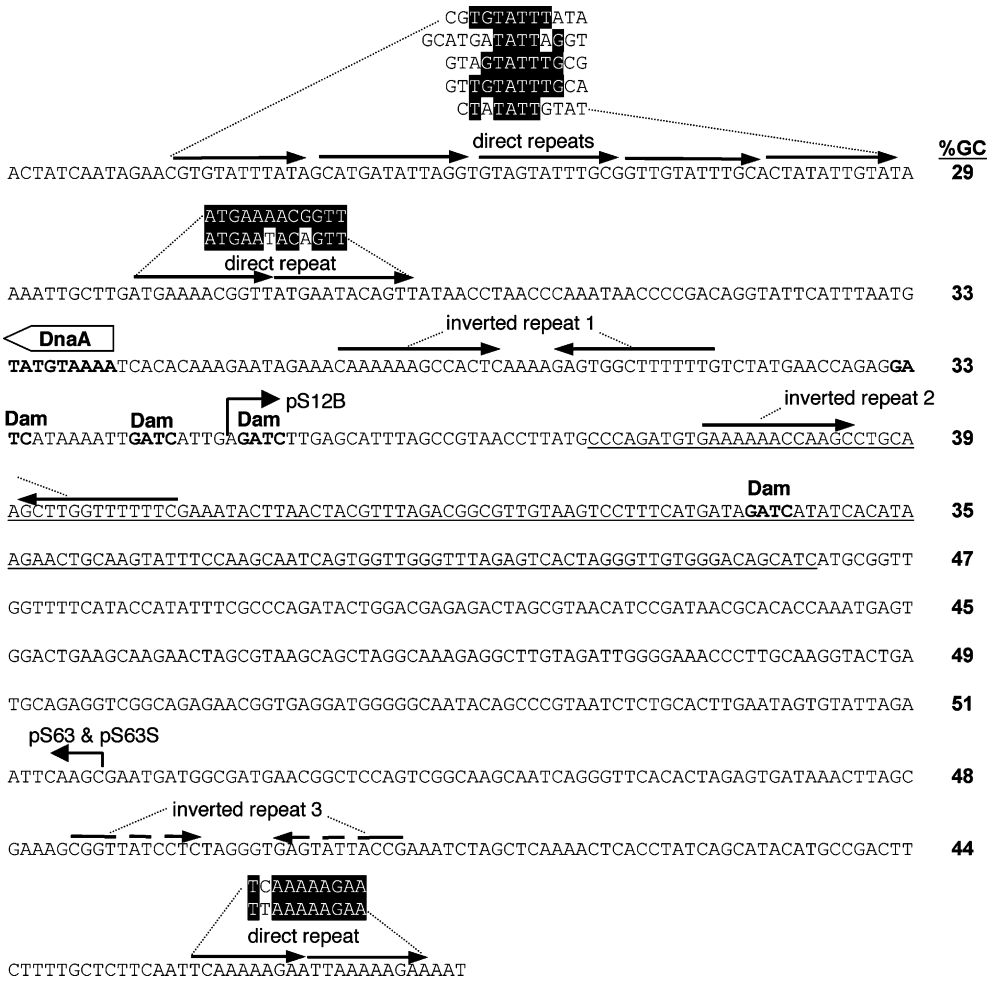


Fig. 2. Sequence analysis of pES213 origin of replication. The 5′–3′ DNA sequence of the pES213-derived insert in pSS1 is shown. Boldface letters correspond to putative Dam methylation or DnaA binding sites. Underlined sequence corresponds to a region of 90% identity to a *V. shiloi* plasmid (GenBank AF009904). The %GC content of each line (75 bp) is shown at the right. Bent arrows indicate where inserts end and the direction they extend in clones pS63 and pS63S, or pS12B, which do not replicate in *V. fischeri*. Alignments and highlighting illustrate sequence matches in putative direct repeats. Discontinuous arrows indicate mismatches in one imperfect inverted repeat.

replication origins (Del Solar et al., 1998; Helinski et al., 1996), and found several features typical of θ-type replicons (Fig. 2), including; (i) a relatively A/T rich region adjacent to a putative DnaA binding site, (ii) inverted repeats, (iii) potential direct repeats spaced approximately one DNA helical turn apart, and (iv) a high concentration of Dam methylation sites. The two inverted repeats proximal to the DnaA binding site contain runs of A and T nucleotides at the 5′ and 3′ ends, respectively, typical of bidirectional transcription terminators (Fig. 2). One of these was also highly conserved in a *V. shiloi* plasmid sequence (Fig. 2). These sequence analyses corroborate our functional identification of this fragment as a replication origin.

3.5. Identifying *V. fischeri recA* and generating *recA* mutants

To test the relationship between RecA and pES213, and to prevent plasmid–plasmid recombination in certain experiments described below, we

identified the *V. fischeri recA* gene and generated mutants lacking RecA activity. We utilized a pre-existing library (Aeckersberg et al., 2001) of ES114 DNA cloned into pVO8 (Visick and Ruby, 1997), to complement the UV-sensitivity of *E. coli recA* mutant DH5α. A complementing plasmid, pVf100, was isolated from the library (data not shown) and mutagenized in vitro with Tn*5*-<Kan-2>. We screened these mutants for loss of ability to confer UV-resistance on DH5α and thereby identified pVf101, which contains a transposon insertion 47 bp upstream of a *recA* homolog apparently disrupting *recA* expression (data not shown). The putative *V. fischeri recA* gene encodes a protein that is 78, 88, and 92% identical to RecA from *E. coli* (Sancar et al., 1980), *Vibrio anguillarum* (Tolmasky et al., 1992), and *Vibrio cholerae* (Goldberg and Mekalanos, 1986), respectively. Subsequent sequencing of the *V. fischeri* genome revealed no other *recA* homologs and indicated that *V. fischeri recA* is not linked to a *recX* homolog.

To generate a *recA* mutant, we crossed the *recA*::Tn*5*-<Kan-2> allele from pVf101 onto the ESR1 chromosome, generating mutant JP100. We also cloned a fragment internal to the *V. fischeri recA* gene in a suicide vector and mobilized this into strains ES114 and ESR1, selecting for single recombinants and generating disrupted *recA* alleles to produce strains EVS510 and AKD512, respectively. JP100, EVS510, and AKD512 were 100- to 1000-fold more sensitive to UV-light than their parental strains (Table 4), consistent with a lack of RecA function. Based on the position of putative ORFs and transcription terminators, *V. fischeri recA* appears monocistronic, and we therefore did not expect the insertions in *recA* to disrupt expression of other genes. Nevertheless, we confirmed that the UV-sensitivity of strains JP100, AKD512, and EVS510 was due to the disruption of *recA*, and not effects on other adjacent genes, by complementing this phenotype with *recA* provided in *trans* (Table 4). We also found that JP100, AKD512, and EVS510 were defective relative to their respective parent strains in an assay for homologous recombination between the chromosome and an introduced suicide plasmid (Table 4).

We used the *recA* mutants to test the role of RecA in pES213 replication. pES213C was introduced into *recA* mutants JP100 and EVS510, as well as their parental strains ESR1 and ES114, and these were grown in the absence of antibiotic selection for 30 generations with less than 1% of the cells of any strain losing the plasmid. Thus, RecA was not required for, and did not appear to affect, the replication and stable maintenance of pES213-derivatives. We were therefore able to use the *recA* mutants to prevent homologous recombination in experiments examining aspects of pES213 mobilization and maintenance.

Table 4
Phenotypes of *recA* mutants

V. fischeri strain	% survival after UV-exposure	Homologous recombination[a]
ESR1	26	2.4
JP100 (ESR1 *recA*-Tn*5*-<Kan-2>)	0.020	<0.026
AKD512 (ESR1 *recA*::pEVS140XNP)	0.083	<0.037
ES114	13	1.9
EVS510 (ES114 *recA*::pEVS140XNP)	0.13	<0.076
JP100 pDMA5 (vector)	0.04	nt[b]
JP100 pEVS131(vector +*recA*)	25	nt
AKD512 pDMA5 (vector)	0.012	nt
AKD512 pEVS131 (vector +*recA*)	26	nt
EVS510 pDMA5 (vector)	0.017	nt
EVS510 pEVS131 (vector +*recA*)	35	nt

[a] Chloramphenicol-resistant colonies per 10^7 recipient cells following conjugation of pEVS61, a suicide plasmid that contains regions of the *V. fischeri* ES114 chromosome surrounding *hvnA* (Stabb et al., 2001), from *E. coli* into strain ES114.
[b] nt indicates not tested.

3.6. Interstrain transmission of pES213 in *V. fischeri*

We noticed that some pES213 derivatives could be mobilized between strains of *V. fischeri* in the absence of a RP4-derived conjugative helper. The frequency of pES213Kn transfer between ES114 and its marked derivative DM135, either on agar plates or in the densely packed *E. scolopes* light organ, was approximately 10^{-4}. Certain plasmids may exclude entry of related plasmids, and plasmids of the same incompatibility groups often cannot stably coexist in the same cell. We therefore tested whether transfer of pES213Kn was influenced by pES213Tp, which is isogenic with respect to pES213Kn except that these plasmids encode different drug resistance markers (Table 3). The transfer frequency of pES213Kn was unaffected by the presence of pES213Tp in recipient cells, even when *recA* recipients were used so that pES213Kn and pES213Tp were forced to replicate independently in the same cell. Thus, in this experiment, the presence of one pES213 derivative did not block entry and retention of a second pES213 derivative.

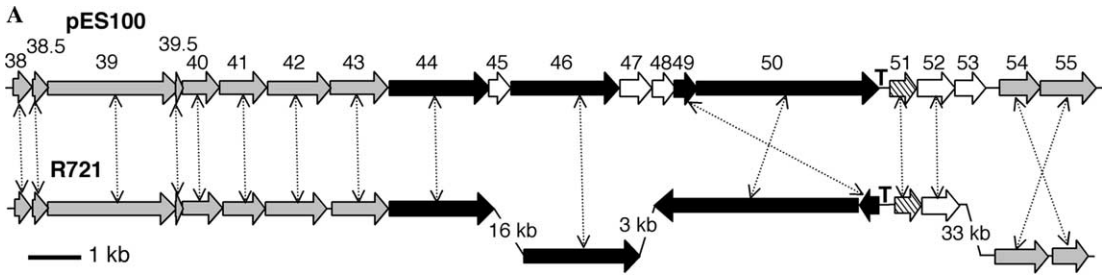
Interestingly, we discovered that different *V. fischeri* strains varied in their ability to serve as donors for the transfer of pES213 derivatives. A strain's proficiency as a donor in conjugations was strongly correlated with the presence of plasmids with homology to pES100 (Table 5), leading us to speculate that these large plasmids may be self-transmissible and able to mobilize pES213. Consistent with this hypothesis, pES100 encodes a putative conjugative relaxase protein ORF VFB50, and only *V. fischeri* strains with a VFB50 homolog acted as effective donors (Table 5). We cloned a fragment internal to the VFB50 gene in a suicide vector and mobilized this into strain ES114, to select for single recombinants with ORF VFB50 disrupted. The proficiency of the resulting mutant, EVS158 (Table 2), as a conjugative donor was reduced over 10,000-fold relative to ES114, to undetectable levels. These observations indicated a mechanistic connection between pES100 and transfer of pES213Kn. We therefore examined pES100 and pES213 sequences and their possible role(s) in plasmid transfer more closely.

Further examination of the pES100 sequence in the *V. fischeri* genome database revealed the components of a conjugative transfer system (Figs. 3A–C). Two small proteins of the putative type IV DNA secretion apparatus were not evident in the automated genome annotation, but closer examination revealed these small ORFs, which we designated ORFs VFB38.5 and VFB39.5, at a conserved

Table 5
Donor effect on *Vibrio–Vibrio* plasmid transfer

Donor[a]	pES100-like plasmid in donor[b]	ORF VFB50 relaxase homolog in donor[c]	pES213Kn transfer frequency to EVS135 ($\times 10^{-6}$)[d]
ES114	+	+	260
ES602	+	+	0.54
ES657	+	+	0.37
ES79	+	+	<0.01[e]
ES191	–	–	<0.01
ES66	–	–	<0.01
ES560	–	–	<0.01
ES566	–	–	<0.01
ES235	–	–	<0.01
ES240	–	–	<0.01
ES334	+	–	<0.01

[a] All donors are wild-type *V. fischeri* strains.
[b] Determined by plasmid isolation and Southern blotting by Boettcher and Ruby (1994).
[c] Determined by PCR amplification with primers EVS109 and EVS110.
[d] Kanamycin-resistant recipients (kanamycin and erythromycin resistant) per recipient (erythromycin resistant). Control experiments showed that spontaneous antibiotic-resistant donors or recipients did not significantly affect the reported transfer frequencies.
[e] Using strain ES79 as a donor, transfer was usually below the limit of detection, however, low levels of transfer ($0.01–0.1 \times 10^{-6}$) were observed in some experiments.



B

VirB7 homologs

39.5 MNNITILCLSLLILSGCSSVSPHACPS----YQQAPIEVINDAQ
R721 MKHPLIIASALLISACSHTPNPVCKKGGWFELNTAIEQLKAGDY

VirB3 homologs

38.5 MSEPIFKACTRFSMLFSVPLVPLMLSNAIIFLVCMWANYFGLGLW-LFILTHFNYLTL-RFITKL---DDAN------LSLLGLRFKCRRMNRNRSFYGATVYSPLPFKKR
R721 ESFMTYNGFNRFALVSGIPLMLLFTAFFAVLTCFPAIF----LWGMKGLILEAICALFLFIVKLACENDPNALRVIRLNLMGLLLKIKHRDLIIG------YSSVR

C

pES100 ORF	Proposed function (homolog, plasmid)
VFB38	type IV secretion (VirB2, pTiA6)
VFB38.5	type IV secretion (VirB3, pTiA6)
VFB39	type IV secretion (VirB4, pTiA6)
VFB39.5	type IV secretion (VirB7, pTiA6)
VFB40	type IV secretion (VirB8, pTiA6)
VFB41	type IV secretion (VirB9, pTiA6)
VFB42	type IV secretion (VirB10, pTiA6)
VFB43	type IV secretion (VirB11, pTiA6)
VFB44	couple DNA to secretion (TraG, RP4)
VFB45	ssDNA-binding protein
VFB46	Topoisomerase (TraE, RP4)
VFB47	outer membrane protein (TraP, pIPO2T)
VFB48	unknown
VFB49	relaxosome component (MbeC, ColE1)
VFB50	relaxase (MbeA, ColE1)
VFB51	unknown transfer function
VFB52	unknown
VFB53	unknown
VFB54	type IV secretion (VirB5, pTiA6)
VFB55	type IV secretion (VirB6, pTiA6)

Fig. 3. Sequence analysis of the pES100 conjugative apparatus. (A) Organizational comparison of pES100 and *E. coli* plasmid R721. Thick horizontal arrows represent ORFs with putative functions in type IV secretion (gray), DNA nicking and processing (black), other transfer function (hatched), or no apparent role in conjugation (open). Gene homologs on the two plasmids are connected by dashed lines with double-headed arrows. Numbers above pES100 ORFs correspond to VFB38 through VFB55 assigned by the *V. fischeri* genome project, with the exception of VFB38.5 and VFB39.5 which were discovered and annotated in this study. "T" indicates putative *oriT*. (B) Amino acid alignment of the small ORFs VFB38.5 and VFB39.5 above their respective homologs in R721. Nine N-terminal residues of the R721 VirB3 homolog are not shown. (C) Summary of proposed functions for pES100 ORFs. For consistency and clarity, homologs in parentheses represent well-studied plasmid systems and not necessarily the closest database match for each ORF.

location in the gene cluster (Fig. 3A). We noticed significant similarity between the arrangement and sequence of ORFs on pES100 and genes involved in the self-transmissibility of *E. coli* plasmid R721 (Fig. 3A), which made the latter a useful template for examining pES100 sequences. For example, our designation of ORFs VFB38.5 and VFB39.5 as VirB3 and VirB7 was bolstered by their similarity in sequence (Fig. 3B) and orientation (Fig. 3A) to genes on R721.

We also found that pES100 has a region homologous to the R721 origin of transfer (*oriT*), and that pES213 has two such sequences, one on each DNA strand, which we designated *oriT* and *oriT′* (Fig. 4A). The region surrounding *oriT* and *oriT′* in pES213 was critical for interstrain plasmid transfer (Figs. 1B and C), further suggesting a role for this region in conjugative transfer. The nucleotides most-highly conserved between the putative *oriT′*s of pES100, pES213, and R721 include an imperfect inverted repeat, and the nicking site for R721 transfer (Fig. 4A). Interestingly the pES213 ORFs TraY and TraZ resembled the N- and C-termini, respectively, of ORF VFB51 from pES100 and YciA from R721 (Fig. 4B). PCR amplification of *traYZ* using a high-fidelity polymerase, and subsequent sequencing of the product, confirmed that these are two distinct ORFs, despite resembling single ORFs in pES100 and R721. Although pairwise BLAST comparisons of TraY-TraZ, ORF VFB51, and YciA revealed only 23–25% identity and 43–51% similarity, there were small clusters of

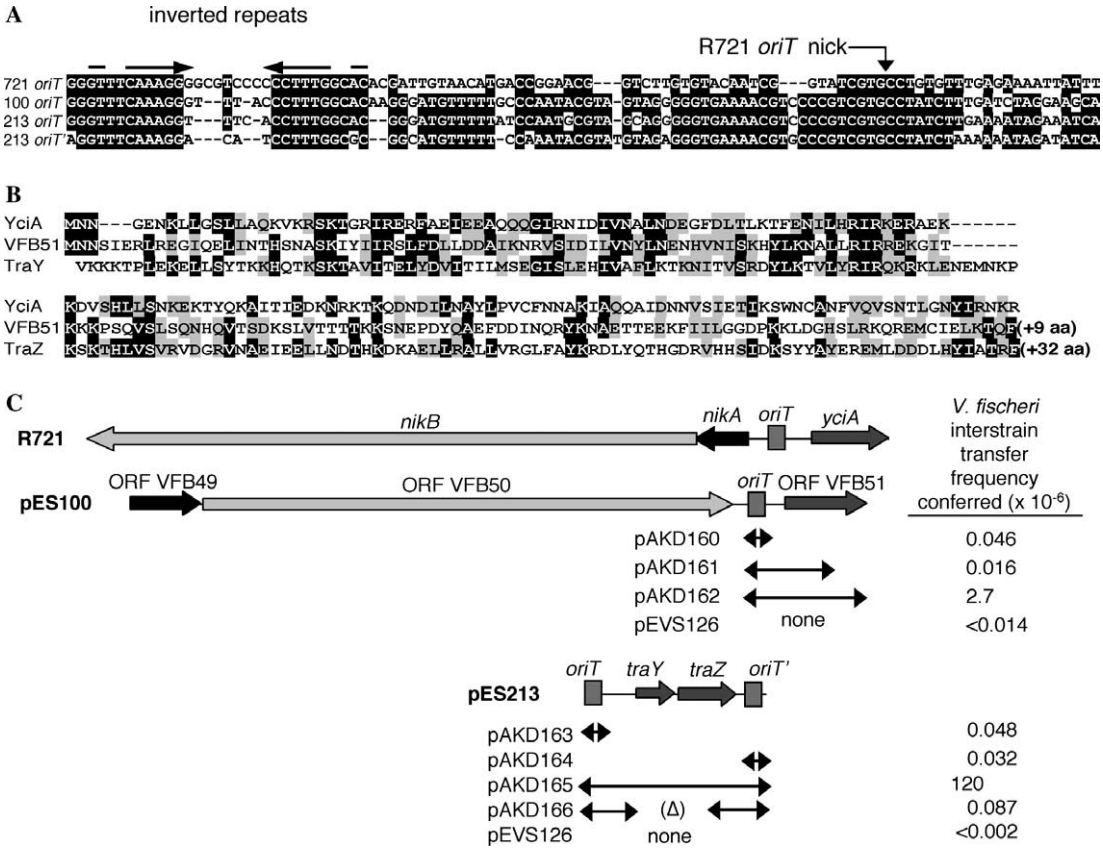


Fig. 4. Analysis of pES100 and pES213 transfer origins. (A) Nucleotide alignment of putative transfer origins in *E. coli* plasmid R721 (721 *oriT*), pES100 (100 *oriT*), and pES213 (213 *oriT* and 213 *oriT*′). The site of nicking in R721 (GenBank AP002527) is indicated by a bent arrow. Arrows above inverted repeats are discontinuous where the repeat is not homologous. (B) Amino acid alignment of YciA (R721), ORF VFB51 (pES100), and TraY/TraZ (pES213). White letters on black indicate conserved residue, gray shading indicates residues similar in BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992). (C) Schematic representation of genes and sites near origin of transfer in R721, pES100, and pES213. Similar shading of arrows or boxes indicates sequence similarity between plasmids. Direction of cross hatches indicates orientation of *oriT* sites (e.g., *oriT* and *oriT*′ in pES213 are on opposite strands). Double-headed arrows are aligned beneath corresponding regions of pES100 or pES213 cloned into pEVS126, and the ability of these clones to confer *Vibrio–Vibrio* transfer from strain ES114 to strain ESR1 in the absence of an RP4-derived helper is shown to the right of each clone.

residues conserved among all three (Fig. 4B). Moreover, the orientation of these ORFs was conserved; ORF VFB51 and *yciA* were immediately adjacent to the *oriT* of pES100 and R721, respectively, while *traYZ* was bracketed by *oriT* and *oriT*′ in pES213 (Fig. 4C).

3.7. Genes involved in pES213 transfer

TraY-TraZ and ORF VB51 also displayed conserved function, each enhancing the frequency of *Vibrio–Vibrio* plasmid transfer. Regions surrounding these ORFs and the nearby transfer origins were cloned into p15A-based vector pEVS126, and scored for their ability to confer *Vibrio–Vibrio* transfer from strain ES114 to strain ESR1 (Fig. 4C). Plasmids with inserts that bear the transfer origins and no ORFs (pAKD160, pAKD161, pAKD163, pAKD164, and pAKD166) were transmissable at frequencies barely above the limit of detection (Fig. 4C); however, when clones also included ORF VFB51 or TraY–TraZ (pAKD162 and pAKD165), transfer frequencies were enhanced 100- to 1000-fold (Fig. 4C). Thus, ORF VFB51 or

TraY–TraZ enhanced transmissibility when provided in *cis* on pAKD162 and pAKD165, respectively, even though ORF VFB51 was also present in *trans* in both the donor and recipient strains (on pES100) in each of these matings.

We considered the possibility that the transfer of plasmids pAKD160, pAKD161, pAKD162, pAKD163, pAKD164, pAKD165, and pAKD166 resulted from homologous recombination between these plasmids and pES100, which may be self-transmissable. For example, pAKD160 might recombine with pES100, the two-plasmid recombinant could mobilize itself to the recipient, and then the two plasmids could resolve themselves again by homologous recombination. This seemed an unlikely model, because the transfer frequencies conferred by inserts do not correlate with their expected behavior as recombination substrates, based on length and degree of homology to pES100. Furthermore, in our experience, we would not expect any of the pES213-derivatives to recombine with pES100 at a significant frequency. Nonetheless, to test this model we used *recA* recipients, which would not allow resolution of any theoretical two-plasmid-recombinant intermediate by homologous recombination. Based on restriction analyses using *Hin*dIII and *Pst*I, we found pAKD160, pAKD161, pAKD162, pAKD163, pAKD164, pAKD165, and pAKD166 could each be identified as distinct from (i.e., not recombined with) pES100 in *recA* recipient strain AKD512. Using this approach, we also isolated one rare two-plasmid recombinant, a hybrid of pES100 and pAKD162, and found that it did not resolve back into the original two plasmids in *recA* mutant AKD512, even after several generations in culture, indicating that there is no significant *recA*-independent path for resolution of such plasmid recombinants (data not shown). Thus, if pES100 is enabling or enhancing the transfer of pAKD160, pAKD161, pAKD162, pAKD163, pAKD164, pAKD165, and pAKD166, it is performing this function primarily in *trans*.

In the course of these experiments, we noticed an unusual dependence on RecA for plasmid transfer. Although *recA* mutant AKD512 and its parent ESR1 were equally proficient as plasmid recipients, the same mutant *recA* allele rendered EVS510 a much less efficient donor than its parent ES114 (Table 6). Providing *recA* on a complementing plasmid largely restored donor proficiency to the *recA* mutant, and the parent vector (pDMA5) did not (Table 6). Thus, the loss of donor efficiency was due to the disruption of *recA* and not effects of the insertion in *recA* on other adjacent genes. Although *recA* was not absolutely required in the donor strain, transfer frequencies were attenuated 1000- to 2500-fold when a *recA* donor was used. We considered the possibility that reduced growth of *recA* donors could result in fewer donor cells relative to recipients, however, *recA* mutants displayed only a 10–15% reduction in growth rate, and their effectiveness as donors was still attenuated at least 100-fold even if transfer frequencies were calculated on a per donor basis (data not shown). RecA was also required in the donor even when the recipient also lacked RecA (Table 6), indicating that growth attenuation of the donor relative to the recipient cannot account for the inefficiency of *recA* mutants as donors.

Table 6
recA dependence of *Vibrio–Vibrio* plasmid transfer

Donor	Recipient	pES213Kn transfer frequency ($\times 10^{-6}$)[a]
ES114	ESR1	210
ES114	AKD512 (*recA*$^-$)	260
EVS510 (*recA*$^-$)	ESR1	0.083
EVS510 (*recA*$^-$)	AKD512 (*recA*$^-$)	0.28
EVS510 (*recA*$^-$)pDMA5 (vector)	ESR1	0.25
EVS510 (*recA*$^-$)pEVS131 (vector +*recA*)	ESR1	96

[a] Kanamycin-resistant recipients (kanamycin and rifampicin resistant) per recipient (rifampicin resistant). Control experiments showed that spontaneous antibiotic-resistant donors or recipients did not significantly affect the reported transfer frequencies.

3.8. Maintenance of pES213

Plasmids employ several strategies to promote their retention (Helinski et al., 1996), including *par* systems that ensure each daughter cell receives a plasmid during cell division and toxin–antidote systems that kill plasmid-free daughter cells or any cells lacking the plasmid. Analyses of pES213 sequences revealed no homologs to known *par* systems, so we investigated whether pES213 encodes an exported bacteriocin. Supernatants from cultures of ES213, or of ES114 carrying pES213 derivatives, did not show inhibitory effects when spotted on lawns of other *V. fischeri* strains. Similarly, in mixed cultures the ratio of pES213C-carrying and plasmid-free cells remained nearly constant over several generations. Some plasmids encode a stable intracellular toxin and a relatively unstable antidote, so that if the plasmid is lost from the cell the antidote degrades before the toxin does and the plasmid-free cell is killed, however viable cells that had lost pES213C, pES213Kn, or pES213Tp could be identified, arising at a frequency of $\sim 10^{-4}$ per generation, indicating that a toxin–antidote system is either absent, not expressed under these conditions, or retarding cells without killing them.

Some plasmids promote their inheritance simply by maintaining a large number of copies per cell, so that random partitioning during cell division is unlikely to yield plasmid-free daughter cells. We therefore tested the copy number of pES213Kn using real-time PCR. Our strategy was to quantitate the relative abundance of the *aph* (kanamycin-resistance) gene present in ES114 carrying pES213Kn and in EVS401, which contains *aph* in single copy on chromosome 2. By comparing the two strains, we determined that pES213Kn was maintained in 9.4 ± 2.4 (standard error) copies per genome. Similar results were obtained by analysis of pES213C. This copy number could account for the stability of pES213 derivatives (see Section 4).

3.9. Prevalence of plasmids similar to pES213 in plasmid-carrying symbiotic V. fischeri strains

pES213A DNA was labeled and used as a hybridization probe under high-stringency conditions to detect plasmids similar to pES213 in ES213 and 10 other symbiotic *V. fischeri* strains that contain small plasmids (Boettcher and Ruby, 1994). All 10 strains harbored plasmids with significant similarity to pES213 (Fig. 5A). Both 1-kb ladder and additional plasmid fragments that did not hybridize to the probe were apparent in the gel prior to blotting (data not shown) and served as negative controls. Based on fragment sizes and hybridization signal, ES213, ES209, and ES334 could contain pES213, although all strains appeared to harbor multiple plasmids [(Boettcher and Ruby, 1994) and data not shown]. Strains ES12, ES79, ES209, ES334, ES595, and ES620 contained plasmids with a *Bgl*II fragment that hybrized to pES213 and co-migrated with pES213 "fragment c" (Fig. 5A), which contains core origin of replication sequences including the putative DnaA binding site, inverted repeat 1, direct repeats, and the closely clustered Dam-methylation sites (Fig. 2). When we tested for the presence of *oriV* sequences by PCR, the plasmid preps from the same six strains clearly showed strong amplification of a product co-migrating with the pES213 *oriV* (Fig. 5B). Plasmid preps from strains ES235, ES240, ES566, and ES602 appeared to have their own relatively conserved fragment pattern but lacked the *oriV*-containing "fragment c" (Fig. 5A). These preps also yielded weaker PCR amplification of different-sized products with the pES213-*oriV* specific primers (Fig. 5B). We cloned and sequenced a PCR product from an ES240 plasmid and found that it was homologous to the pES213 *oriV*, including a stretch of 146 bp encompassing inverted repeats 1 and 2 (Fig. 2) that was 98% identical to pES213. This conserved *oriV* sequence was also found on PCR products amplified from an ES566 plasmid(s). Finally, we used PCR to test for the presence of the *oriT-traYZ-oriT′* region in this group of plasmids, and found that using each of the *V. fischeri* small-plasmid preps as template DNA we obtained a PCR product with the same size as *oriT-traYZ-oriT′* (Fig. 5C). We cloned and sequenced the PCR products from the ES240, ES566, and ES620 plasmid templates and found that they were each 97% identical to the *oriT-traYZ-oriT′* region of pES213. We obtained no *oriV*- or *oriT-traYZ-oriT′*-containing PCR

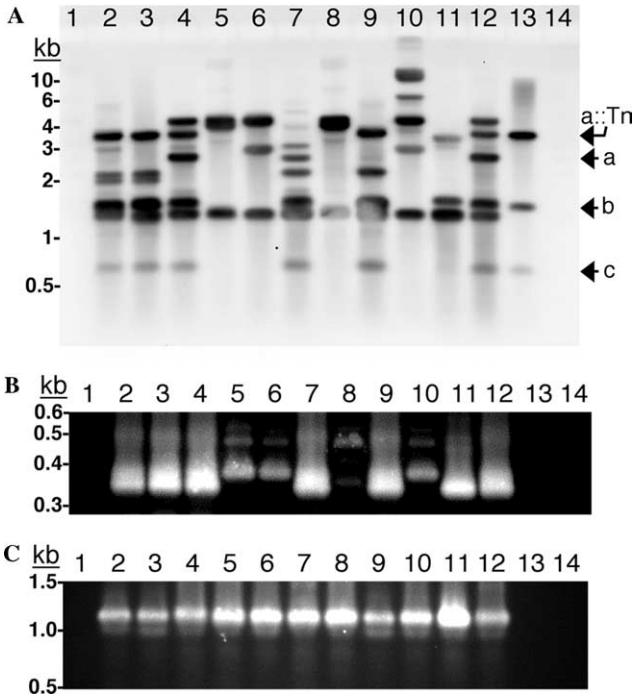


Fig. 5. Distribution of plasmids similar to pES213 in *V. fischeri* isolates from *E. scolopes*. (A) Southern blot, using labeled pES213A DNA to probe: 1-kb-ladder (lanes 1 and 14), *Bgl*II-digested plasmid preparations from *V. fischeri* strains ES12, ES79, ES209, ES235, ES240, ES334, ES566, ES595, ES602, ES620, and ES213 (lanes 2–12, respectively), and *Bgl*II-digested pES213A (lane 13). Arrows indicate the predicted migration of three pES213 *Bgl*II fragments (a–c) and of the transposon-containing fragment in pES213A (a::Tn). Approximately 1 μg of 1-kb-ladder and 0.5 μg of digested plasmid were loaded in each lane prior to electrophoresis and blotting. (B) PCR amplification from plasmid templates using primers specific to the pES213 *oriV* region (EVS106 and EVS113) and 500-pg template DNA. Templates were: lane 1, pES100; lanes 2–12 the same as in (A); lane 13 pUC19; and lane 14 pACYC184. (C) PCR amplification of *oriT-traYZ-oriT′* using primers AKD2b and AKD2c. Templates were the same as in (B).

products from control templates pES100, pUC19, or pACYC184 (Figs. 5B and C). We conclude that pES213 represents a family of plasmids with conserved replication and transfer functions that is common among *V. fischeri* isolates from *E. scolopes*.

4. Discussion

In this study, we report the characterization of a small plasmid endogenous to *V. fischeri*. We have isolated derivatives of pES213, shown that this plasmid represents a group of plasmids common among *V. fischeri* isolates from *E. scolopes*, and described the plasmid-encoded functions that enable it to replicate and spread in *V. fischeri* populations. Our results complement the recent *V. fischeri* genome sequencing project, elucidate novel plasmid functions, and will form the basis for new genetic tools in *V. fischeri* based on native vectors. Furthermore, we show evidence (discussed below) that pES213 utilizes conjugative transfer functions from the relatively large plasmid pES100 to promote its transfer between *V. fischeri* strains, which would provide a mechanistic explanation for the previously described frequent co-occurence of these small and large plasmid types in *V. fischeri* strains.

To isolate pES213 derivatives in *E. coli* we had to overcome two obstacles. First, the native host for pES213 harbors multiple plasmids, making restriction mapping of any one plasmid difficult. Second, even if a restriction map were available it would be impossible to know a priori where a selectable marker could be introduced without

disrupting important plasmid functions. We therefore employed a strategy similar to that used by Agron et al. (2002), whereby plasmid DNA was transposon mutated in vitro to introduce a selectable marker, and a tagged self-replicating plasmid was subsequently recovered in *E. coli*. We then randomly subcloned fragments of this plasmid into pEVS122, which enabled us to determine the sequence of pES213 and the regions involved in plasmid replication and *Vibrio–Vibrio* plasmid transfer (Fig. 1).

A minimal 863-bp fragment of pES213 capable of directing replication in *V. fischeri* was isolated and characterized. This 863-bp sequence does not bear striking similarity to replication origins from other *Vibrio* plasmids (Bidinost et al., 1999; Di Lorenzo et al., 2003; Powers et al., 2000; Sobecky et al., 1998), but does contain attributes (Del Solar et al., 1998; Helinski et al., 1996) typical of origins from plasmids that replicate via a θ-type mechanism (see Section 3 and Fig. 2). Although many θ-type vectors utilize a plasmid-encoded Rep protein to replicate, none of the ORFs on pES213 was required for replication. The minimal 863-bp replication-conferring fragment contains only one putative ORF, designated ORF5, and a frame-shifting 4-bp insert in this ORF did not affect replication. Furthermore, nucleotide sequence from a region of the origin overlapping ORF5 was conserved in a *V. shiloi* plasmid (Fig. 2), but ORF5 itself was not conserved, which could indicate that this small putative ORF may not encode an expressed protein.

Because pES213 was isolated from *V. fischeri*, it was not surprising that its derivatives were relatively stable in this bacterium. The frequency of plasmid loss, approximately 10^{-4} per generation, could be accounted for by random segregation of plasmids. In theory, dividing cells containing thirteen randomly-segregating plasmids would yield a plasmid-free daughter cell at a 10^{-4} frequency ($0.5^{13} = 1.2 \times 10^{-4}$). Using real-time PCR we estimated that there are an average of 9.4 pES213Kn copies per genome. If we assume an average of one and a half genomes per actively growing cell and a low number of unresolved dimeric plasmid replication intermediates, we reach an estimate very close to thirteen segregating plasmids per dividing cell. Interestingly, when we examined cells containing both pES213Tp and pES213Kn, we found that the former, 481-bp smaller plasmid was retained at a higher frequency than the larger plasmid (data not shown), possibly because it is replicated faster. If correct, this would indicate a selective advantage for maintaining small plasmid size. We did not detect any specific mechanisms for plasmid retention (e.g., par or toxin–antidote systems), and we can rule out the possibility of a constitutive exported toxin, because supernatant from strain ES213 did not inhibit other *V. fischeri* strains. However, it is possible that some retention system was disrupted by the transposon insertion in our pES213 derivatives.

We also found that pES213 derivatives can be transferred between certain *V. fischeri* strains (Table 5). Our data suggest that pES213 contains *cis*-acting elements required to utilize *trans*-acting mobilization factors from pES100, which encodes conjugation machinery similar to that on *E. coli* plasmid R721 (Figs. 3 and 4). For example, a mutant with a disrupted relaxase on pES100 was attenuated >10,000-fold as a conjugative donor for pES213 derivatives. We cannot rule out the possibility that the inability of this mutant to mobilize pES213 derivatives results from effects of the insertion on flanking loci rather than the disruption of the relaxase gene (VFB50) itself, however, VFB50 is embedded among putative conjugative genes (Figs. 3 and 4) so either scenario would equally support the conclusion that the conjugative apparatus of pES100 is important for pES213 transfer.

Based on other conjugation systems (Frost et al., 1994; Grohmann et al., 2003; Pansegrau et al., 1994; Pansegrau and Lanka, 1996) we expected a small non-coding *oriT* sequence in *cis* would effectively direct plasmid transfer, however, we discovered an unanticipated role for TraY-TraZ and ORF VFB51, from pES213 and pES100, respectively. These ORFs, which are similar in amino acid sequence and position relative to *oriT* (Figs. 4B and C), are apparently required in *cis* for efficient *Vibrio–Vibrio* plasmid transfer. Although ORF VFB51 was present in both donor and recipient cells on pES100, its presence in *cis* on a p15A-derived vector (pAKD162) increased the transfer

frequency more than 100-fold relative to vectors (pAKD160 and pAKD161) that carried *oriT* alone (Fig. 4C). Similarly, removal of *traYZ* from between *oriT* and *oriT′* in the pES213 sequence decreased transfer frequency over 1000-fold (Fig. 4C). The importance of this region in *cis* could stem not from any protein products but rather from the DNA itself, however, it is worth noting in this regard that in contrast to the low but evident amino acid similarities in these predicted genes (Fig. 4C), comparisons of the *yciA*, VFBB51, and *traYZ* DNA sequences did not reveal any apparent significant nucleotide conservation. Future experiments aimed at understanding the novel involvement of ORFs in *cis* on these mobilized plasmids may elucidate previously undescribed mechanisms contributing to DNA transfer.

We also found that *recA* is required in the donor, but not the recipient, for efficient *Vibrio–Vibrio* plasmid transfer (Table 6). RecA has broad effects on gene expression and DNA topography, many of which influence plasmid biology, yet we are unaware of a precedent for the requirement of RecA in a conjugative donor. It is well known though that certain phages exit host cells after RecA cleaves a phage-encoded c1-type repressor protein, and pES100 encodes a putative c1-type repressor (ORF VFB18). It is tempting to speculate that RecA is required in donors to process the pES100 c1 protein and thereby derepress genes involved in conjugative export. However, there are many other explanations for our data that are also consistent with known functions of RecA. For example, RecA can contribute to plasmid multimerization (Biek and Cohen, 1986; James et al., 1982; Summers and Sherratt, 1984), and perhaps pES213 mobilization requires plasmid multimer as the donor substrate. Also, *recA* mutants of *V. cholerae* display altered adherence properties (Kumar et al., 1994), which could be important in establishing a mating pair. Future work will help distinguish between these and other possible roles for RecA in conjugation. Although RecA contributes to plasmid stability in other systems (Biek and Cohen, 1986; Summers and Sherratt, 1984; Vartholomatos et al., 1993), we have ruled out the possibility that RecA is required simply to stabilize plasmids in the donor, because *recA* mutations did not affect stability of pES213-derivatives.

Given the apparently unique requirements of *traYZ* (or ORF VFB51) in *cis* and RecA in the donor for plasmid transfer, we examined the relationship of pES213 and pES100 to known groups of mobilizable plasmids more closely. Francia et al. (2004) recently proposed using relaxase sequences as a logical way to group mobilizable plasmids. pES213 lacks a relaxase, however, the putative relaxase of pES100, ORF VFB50, apparently places this plasmid in the ColE1-3H family, a conclusion supported by analyses of ORF VFB49. ORF VFB50 and NikB of R721 each contain the three His residues in the family-defining Hx(D/E)…HxH portion of "motif III," however, they also lack the (D/E) residue thought to be functionally important. ColE1-3H family members are diverse and relatively few have been subjected to experimental analyses (Francia et al., 2004), so it is perhaps unsurprising that our studies revealed apparently novel observations.

Advances in the understanding of *V. fischeri* genetics, and the concomitant generation of new genetic tools, will make important contributions to studies of bioluminescence, quorum-sensing gene regulation, and beneficial animal–bacteria interactions. The results of this study provide two potential avenues for genetic-tool development. First, pES213 is native to *V. fischeri* and is rarely lost from cells, making pES213-based derivatives attractive as vectors for the stable introduction of genes for mutant complementation, strain tagging, or gene-expression studies. pES213-derived plasmids could be especially useful because certain common "broad host range" replicons (e.g., RK2) apparently do not replicate in *V. fischeri* ES114, and the p15A-based vectors currently in use have limited stability during growth in the light organ. Second, if the pES213 *oriT-traYZ-oriT′* sequence were integrated in the ES114 genome, the conjugation machinery of pES100 might direct chromosomal transfer, initiating from this insert, to other *V. fischeri* strains. Such inserts could be utilized in combination with selectable markers at defined loci distributed throughout the genome to physically map spontaneous mutations in *V. fischeri*. Further characterization of pES213, pES100, and other *V. fischeri* plasmids will enhance these efforts.

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