

NEW GENETIC TOOLS FOR USE IN THE MARINE BIOLUMINESCENT BACTERIUM *VIBRIO FISCHERI*

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Introduction

In recent years, tools for the genetic analysis of marine *Vibrio* species have become increasingly established (1). In particular, transposon mutagenesis has been applied as a powerful technique. However, not all of the techniques that have been developed in one species of *Vibrio* can be successfully applied to another. For example, phage P1 is an effective delivery system for transposon mutagenesis of *V. harveyi* (1), but not for *V. fischeri* (Ruby, unpublished data), a species whose symbiotic association with the squid *Euprymna scolopes* has become an emergent model for the study of symbiosis genes (2). The most efficient method for the introduction of DNA into *V. fischeri* is through conjugative matings with *Escherichia coli*, which result in a high efficiency of plating both for the transfer of plasmids (3) and for transposition (4).

To date, two different transposons have been reported for the study of *V. fischeri* genes in the squid symbiont strain ES114, and its derivatives (2). Mutagenesis with the temperature sensitive MudI1681 (5) transposon yielded a pool of mutants with random chromosomal insertions, a subset of which were flagella mutants (4). Although insertions with this transposon were readily obtained, there was a tendency for the delivery vector to be maintained, resulting in multiple insertions under conditions where a low temperature was not strictly maintained. Mini-Tn5 Cm (6), a derivative of Tn5 in which the transposase is external to the transposon, has also been useful in generating a transposon-insertion library of *V. fischeri* (2). This transposon is one of a set of Tn5-derived transposons that contain many useful features, including a multiple cloning site internal to the transposon and the suicide origin, oriR6K, in the delivery vector. We have had some difficulty, however, in obtaining expression from Tn5 derivatives carrying either promoterless *lac* or *lux* constructs introduced into *V. fischeri* (unpublished data).

A new transposon system (7) that has become available is based on Tn10 and has some of the same useful features as the mini-Tn5 system—a transposase gene external to the transposon, a multiple cloning site internal to the transposon, an origin of replication that is suicidal in *V. fischeri*, and a conjugation-based delivery system. In this paper we describe two derivatives of this Tn10-derived transposon that we have constructed, as well as other genetic tools that we expect will be useful for the genetic analysis of *V. fischeri*.

Materials and Methods

Reagents: Restriction enzymes and T4 DNA ligase were obtained from either Promega (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). X-gal (5-bromo-4-chloro-3-indoyl- β -D galactopyranoside) was purchased from 5 Prime 3 Prime (Boulder, CO, USA)

Procedures: Standard cloning techniques (8) were used to construct the plasmids described in this paper: parent plasmids were digested with appropriate restriction enzymes, the DNA was agarose gel purified, and the correct fragments were extracted from the agarose using the GeneClean kit (Bio101). The fragments were ligated together and the resulting mix was used to transform CaCl₂ competent *E. coli* cells (either strain DH5 α or CC118 λ pir). For conjugations, plasmids were transferred into S17-1 λ pir *E. coli* cells by electroporation (8).

Results and Discussion

Mini-Tn10 derivatives. Mini-Tn10-carrying plasmids pKV32 and pKV34 were derived from pBSL181 (7). Plasmid pKV32 (Fig. 1A) was constructed by the insertion of the 7-kb BamHI fragment carrying the promoterless *lacZYA* genes from pMC903 (9) into pBSL181 digested with BamHI. S17-1 λ pir cells carrying pKV32 were conjugated with *V. fischeri* strain ESR1 (4), a rifampicin-resistant derivative of ES114 (2). The cells were spread on LBS plates

containing rifampicin, chloramphenicol, and X-gal. After incubation at 28°C, a small number of blue and white colonies arose at approximately equal frequencies. These data suggested to us that the transposon was functional in *V. fischeri* and that promoterless genes internal to the transposon could be expressed by promoters upstream of the inserted DNA.

Plasmid pKV34 (Fig. 1B) carries the promoterless *luxAB* genes from *V. harveyi* inside the Tn10 transposon. It was constructed by the ligation of a 3-kb *Bam*HI-*Pvu*II (*luxAB*) fragment from pHV100 with pBSL181 digested with *Bam*HI and *Sma*I. A number of colonies, appearing at a frequency similar to that obtained above, arose when S17-1 λ pir cells carrying plasmid pKV34 were conjugated with KV150, a *luxA* mutant of ESR1 (10).

A subset of the colonies obtained were examined for luminescence in culture. Specific luminescence levels varied from just above background (the *luxA*-deletion parent emits no luminescence) to greater than the level observed for *luxA*⁺ strain ESR1. These data suggest that the promoterless *luxAB* genes are located downstream (and are being transcribed) from promoters of varying strengths in the chromosome of *V. fischeri*. The mutants were checked by Southern analysis to determine the number and location of the transposon insertions. All pKV34.

strains checked, except one that apparently maintained the suicide vector, had a single transposon insertion. The location of the insertions was different for each strain, suggesting that the transposition of Tn10 in *V. fischeri* may be random. Thus, both pKV32 and pKV34 are useful constructs both for the introduction of gene fusions into the chromosome of *V. fischeri*, and for the study of gene expression. Similar constructs can also be made that encode sequences for protein fusions.

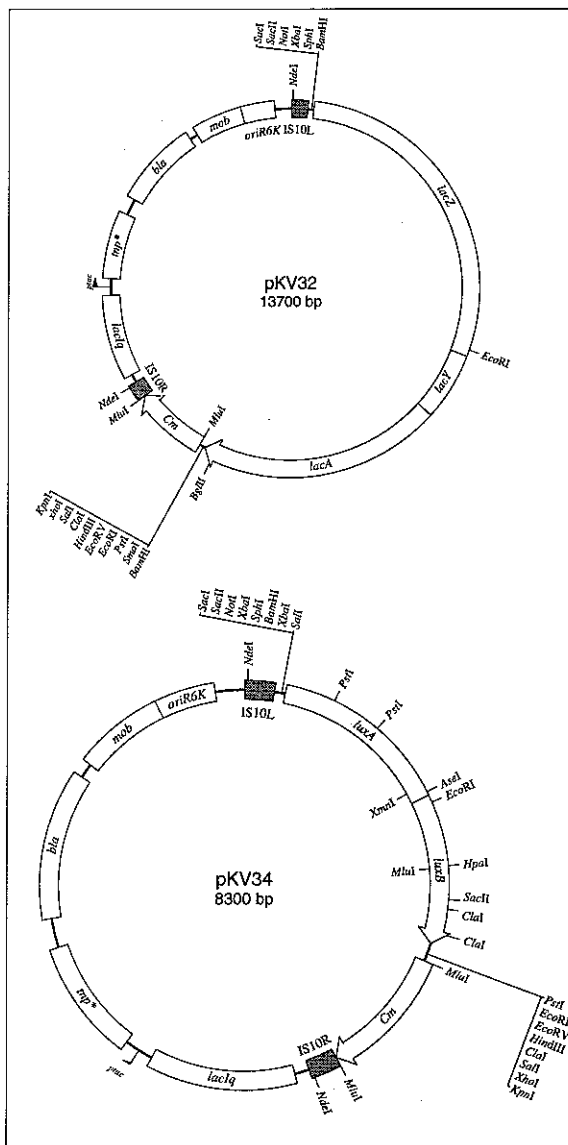


Figure 1. (A) Plasmid map of pKV32. (B) Plasmid map of the transposon insertions. All pKV34.

Tn7. Transposon Tn7 in *E. coli* inserts into a single chromosomal site (11). We investigated the ability of Tn7 to insert into the chromosome of *V. fischeri*. Plasmid pSUP2017 is a mobilizable vector that encodes chloramphenicol resistance and carries Tn7 (12). Tn7 itself encodes resistance to trimethoprim, an antibiotic to which *V. fischeri* is sensitive. When this plasmid is introduced into ESR1, both chloramphenicol-resistant and

trimethoprim-resistant colonies can be obtained. Strains that became chloramphenicol sensitive but remained trimethoprim resistant were isolated, suggesting that the Tn7 transposon was able to transpose into native sequences from the vector, which was subsequently lost. Preliminary Southern analysis (unpublished data) indicated that the Tn7 transposon targeted the same site in each of 6 isolates. These data support the conclusion that Tn7 inserts into a single site in *V. fischeri*; we predict that Tn7 will be useful as a vector for introducing genes in single copy in the chromosome.

Useful vectors. In addition to being sensitive to the antibiotics chloramphenicol and trimethoprim, *V. fischeri* strain ES114 is also sensitive to erythromycin, tetracycline, gentamycin, and kanamycin. Of these, only chloramphenicol, trimethoprim and erythromycin have proven useful for our studies. The tetracycline resistance gene encoded by pACYC184 is unable to confer resistance to *V. fischeri*, and the same is true for the single gentamycin resistance gene that we have checked (unpublished data). While kanamycin can and has been used as an antibiotic, there is a high background of spontaneously resistant colonies regardless of the concentration of kanamycin used. *V. fischeri* cells are also resistant to ampicillin, making most of the commonly available vectors of no

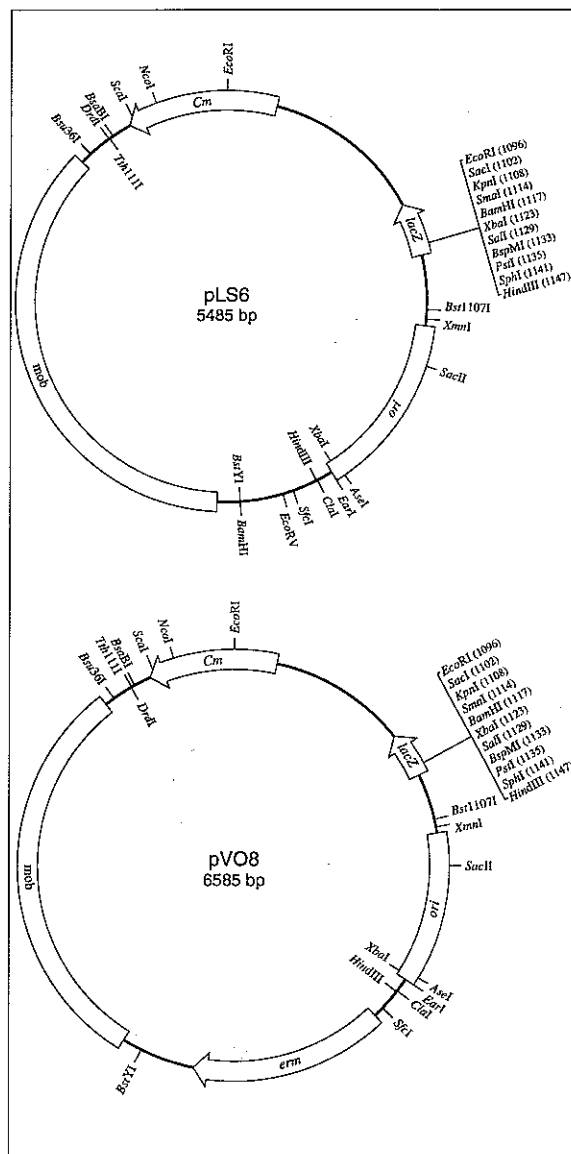


Figure 2. (A) Plasmid map of pLS6. (B) Plasmid map of pVO8.

practical use. In addition, the high-copy-number vectors such as pBluescript (Stratagene, Inc.) that replicate in *E. coli* are relatively unstable in *V. fischeri*.

Derivatives of pACYC184 (8) (or any vector that contains the p15A origin of replication) appear to replicate fairly well in *V. fischeri*, and thus are useful for complementation or overexpression studies. For this reason, we have constructed modified vectors that can be used in *V. fischeri*. Plasmid pLS6 (Fig. 2A) was derived in two steps from pSUP102, a mobilizable version of pACYC184. The first construction step abolished the tetracycline resistance gene of pSUP102 by a deletion of a 1.6-kb BstYI restriction fragment. The second step resulted in an insertion of the multiple cloning site and *lacZ* cassette from pUC19 (8), thereby increasing the ease of cloning genes of interest into this vector. Plasmid pLS6 was further modified to contain the gene for erythromycin resistance. This vector, pVO8 (Fig. 2B), will be useful for complementing the chloramphenicol-resistant mutants obtained by transposon mutagenesis.

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References

1. Silverman M, Showalter R, McCarter L. Genetic analysis in *Vibrio*. *Methods Enzymol* 1991; 204:515-36.
2. Ruby, E G. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri*-*Euprymna scolopes* light organ symbiosis. *Annu Rev Microbiol* 50:591-624.
3. Dunlap PV, Kuo A. Cell-density modulation of the *Vibrio fischeri* luminescence system in the absence of autoinducer and LuxR protein. *J Bacteriol* 1992; 174:2440-8.
4. Graf J, Dunlap PV, Ruby EG. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. *J Bacteriol* 1994; 176:6986-91.
5. Castilho BA, Olfson P, Casadaban MJ. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. *J Bacteriol* 1984; 158:488-95.
6. de Lorenzo V, Herrero M, Jakubzik U, Timmis KN. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* 1990; 172:6568-72.
7. Alexeyev MF, Shokolenko IN. Mini-Tn10 transposon derivatives for insertion mutagenesis and gene delivery into the chromosome of Gram-negative bacteria. *Gene* 1995; 160:59-62.
8. Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. Casadaban MJ, Chou J, Cohen SN. *In vitro* gene fusions that join an enzymatically active β -galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. *J Bacteriol* 1980; 143:971-80.
10. Visick KL, Ruby EG. Construction and symbiotic competence of a *luxA*-deletion mutant of *Vibrio fischeri*. *Gene* 1996; 175:89-94.
11. Craig, NL. Transposon Tn7. In: Howe, MM., editor. *Mobile DNA*. Washington, DC: Am Soc Microbiol, 1989:211-26.
12. Simon, R. Priefer, U, Puhler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* 1:784-91.

PHENOTYPIC BIOLUMINESCENCE AS AN INDICATOR OF COMPETITIVE DOMINANCE IN THE *EUPRYMNA-VIBRIO* SYMBIOSIS

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Introduction

The study of coevolutionary relationships among symbiotic associations has been an important avenue for understanding the establishment and development of two independent, but closely integrated organisms (1-3). Most studies investigating the evolutionary relatedness amongst host-symbiont pairs have alluded to the parallelisms that occur with the onset of a particular host speciation event that eventually leads to the isolation of the symbiont population (4). Hence, symbionts from an "ancestral" host species may have similar biochemical, physiological, or molecular characteristics that group them within the same "species". Yet, the delineation between strains or biovars within a species is much harder to define; strain characteristics that would normally define a particular bacterial population may be consistent throughout the entire group of symbionts. Although sequencing hypervariable regions of specific loci is a technique that can genetically differentiate between the strains (5), there are a few ways that one can phenotypically distinguish similar strains from one another.

The sepiolid squid-bioluminescent bacterial association offers several advantages as a model system to study the coordinated influence of luminous bacteria on the coevolution and speciation between partners (6). Both the bacteria and the host squid can be cultured separately, allowing the specific comparison between different symbiotic strains during colonization of a particular host squid light organ. Initiation, colonization, and persistence of each strain can then be monitored individually, or, in a competition experiment, where two strains compete for dominance in a particular species of host squid (7). Previous research has clearly indicated that symbiotic bacterial strains can be genetically differentiated from each other, either through restriction fragment length polymorphisms from specific DNA fragments (Lee and Ruby, unpubl. data), or through direct sequencing of loci that are variable between strains (8). Although molecular probes designed from these types of experiments can be used to distinguish and identify various strains in each individual light organ, this method is laborious and is limited by the number of colony blots one utilizes per infection assay (most infection/competition experiments utilize approximately 100 juveniles) (9). It has been recently discovered that variation in luminescence intensity of individual strains of symbiotic bacteria can be utilized to phenotypically distinguish strains from each other in an infected juvenile sepiolid light organ (8, Figure 1). This phenotypic