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Genetic Manipulation of *Vibrio fischeri*

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Abstract

Vibrio fischeri is a non-pathogenic organism related to pathogenic *Vibrio* species. The bacterium has been used as a model organism to study symbiosis in the context of its association with its host, the Hawaiian bobtail squid *Euprymna scolopes*. The genetic tractability of this bacterium has facilitated the mapping of pathways that mediate the interactions between these organisms. The protocols included here describe methods for the genetic manipulation of *V. fischeri*. Following these protocols, the researcher will be able to introduce linear DNA via transformation to make chromosomal mutations, to introduce plasmid DNA via conjugation and subsequently eliminate unstable plasmids, to eliminate antibiotic resistance cassettes from the chromosome, and to randomly or specifically mutagenize *V. fischeri* with transposons.

Keywords

Vibrio fischeri; transformation; conjugation; mutagenesis; plasmid

INTRODUCTION

Vibrio fischeri is a Gram-negative, comma-shaped, bioluminescent marine bacterium (Allen & Baumann, 1971; Lyell et al., 2017). It serves as a model system for the study of a variety of traits, including light production and biofilm formation, as well as the regulatory processes that control these energy intensive phenotypes (Christensen & Visick, 2020; Tischler, Hodge-Hanson, & Visick, 2019). Both light production and biofilm formation are linked to the symbiotic lifestyle that *V. fischeri* shares with its host, *Euprymna scolopes* – the Hawaiian bobtail squid (Mandel & Dunn, 2016; Eric V. Stabb & Visick, 2013). In this relationship, the bacteria produce light as camouflage for the squid, a phenomenon called counterillumination (Jones & Nishiguchi, 2004), while the squid provides a protected niche and nutrients for the bacteria in a specialized light-emitting organ, or light organ (Graf & Ruby, 1998; Jones & Nishiguchi, 2004).

Historically, *V. fischeri* has been considered a genetically tractable organism, but within the last ~10 years, manipulation of this organism has greatly improved, resulting in a comprehensive genetic toolbox with optimized protocols (Christensen & Visick, 2020). Consequently, *V. fischeri* is now a powerful model system for dissecting genetic pathways. Two major methods are used to introduce DNA into *V. fischeri*, transformation and conjugation. Transformation can be used to introduce either linear DNA (*e.g.*, sheared chromosomal DNA or PCR amplified DNA) with high efficiency or circular plasmid DNA

with much lower efficiency. Conjugation is primarily used to efficiently introduce plasmid DNA. While transduction is often used in other Gram-negative species to horizontally transfer DNA between bacteria, there has been only one reported generalized transducing phage, rp-1 (Graf & Ruby, 2000; Levisohn, Moreland, & Nealson, 1987), which is no longer used. However, the combination of transformation and conjugation approaches has facilitated the generation of mutant strains with unmarked or marked gene deletions, allelic replacements, engineered epitope tags, and gene complementation. Additionally, transposon mutagenesis, traditionally performed using a plasmid-borne transposon delivered by conjugation, has been used as a powerful technique to identify genes involved in a phenotype of interest in an unbiased manner.

V. fischeri is naturally competent, meaning that the bacterium encodes genetic determinants for DNA uptake from the environment. For this organism to take up DNA in a laboratory setting, however, its competence machinery must be induced either through special growth conditions or by genetic activation of the competence pathway (Pollack-Berti, Wollenberg, & Ruby, 2010). *V. fischeri* encodes machinery for homologous recombination that allows exogenous DNA with homology to the chromosome to be incorporated into the genome. Thus, with appropriately designed DNA constructs containing homology to the *V. fischeri* genome, DNA can be removed or inserted via transformation.

Below, we describe the steps that should be taken when planning to generate a mutant, methods for conjugation and transformation, and specific applications of these methods that can permit generation of a strain of interest.

STRATEGIC PLANNING

Strategic planning is necessary to successfully obtain a desired strain. What is the goal? Are you deleting a gene or multiple genes, creating an allelic variant, inserting DNA for an epitope tag, or introducing a promoter in front of a gene of interest? What will you use as the parent strain for the strain construction? How will you distinguish and isolate this mutant from its parent? The answers to these questions will vary depending on the study that you propose to conduct but will ultimately be critical to obtain the strain of interest. The following section describes some factors that you should consider when deciding what procedures to perform.

As a starting point, you will need to choose a strain to genetically manipulate. There are many available isolates of *V. fischeri* with distinct phenotypes, and genetic manipulation of these strains can uncover the determinants of these phenotypes. *V. fischeri* strain ES114 (ATCC 700601) (Boettcher & Ruby, 1990) has been commonly used as a parent strain for genetic manipulation and phenotypic characterization. Thus, there are established protocols for how to grow and perform phenotypic characterization of ES114 and its mutant derivatives (Christensen & Visick, 2020). As such, the protocols described herein primarily consider this strain. However, there is value in using other *V. fischeri* strains such as FQ-A001, MB13B2, or the fish symbiont MJ11 (Bongrand & Ruby, 2019; Koehler et al., 2018; Mandel, Wollenberg, Stabb, Visick, & Ruby, 2009; Sun et al., 2016). If using a strain of *V.*

fischeri other than ES114, these protocols may need to be optimized for the strain(s) of interest.

Perhaps you would like to use a "reverse genetics" approach to identify the function of a specific gene or determine when that gene is expressed. Transformation can be used to delete a gene, make an allelic variant of a gene, or introduce sequences for an epitope tag into a gene. However, for the introduced DNA to be delivered to the desired location, it must contain regions of identity with sequences flanking that location. Thus, using a sequenced strain like ES114 facilitates rational design of DNA constructs. Furthermore, to obtain the correct mutant strain from a mixture that includes the unmutated parent, the DNA must include a selectable marker, such as an antibiotic resistance cassette (*i.e.*, contain a promoter, ribosome binding site, and the gene encoding antibiotic resistance). A set of antibiotic resistance cassettes for use in *V. fischeri* can be found in (Visick, Hodge-Hanson, Tischler, Bennett, & Mastrodomenico, 2018). Once an antibiotic-resistant colony is obtained, it should be checked for the successful recombination at the site of interest. This is often confirmed by PCR using primers that are complementary to sequences that flank the site of recombination.

Until recently, if multiple genes were to be sequentially mutated, the maximum number of mutations was limited by the number of antibiotic cassettes. Now, the Flp-FRT system originally from *Saccharomyces cerevisiae* has been adapted for use in *V. fischeri* and can permit elimination (flipping) of antibiotic resistance cassettes that are flanked by two 34-bp FRT sites (5'-*gaagttcctattctctagaaagtataggaacttc*-3') (Datsenko & Wanner, 2000; Hoang, Karkhoff-Schweizer, Kutchma, & Schweizer, 1998; Schlake & Bode, 1994; Visick et al., 2018). While this method has caveats, mutations initially marked by the same antibiotic resistance cassette can be combined by eliminating one cassette in a strain and then transforming the now antibiotic-sensitive mutant with DNA containing the second (marked) mutation.

Additionally, *V. fischeri* has been used for "forward genetic" screens to identify genes required for phenotypes like luminescence and biofilm formation (Lyell, Dunn, Bose, & Stabb, 2010; Singh, Brooks, Ray, Mandel, & Visick, 2015). Specifically, transposon mutagenesis can be used to rapidly identify mutations in genes that are important for a phenotype of interest. There are many different transposons that can be used, but the chosen transposon is dictated by the experimental question being posed. For example, certain transposons will simply interrupt a gene and/or its regulatory region, while other transposons will introduce a promoter to drive expression of a nearby gene or contain a reporter gene such as green fluorescent protein (GFP) that permits analysis of specific properties such as transcription of a gene or localization of the resulting fusion protein. Both Tn*5* and mariner transposons have been used successfully in *V. fischeri* (Brooks et al., 2014; Lyell, Dunn, Bose, Vescovi, & Stabb, 2008). Many derivatives of the useful Tn*5* transposon have been generated for different purposes (Ondrey & Visick, 2014; Stoudenmire, Black, Fidopiastis, & Stabb, 2019). In addition, the mariner transposon has been developed for use with INSeq methodology, an approach that combines mutagenesis with sequencing to permit large scale evaluation of the phenotypes of interest (Brooks et al., 2014; Goodman, Wu, & Gordon, 2011).

Plasmids can be used to provide additional genetic information to the cell, either stably or transiently, and/or to insert or remove genetic material from the genome. There are three broad classes of useful plasmids (Table 1): (1) a stable plasmid, (2) an unstable plasmid that is transiently maintained as a replicating plasmid via selective pressure with an antibiotic in the medium, and (3) a "suicide" plasmid that cannot replicate in *V. fischeri* but can integrate into the genome if homologous sequences are present and/or can deliver genetic information to the genome. Replicating plasmids can have many uses, such as complementation, overexpression of a gene, or introducing reporters like GFP or RFP to distinguish different strains. Plasmids can also have transient uses in strain construction. For example, a plasmid carrying the gene for TfoX can induce competence, but once the strain is made, the plasmid can be lost by growing the strain without selective pressure (*e.g.*, in the absence of antibiotics). Finally, a suicide plasmid can facilitate genetic manipulation of the genome via integration into the chromosome or, for example, serving as a delivery vehicle for a transposon.

BASIC PROTOCOL 1: TRANSFORMATION OF *V. fischeri* WITH LINEAR DNA

V. fischeri is naturally competent, which means that it can take up linear DNA from the environment (Fig. 1). The bacterium can also perform homologous recombination to introduce the exogenous DNA into its genome. The source of the DNA is typically sheared chromosomal DNA or PCR-amplified DNA that can introduce novel sequences into *V. fischeri* or cause the deletion or modification of native sequences. The DNA should include sequences homologous to a region of the *V. fischeri* genome (see Critical Parameters below). To select for the mutant of interest after transformation, the exogenous DNA to be introduced must also contain a selectable marker, such as an antibiotic resistance gene. A generalizable approach and accompanying set of tools has been developed to rapidly generate mutants in a uniform manner (Visick et al., 2018). These tools include a strain engineered to facilitate insertion into an inert site in the chromosome for purposes such as complementation, as well as a plasmid set containing FRT-flanked antibiotic resistance cassettes, antibiotic resistance-linked promoters to drive gene expression, and epitope tags, all with convenient linkers to facilitate splicing by overlap extension (SOE) PCR (Visick et al., 2018).

Regardless of the DNA source, transformation allows different alleles to be introduced into *V. fischeri* to permit genetic comparisons. *V. fischeri* encodes machinery for competence, but in the laboratory, the competence pathway must be induced. Based on findings in *V. cholerae*, this is commonly accomplished using plasmids that overexpress the competence regulator TfoX (see Basic Protocol 2), which induces the production of proteins that facilitate the uptake and recombination of exogenous DNA (Meibom, Blokesch, Dolganov, Wu, & Schoolnik, 2005; Pollack-Berti et al., 2010). Alternatively, competence can be induced by chitohexose, which naturally induces production of TfoX (Pollack-Berti et al., 2010). In either case, competence is achieved when the cells (TfoX-overexpressing or chitohexose-exposed) are grown in a defined minimal medium. The following protocol describes the steps involved in inducing competence and recombination using a TfoX-overexpressing strain (Brooks et al., 2014; Pollack-Berti et al., 2010).

Materials

V. fischeri (*e.g.*, ES114 [ATCC 700601]) with competence plasmid on plasmid-selective LBS agar plate or frozen stock

TMM (*made fresh prior to use*, see recipe in Reagents and Solutions)

LBS broth (see recipe in Reagents and Solutions)

LBS agar plate containing selective antibiotic (LBS-antibiotic) (see recipe in Reagents and Solutions)

PCR DNA (~1000 ng) or genomic DNA (~500 ng) containing a selectable marker

Pipettes and sterile tips

Sterile applicator sticks

Sterile capped glass culture tubes (routinely 18 × 150 mm)

Sterile 125 mL baffled flask

28°C shaking incubator

24°C shaking incubator

2 mL microcentrifuge tube

Glass rod or L-shaped plate spreader

28°C incubator

1a. *From agar plate*: Pick a single colony of *V. fischeri* carrying competence plasmid with an applicator stick.

This plate should contain antibiotic to select for the competence plasmid.

This strain can be made by conjugating either plosTfoX or plosTfoX-kan into the *V. fischeri* recipient (Basic protocol 2).

1b. *From frozen stock*: Scrape some *V. fischeri* cells carrying the competence plasmid from the frozen culture stock with an applicator stick.

2. Inoculate 5 mL of TMM with plasmid-selective antibiotic in a sterile 18 × 150 mm, capped glass culture tube by suspending the cells from the stick. Remove the stick and cap the test tube.

3. Grow the culture overnight at 28°C with aeration by shaking at 220 rpm.

A shorter overnight (i.e., 12–14 hours) will greatly enhance recovery of the cells.

4. Subculture a volume of overnight culture to achieve a final OD_{600} of 0.05 into a 125 mL baffled flask containing 20 mL of TMM with antibiotic to maintain plasmid.

For example, if the overnight culture achieves an OD_{600} of 1, dilute 1000 μL of overnight culture into the 20 mL medium.

5. Grow the culture at 24°C with aeration by shaking at 220 rpm. After 4–5 hours, assess the optical density of the culture with a goal of reaching an OD_{600} of about 0.5.

If the culture is below an OD_{600} of 0.5 after 5 hours, check the optical density hourly. If the desired OD_{600} is not achieved within 7–8 hours, the experiment should be abandoned and restarted on a subsequent day, as the poorly growing cells are unlikely to become competent.

A higher OD_{600} enhances probability of obtaining transformants. After the OD_{600} reaches between 0.8 – 1, the efficiency of transformation may decrease.

6. Once the cells reach the appropriate density, transfer 500 μL of culture to a 2 mL microcentrifuge tube in duplicate. To one tube, add 5–10 μL of linear DNA and vortex briefly. To the other tube, do not add DNA; this will serve as the negative control.

5 μL of a chromosomal DNA stock (100 ng/μL) is typically sufficient.

Volumes of linear DNA up to 50 μL have yielded transformants.

There is generally a positive correlation between the amount of DNA added and transformants yielded.

7. Incubate the culture-DNA mixture and negative control tube at room temperature (~22°C) for 30’ without agitation.

During this time, the cells will take up DNA from their environment.

8. After 30’, add 500 μL LBS medium to each tube. Mix by pipetting and transfer each suspension to a sterile 18 × 150 mm glass tube.

9. Recover the cells by shaking the tubes at 28°C for 90’.

During this time, the cells will incorporate the DNA into their genome. If the DNA contained an antibiotic resistance cassette, the protein conferring resistance will be expressed.

10. With a glass rod or L-shaped spreader, spread 100–200 μL of recovered cells on an LBS-antibiotic plate. Do the same for the negative control. Incubate the plates inverted overnight at 28°C.

Let the culture tubes with remaining transformation reactions sit overnight on the benchtop. If no colonies develop, these cultures can be used in an additional round of plating the next day.

Depending on the efficiency of transformation and/or the quality/quantity of DNA, a larger volume of culture may need to be plated to obtain transformants.

11. Assess the plate for the growth of successful transformants that have acquired resistance. Re-streak 4 representative colonies on an LBS-antibiotic plate and incubate overnight at 28°C.

Spontaneous mutants will arise in the presence of certain antibiotics like trimethoprim. Thus, it is crucial to have a negative control for comparison.

12. Once colonies arise, confirm introduction of DNA into *V. fischeri* genome.

Perform a PCR reaction using primers that amplify across the expected region of insertion/deletion. Compare the transformants to the parent strain to observe a shift in size via DNA gel electrophoresis.

BASIC PROTOCOL 2: PLASMID TRANSFER INTO *V. fischeri* VIA CONJUGATION

Conjugation allows the transfer of DNA directly from one bacterium to another. It can be used to introduce stable or unstable plasmids, or it can facilitate delivery of DNA specifically or randomly into the genome (*e.g.*, transposon mutagenesis) by introducing a suicide plasmid. In its simplest form, a biparental mating, a plasmid is transferred into *V. fischeri* from an *E. coli* encoding conjugation machinery in its genome (*e.g.*, strain S17–1). However, it can also occur in a stepwise progression between multiple strains (Fig. 2). This protocol describes a triparental mating, where two *E. coli* strains are used to introduce a plasmid into a *V. fischeri* recipient. The donor *E. coli* harbors a plasmid of interest that is to be moved into *V. fischeri*, while the helper *E. coli* carries a plasmid that (*e.g.*, pEVS104) contains genes necessary to make a conjugative pilus (E. V. Stabb & Ruby, 2002). The conjugative plasmid is transferred from the helper strain into the donor strain, and then the donor strain can transfer the plasmid of interest into *V. fischeri*. The advantage of a triparental mating is that the donor plasmid can be present in any desired *E. coli* strain; no specific genotype is required.

For any conjugation approach, it is necessary to select for the recipient that has acquired the plasmid of interest (the transconjugant), select against the plasmid-less *V. fischeri* cells, and to counterselect against the helper and/or donor *E. coli*. Any commercial *E. coli* can be transformed with the desired plasmid (donor *E. coli*) or helper plasmid (helper *E. coli*). However, to facilitate counterselection against the helper and/or donor *E. coli*, *E. coli* auxotrophs can be used that require a supplement to grow, such as diaminopimelic acid (DAP) or thymidine (Table 2). Furthermore, incubation on media containing antibiotics, which will prevent growth of the plasmid-less *V. fischeri* recipient, is carried out at the optimal *V. fischeri* temperature of 28°C or at room temperature to reduce *E. coli* growth.

Materials

Recipient *V. fischeri* grown on LBS agar plate or frozen stock

Donor *E. coli* strain on LB agar plate with appropriate supplements or frozen stock

Helper *E. coli* strain on LB agar plate with appropriate supplements or frozen stock

LB broth (see recipe in Reagents and Solutions)

LBS broth (see recipe in Reagents and Solutions)

LBS agar plate (see recipe in Reagents and Solutions)

LBS agar plate containing plasmid-selective antibiotic (LBS-antibiotic$_{plasmid}$) (see recipe in Reagents and Solutions)

Pipettes and sterile tips

Sterile applicator sticks

Sterile capped glass culture tubes (routinely 18 × 150 mm)

1.5 mL microcentrifuge tubes, sterile

Microcentrifuge

Glass rod or L-shaped plate spreader

28°C shaking incubator

28°C incubator

37°C incubator or warm room with orbital shaker

1a. *From agar plate*: Individually pick a single colony of *V. fischeri*, a single colony of helper *E. coli,* and a single colony of donor *E. coli* using sterile applicator sticks.

1b. *From frozen stock*: Scrape some *V. fischeri* cells from the frozen culture stock with an applicator stick. Do the same for the donor *E. coli* and helper *E. coli* strains.

Using frozen or plate-grown bacteria will not significantly impact this procedure.

2. Inoculate *V. fischeri* in 5 mL of LBS in a sterile 18 × 150 mm, capped glass culture tube by suspending the cells from the stick. Remove the stick and cap the test tube.

Individually inoculate donor *E. coli* in 5 mL of LB with appropriate supplements and helper *E. coli* in 5 mL of LB with appropriate supplements in sterile 18 × 150 mm, capped glass culture tubes by suspending the cells from the stick. Remove the sticks and cap the test tubes.

Appropriate supplements for the *E. coli* include any nutrients needed for auxotroph growth (*e.g.*, thymidine or DAP) and antibiotics to maintain plasmids.

3. Grow the *V. fischeri* culture overnight at 28°C with aeration by shaking at 220 rpm.

4. Grow the *E. coli* cultures overnight at 37°C with aeration by shaking at 220 rpm.

5. Subculture 50 μL of *V. fischeri* overnight culture into 5 mL LBS. Grow the culture at 28°C with aeration by shaking at 220 rpm.

6. Individually subculture 100 μL of the donor *E. coli* in 5 mL LB with appropriate supplements and 100 μL of the helper *E. coli* in 5 mL LB with appropriate supplements. Grow the cultures at 37°C with aeration by shaking at 220 rpm.

7. After approximately 3 hours, combine 1 mL *V. fischeri* recipient culture and 250 μL of each *E. coli* culture in a 1.5 mL microcentrifuge tube. For a donor-only control, add 250 μL of donor culture alone to a 1.5 mL microcentrifuge tube. For a recipient-only negative control, add 1 mL of recipient culture alone to a 1.5 mL microcentrifuge tube.

At the indicated time point, the three cultures should have achieved substantial turbidity, with an OD_{600} of about 1, although the exact density is generally unimportant as long as the culture is healthy. Less dense cultures, resulting from slower growing cultures or shorter incubation periods, can be used to achieve successful conjugations. The key for success is obtaining healthy cultures in the exponential phase of growth. Cells from less dense cultures can be concentrated using a microcentrifuge. The resulting cell pellet can be resuspended in half of the volume or less to achieve a final cell density similar to a more turbid culture, then combined with other cell types as described above.

8. Centrifuge for 2’ at 13,500 rpm at room temperature to pellet the cells. Decant or pipette off the supernatant, leaving around 10–20 μL of fluid with the cell pellets.

Concentrating the cells brings them into close proximity, permitting conjugation to occur.

Make sure the centrifuge is balanced by including microfuge tubes with equivalent volumes of water as appropriate.

9. Use a pipette to gently resuspend the cells in the remaining liquid and spot the cell suspension on a pre-warmed LBS plate. Repeat for the donor-only and recipient-only controls.

10. Let the liquid absorb into the plate. Then, place the plate in a 28°C incubator for 3 hours or overnight. The cells will grow within the spots of both the conjugation and controls.

Longer incubation will increase the probability of conjugation, though usually 3 hours is sufficient for the transfer of a stable plasmid.

11. Use a pipette tip or sterile stick to scrape half of the colony and streak those cells onto an LBS-antibiotic$_{plasmid}$ plate to select for the plasmid-containing recipient. Repeat for the donor-only and recipient-only colonies.

This approach is used primarily when all the resulting recipients will be essentially clonal, such as when introducing a replication-competent (stable or unstable) plasmid into *V. fischeri*. The streaking readily separates *V. fischeri* from the donor and helper *E. coli* strains, permitting a rapid isolation of the desired recipient, with the first isolation step occurring within hours of mixing the strains. The *V. fischeri* colonies will be initially larger than the *E. coli* colonies; with prolonged growth, the two will be of a similar size but may be distinguished by colony color, as *V. fischeri* produces colonies with a yellowish color. The identity of the resulting colonies may also be confirmed by assessing growth at 37 degrees, a temperature that is non-permissive for *V. fischeri* growth.

The spot plate from step 10 may be left at room temperature or stored in the refrigerator overnight. Then, if no colonies have arisen on the streak plate, the spot plate can be incubated longer and/or step 11 can be repeated.

12. Place the plate inverted in a 28°C incubator or at room temperature overnight. Colonies should arise within 24 hours.

These temperatures are suboptimal for the growth of the *E. coli*, whose growth optimum is 37°C, resulting in decreased *E. coli* growth and an enrichment for *V. fischeri*.

If no colonies arise, leave the plates for 48 hours. Be aware, however, that this prolonged incubation will also result in increased growth of *E. coli*.

13. Restreak any colonies from the LBS-antibiotic$_{plasmid}$ plate onto a new LBS-antibiotic$_{plasmid}$ plate to purify the *V. fischeri* away from any *E. coli* growth. Incubate the plate inverted in a 28°C incubator overnight.

14. Place the plate in the refrigerator or, to prepare a frozen glycerol stock, pick a single colony from the plate with a sterile stick and inoculate a tube containing 5 mL LBS with selective antibiotic.

SUPPORT PROTOCOL 1: REMOVING FRT-FLANKED ANTIBIOTIC RESISTANCE CASSETTES FROM THE *V. fischeri* GENOME

For certain experiments, multiple mutations must be introduced into the same strain. In some cases, each mutation may be marked by a different antibiotic resistance cassette. However, if two mutations have been independently made with the same resistance marker, removal of one resistance cassette would allow introduction of the other. By flanking antibiotic resistance cassettes with flippase recognition target (FRT) sites, the resistance markers can be eliminated (flipped) by a flippase enzyme to prepare the strain for new mutations (Datsenko & Wanner, 2000; Hoang et al., 1998; Schlake & Bode, 1994; Visick et al., 2018). Flipping antibiotic resistance cassettes also reduces exogenous DNA present in the genome that may have unforeseen consequences (such as, for example, impairing transcription of neighboring genes). This protocol requires conjugation of the flippase-encoding plasmid into *V. fischeri* and multiple restreak steps to ensure the loss of the antibiotic resistance cassette.

Additional Materials (see also Basic Protocol 2)

V. fischeri with antibiotic resistance cassette flanked by FRT sites on LBS agar plate

Donor *E. coli* carrying a plasmid encoding a flippase (*e.g.,* pKV496) on LB agar plate with appropriate supplements or frozen stock

LBS-antibiotic$_{cassette}$ plate (see recipe in Reagents and Solutions)

1. As described in Basic Protocol 2, perform a conjugation between *V. fischeri* carrying an antibiotic resistance cassette to be flipped, a helper *E. coli*, and a donor *E. coli* carrying a plasmid encoding a flippase.
2. Using a sterile stick, pick a single colony of the *V. fischeri* strain carrying the flippase plasmid.
3. Restreak the cells on an LBS-antibiotic$_{plasmid}$ plate and incubate overnight at 28°C.

   During this time, the flippase will be expressed and will eliminate the antibiotic resistance cassette. The *V. fischeri* should become sensitive to that antibiotic.
4. Pick a colony from the restreak plate using a sterile stick. Make a small patch on an LBS-antibiotic$_{cassette}$ plate. With the same stick, make another patch on an LBS plate. Pick at least eight of the transconjugants in the same manner. Positive (antibiotic-resistant) and negative (antibiotic-sensitive) controls should also be patched onto the plate.

   Patching on the plate with antibiotic first helps ensure that sufficient cells are applied, decreasing the chance of a false negative result. The second patch onto antibiotic-free medium both verifies that there were sufficient cells and permits capture of the desired cells, which cannot grow in the presence of antibiotics.

The plasmid encoding the flippase may be lost at this step, because the plates lack selection for it.

5. Clones with successfully eliminated antibiotic resistance cassettes will fail to grow on the LBS-antibiotic$_{cassette}$ plate.

   If all of the patches retain the resistance marker, the starting strain may be restreaked on LBS-antibiotic$_{plasmid}$ and step 4 repeated.

   The final strain may still include the flippase plasmid, which is undesirable. See Support Protocol 2 for how to eliminate this unstable plasmid.

SUPPORT PROTOCOL 2: ELIMINATING UNSTABLE PLASMIDS FROM *V. fischeri*

Unstable plasmids are useful for inducing a transient effect such as transformation or cassette removal (Support Protocol 1). Subsequent to achieving those goals, elimination of the plasmid is often necessary to obtain an isogenic strain or subsequently introduce a plasmid or mutation that confers the same resistance. The plasmids used for those procedures were chosen for these approaches because they are relatively unstable and can be passively lost by removing selection for the plasmids. However, this same approach can be used for any unstable plasmid.

Materials

V. fischeri carrying unstable plasmid on LBS agar plate or from frozen stock

LBS plate (see recipe in Reagents and Solutions)

LBS-antibiotic$_{plasmid}$ plate (see recipe in Reagents and Solutions)

Sterile applicator sticks

28°C incubator

1. With a sterile stick, either pick a colony of *V. fischeri* or scrape some *V. fischeri* cells from a frozen stock.
2. Use the stick to apply cells onto an LBS agar plate, drawing a short streak across the edge of the plate. With a fresh stick, drag through the streak and draw additional streaks across one quadrant of the plate. Repeat this once more to isolate single colonies.

   These streaks dilute the cells such that in some areas of the plate, single cells are deposited and grow into isolated individual colonies.
3. Incubate the plate overnight in a 28°C incubator.

   The cells should lose the plasmid when grown without selection.
4. Patch the *V. fischeri* onto an LBS-antibiotic$_{plasmid}$ plate. With the same stick, make a small streak on a corresponding LBS plate and repeat step 2 to

isolate single colonies. Patch at least four to eight colonies on these same plates.

5. Incubate the plates overnight in a 28°C incubator.

6. If the cells are sensitive to the antibiotic to which the plasmid confers resistance, the cells should grow only on the LBS plate.

   Usually, performing these steps will be sufficient to obtain sensitive colonies. However, if the cells are still resistant to the antibiotic for which the plasmid encodes resistance, repeat steps 2 through 5 from the LBS plate one or two more times until sensitivity is achieved.

ALTERNATE PROTOCOL 1: INTRODUCTION OF EXOGENOUS DNA VIA SUICIDE PLASMID

DNA can be inserted into and/or deleted from the genome of *V. fischeri* via three methods. One method involves introducing exogenous, linear DNA into the cells via transformation (Basic Protocol 1). That DNA contains a selectable marker to facilitate selection of the desired DNA flanked by sequences homologous to the chromosome, which through a double recombination event, is then maintained in the resulting mutant strain (Fig. 1). The second method is the introduction of a transposon into the bacterial genome via a suicide plasmid that cannot replicate in *V. fischeri* (see Alternate Protocol 2 for site-specific insertional mutagenesis; Alternate Protocol 3 for random mutagenesis). Only those cells that acquire the transposon, not the plasmid, will stably express antibiotic resistance. The third method, described below, integrates an entire suicide plasmid vector into the genome via a single recombination event. Since the suicide plasmid cannot replicate in *V. fischeri,* the cell will only become resistant to the antibiotic conferred by the plasmid if it recombines into the genome via homologous DNA sequences encoded within the plasmid.

Once an unstable/suicide plasmid is integrated into the genome, there are two possible outcomes (Fig. 3): the plasmid can either (1) be semi-stably maintained in the genome, either interrupting a gene of interest (an approach termed Campbell mutagenesis) or inserting a sequence of interest (such as an epitope tag), or (2) recombine back out and subsequently be lost from the cell. For the latter possibility, this recombination event can result in regeneration of the parental genotype or can leave behind a desired insertion, deletion, or modification (Gene/Allelic replacement). Once a suicide plasmid is integrated into the *V. fischeri* genome, it can be relatively stable, a phenomenon that permits the reliable application of Campbell mutagenesis. Thus, to promote the removal of an integrated plasmid to facilitate gene replacement, suicide plasmids with an inducible toxin have been developed, such as the one described here that contains a *ccdB* toxin gene controlled by an arabinose-inducible promoter (Le Roux, Binesse, Saulnier, & Mazel, 2007). Subsequent induction of the toxin (*e.g.*, with arabinose addition) in the strain with the integrated plasmid will enrich for those relatively rare cells that have undergone the second recombination event (Fig. 3). While this method has the downside of being more laborious, it has the advantage of permitting the introduction or deletion of DNA without the retention of any foreign/extraneous DNA. The procedure below will describe the complete integration and excision

of the plasmid encoding the CcdB toxin. If using a plasmid without *ccdB* (for example, to accomplish Campbell mutagenesis), the glucose can be omitted from the medium where indicated and the procedure can be abbreviated as noted.

Additional Materials (see also Basic Protocol 2)

LBS agar plates containing selective antibiotic and 1% glucose (LBS-antibiotic-gl) (see recipe in Reagents and Solutions)

LBS agar plates containing selective antibiotic (LBS-antibiotic)

LBS agar plates containing 0.2% arabinose

Donor *E. coli* strain carrying suicide plasmid (see Table 1 for possible plasmids)

1. As described in Basic Protocol 2, perform a conjugation up to Step 10 between the *V. fischeri* recipient strain, helper *E. coli* strain, and donor *E. coli* containing the suicide plasmid carrying DNA of interest.
2. Let the liquid absorb into the plate. Then, place the plate inverted in a 28°C incubator and incubate overnight.
3. Scrape the colony off of the agar using a pipette tip or applicator, and suspend the colony in a 1.5 mL-microcentrifuge tube containing 1 mL LBS by vortexing.
4. Pipette and spread 100–200 μL of the cell suspension onto an LBS-antibiotic-gl plate.

   Glucose will repress transcription of the *ccdB* toxin gene and, therefore, must be included in growth media after the conjugation event to maintain viability if using a plasmid carrying the *ccdB* toxin gene. Omit glucose if using a plasmid that does not encode the CcdB toxin.
5. Incubate at room temperature for one to two days until colonies appear.
6. Choose eight colonies and re-streak each of them for single colonies on LBS-antibiotic-gl agar. Incubate the plates at 28°C overnight.

   The colonies that arise have either taken up the plasmid transiently or taken up and integrated the plasmid into the genome.

   Omit glucose if using a plasmid that does not encode the CcdB toxin.
7. Streak a single colony of each of the eight isolates on LBS-gl agar. Incubate the plates at 28°C overnight.

   Strains that have not integrated the plasmid into their genome will lose the plasmid.

   Omit glucose if using a plasmid that does not encode the CcdB toxin.
8. Verify the plasmid is integrated by patching the colonies on LBS-antibiotic-gl. Incubate the plates at 28°C overnight.

   Omit glucose if using a plasmid that does not encode the CcdB toxin.

Include positive (resistant to the antibiotic) and negative (sensitive to the antibiotic) controls on the plate.

9. For the patches that retain antibiotic resistance, use a sterile stick to inoculate from that patch into 5 mL LBS broth containing 0.2% arabinose. Repeat for each resistant patch. Grow the cultures at 28°C for 4 hours.

Cells that retain the integrated plasmid will express the *ccdB* toxin gene and be killed.

If introducing the plasmid is sufficient (such as for Campbell mutagenesis), proceed to step 13 instead of inoculating LBS broth containing arabinose.

If all of the patches are sensitive to the antibiotic, repeat from step 6.

10. Dilute the cultures 1:100 in LBS in a final volume of 1 mL. Plate 100 μL of the dilution onto LBS agar containing 0.2% arabinose, and incubate the plate overnight at 28°C.

11. Once colonies have arisen, restreak onto LBS agar containing 0.2% arabinose. Incubate the plate overnight at 28°C.

Often, the larger colonies correspond to isolates that have lost the plasmid.

12. Pick single colonies. Using the same stick, make patches on LBS-antibiotic and LBS agar. Incubate the plates at 28°C overnight.

Test up to 50 or 100 colonies to identify antibiotic-sensitive colonies that have lost the integrated plasmid.

13. Once antibiotic-sensitive colonies have arisen (or antibiotic-resistant colonies for Campbell mutagenesis), confirm the presence of the mutation by performing a colony PCR using primers to distinguish the mutant DNA region from wild type.

In the event that all of the resulting colonies contain the wild-type sequence, additional colonies can be evaluated. Alternatively, or in addition, the protocol can be repeated from the beginning or from saved intermediates obtained at the beginning of step 9 (strains with stably integrated plasmid).

ALTERNATE PROTOCOL 2: SITE-SPECIFIC TRANSPOSON INSERTION USING A SUICIDE PLASMID

As mentioned above, a transposon can be introduced into *V. fischeri* on a suicide plasmid via conjugation. Since the plasmid cannot replicate, only the cells that acquire the transposon will obtain the antibiotic resistance conferred by the transposon. The site of integration can be specific (*e.g.*, Tn*7*), or the transposon can distribute more randomly across the genome (see Alternate Protocol 3) (Bao, Lies, Fu, & Roberts, 1991; McCann, Stabb, Millikan, & Ruby, 2003). The use of a site-specific transposon like Tn*7* permits the reliable insertion of sequences (*e.g.*, the introduction of a gene for the purposes of complementation) at a known, benign site. Transposition enzymes are required to catalyze the transfer of the transposon

from the suicide plasmid into the host genome. These transposition genes can be encoded on the same plasmid as the transposon or on a different plasmid. For the site-specific Tn*7* transposon, the transposase is encoded on a separate plasmid, thus necessitating the inclusion of an additional strain in the conjugation procedure. In the following example, we will describe a tetraparental mating in which a *V. fischeri* recipient cell is mated with a helper *E. coli* and two donor *E. coli* where one donor *E. coli* contains a plasmid with the site-specific transposon of interest and the other donor contains a plasmid with the transposition genes.

Additional Materials (see also Basic Protocol 2)

Donor *E. coli* strain carrying suicide plasmid containing the transposon (see Table 1) on LB agar plate containing appropriate supplements or from frozen stock

Donor *E. coli* strain carrying suicide plasmid containing transposition genes (pUX-BF13) on LB agar plate containing appropriate supplements or from frozen stock

LBS agar plate containing transposon-selective antibiotic (LBS-antibiotic$_{transposon}$) (see recipe in Reagents and Solutions)

LBS agar plate containing transposon vector-selective antibiotic (LBS-antibiotic$_{vector}$)

1a. *From agar plate*: Individually pick a single colony of *V. fischeri*, a single colony of helper *E. coli,* and a single colony of each donor *E. coli* using sterile applicator sticks.

1b. *From frozen stock*: Scrape some *V. fischeri* cells from the frozen culture stock with an applicator stick. Do the same for the donor *E. coli* strains and helper *E. coli* strain.

Using frozen or plate-grown bacteria will not significantly impact this procedure.

2. Inoculate *V. fischeri* in 5 mL of LBS in a sterile 18 × 150-mm, capped glass culture tube by suspending the cells from the stick. Remove the stick and cap the test tube.

Individually inoculate each donor and helper *E. coli* in 5 mL of LB with appropriate supplements in sterile 18 × 150 mm, capped glass culture tubes by suspending the cells from the stick. Remove the sticks and cap the test tubes.

Appropriate supplements for the *E. coli* include any nutrients needed for auxotroph growth (*e.g.*, thymidine or DAP) and antibiotics to maintain plasmids.

3. Grow the *V. fischeri* culture overnight at 28°C with aeration by shaking at 220 rpm.

4. Grow the *E. coli* cultures overnight at 37°C with aeration by shaking at 220 rpm.

5. Subculture 50 μL of *V. fischeri* overnight culture into 5 mL LBS. Grow the culture at 28°C with aeration by shaking at 220 rpm.

   Individually subculture 100 μL of each donor and helper *E. coli* in 5 mL LB with appropriate supplements. Grow the cultures at 37°C with aeration by shaking at 220 rpm.

6. After approximately 3 hours, combine the *V. fischeri*, helper *E. coli*, and both donor *E. coli* in a 1:1:1:1 ratio in a 1.5 mL centrifuge tube (*e.g.*, 250 μL of each strain). For a donor-only control, add 250 μL of each donor culture to a 1.5 mL microcentrifuge tube. For a recipient-only negative control, add 250 μL of recipient culture alone to a 1.5 mL microcentrifuge tube.

7. Centrifuge for 2’ at 13,500 rpm at room temperature to pellet the cells. Decant or pipette off the supernatant, leaving around 10–20 μL of fluid with the cell pellets.

   Concentrating the cells brings them into close proximity, permitting conjugation to occur.

   Make sure the centrifuge is balanced by including microfuge tubes with equivalent volumes of water as appropriate.

8. Use a pipette to gently resuspend the cells in the remaining liquid and spot the cell suspension on a pre-warmed LBS plate. Repeat for the donor-only and recipient-only controls.

9. Let the liquid absorb into the plate. Then, place the plate inverted in a 28°C incubator and incubate overnight.

   During this time, the transposon should integrate into the genome of *V. fischeri* cells that have taken up both the plasmid containing the transposon and plasmid containing transposition genes.

10. Scrape the colony off of the agar using a pipette tip or applicator stick, and suspend the colony in a 1.5 mL-microcentrifuge tube containing 1 mL LBS by vortexing.

11. Pipette and spread 100–200 μL of the cell suspension on LBS-antibiotic$_{transposon}$ agar.

12. Incubate at room temperature for one to two days until colonies appear.

13. Choose four to eight colonies and restreak each of them for single colonies on LBS-antibiotic$_{transposon}$ agar. Incubate the plates at 28°C overnight.

    The colonies that arise have either taken up the plasmid transiently or taken up and integrated the transposon into the genome.

In some cases, vector sequences flanking the Tn7 transposon are inserted into the genome along with the transposon. The correct strain that has incorporated only the transposon can be identified amongst the isolates by subsequent evaluation of antibiotic resistance as described below.

14. Restreak a single colony of each of the eight isolates on LBS agar. Incubate the plates at 28°C overnight.

Strains that have not integrated the plasmid into their genome will lose the plasmid.

15. Verify the transposon has integrated into the genome by patching the colonies on LBS- antibiotic$_{vector}$ agar and LBS-antibiotic$_{transposon}$ agar. Incubate the plates at 28°C overnight.

For introduction of Tn7 from delivery plasmid pEVS107, insertion of Tn7 only (and not also the delivery plasmid) would be suggested by the isolation of erythromycin resistant, kanamycin sensitive colonies.

16. Once colonies have arisen that are resistant to the antibiotic encoded by the transposon and sensitive to the antibiotic encoded by the delivery vector, confirm the presence of the insertion by performing a colony PCR using primers to distinguish the insertion from wild type sequences.

ALTERNATE PROTOCOL 3: RANDOM TRANSPOSON MUTAGENESIS VIA SUICIDE PLASMID

Transposon mutagenesis is a powerful tool to simultaneously identify multiple genes involved in a phenotype in an unbiased way. If using a transposon such as Tn*5* or mariner, the transposon will insert across the genome in an almost random fashion (Brooks et al., 2014; Lyell et al., 2008). The transposon can be introduced into *V. fischeri* on a suicide plasmid via conjugation in a triparental mating, and as such, the protocol does not vary significantly from that described in Basic Protocol 2. In the case of random transposon mutagenesis, the transposition genes are typically carried on the same plasmid as the transposon, generally within the vector portion such that the transposase gene is not incorporated into the genome, preventing further transposition. It is typically necessary to perform multiple independent conjugations in parallel that are plated on multiple plates to obtain a large number of mutants with insertions in different genes. This approach increases the coverage and depth of transposon mutagenesis.

Additional Materials

Donor *E. coli* strain carrying suicide plasmid containing transposon and transposition genes on LB agar plate containing appropriate supplements or from frozen stock

LBS agar plate containing transposon-selective antibiotic (LBS-antibiotic$_{transposon}$) (see recipe in Reagents and Solutions)

1a. *From agar plate*: Individually pick a single colony of *V. fischeri*, a single colony of helper *E. coli,* and a single colony of donor *E. coli* using sterile applicator sticks.

1b. *From frozen stock*: Scrape some *V. fischeri* cells from the frozen culture stock with an applicator stick. Do the same for the donor *E. coli* and helper *E. coli* strains.

Using frozen or plate-grown bacteria will not significantly impact this procedure.

2. Inoculate *V. fischeri* in 5 mL of LBS in a sterile 18 × 150-mm, capped glass culture tube by suspending the cells from the stick. Remove the stick and cap the test tube.

Individually inoculate donor *E. coli* in 5 mL of LB with appropriate supplements and helper *E. coli* in 5 mL of LB with appropriate supplements in sterile 18 × 150 mm, capped glass culture tubes by suspending the cells from the stick. Remove the sticks and cap the test tubes.

Appropriate supplements for the *E. coli* include any nutrients needed for auxotroph growth (*e.g.*, thymidine or DAP) and antibiotics to maintain plasmids.

3. Grow the *V. fischeri* culture overnight at 28°C with aeration by shaking at 220 rpm. Grow the *E. coli* cultures overnight at 37°C with aeration by shaking at 220 rpm.

4. Subculture 50 μL of *V. fischeri* overnight culture into 5 mL LBS. Grow the culture at 28°C with aeration by shaking at 220 rpm.

5. Individually subculture 100 μL of the donor *E. coli* in 5 mL LB with appropriate supplements and 100 μL of the helper *E. coli* in 5 mL LB with appropriate supplements. Grow the cultures at 37°C with aeration by shaking at 220 rpm.

6. After approximately 3 hours, combine 1 mL *V. fischeri* recipient culture and 250 μL of each *E. coli* culture in a 1.5 mL microcentrifuge tube. Scale up as appropriate to obtain a large set of mutants.

To obtain a saturating mutagenesis with multiple independent insertions within the same gene, it is necessary to carry out step 6 (and subsequent steps) with 10 – 20 sets of samples, depending on desired level of saturation.

7. Centrifuge for 2’ at 13,500 rpm at room temperature to pellet the cells. Decant or pipette off the supernatant, leaving around 10–20 μL of fluid with the cell pellets.

Concentrating the cells brings them into close proximity, permitting conjugation to occur.

8. Use a pipette to gently resuspend the cells in the remaining liquid and spot the cell suspension on a pre-warmed LBS plate.

9. Let the liquid absorb into the plate. Then, place the plate in a 28°C incubator for 8 hours or overnight.

   Incubation for 8 hours produces a good number of transposon mutants without development of too many siblings. The optimal incubation time should be determined empirically.

10. Using a pipette tip or sterile stick, scrape the colony and resuspend in 1 mL LBS. Vortex. Spread a 50 μL aliquot on an LBS-antibiotic$_{transposon}$ plate using a glass rod or L-shaped plate spreader.

    This approach will permit the isolation of large numbers of potentially distinct individual colonies.

    Depending on the efficiency of transposition, it may be necessary to spread a larger volume (up to 200 μL) instead.

11. Place the plate inverted in a 28°C incubator or at room temperature overnight.

12. Quantify the number of colonies obtained.

    An ideal plate should have approximately 500 – 2000 colonies per plate such that the colonies are sufficiently distant from one another.

13. Repeat Steps 1–11 in independent conjugations in parallel until the mutagenesis produces a sufficient number of colonies for the desired application.

    To achieve at least a single transposition event per gene, the number of mutants obtained should minimally equal the number of genes (~4300) in the *V. fischeri* genome, but more is better as the transposon may insert unevenly within the genome. Thus, if 20 plates with 1000 colonies on each are obtained, this would mean each gene is mutated >4 times on average.

REAGENTS AND SOLUTIONS

Arabinose stock, 20% (w/v)

Dissolve 20 g of arabinose in 75 mL of dH_2O. Adjust the volume to 100 mL. Autoclave the mixture. Store at room temperature for up to 3 months.

Artificial seawater stock, 2× (ASW)

197.6 g	$MgSO_4 * 7\ H_2O$
23.2 g	$CaCl_2 * 2\ H_2O$
280.8 g	NaCl
12 g	KCl

Add 4 L dH_2O into an 8 L carboy. Dissolve magnesium sulfate heptahydrate in 1 L dH_2O and add to carboy. Dissolve calcium chloride dihydrate into 1 L dH_2O and add to carboy.

Dissolve sodium chloride and potassium chloride directly into the carboy. Bring the final volume in the carboy to 8 L. Store at room temperature for up to 3 months.

The final concentrations of each salt in this stock are 100 mM $MgSO_4$, 19.7 mM $CaCl_2$, 600 mM NaCl, and 20.1 mM KCl.

Glucose stock, 20% (w/v)

Dissolve 20 g of arabinose in 75 mL of dH_2O. Adjust the volume to 100 mL. Autoclave the mixture. Store at room temperature for up to 3 months.

LB broth

10 g	Tryptone
5 g	Yeast Extract
10 g	NaCl

Add the above reagents and dH_2O to a final volume of 1000 mL in a 2 L flask or beaker and stir to completely dissolve solids. Distribute into clean bottles and autoclave. Store at room temperature for up to 3 months.

LB agar plates

10 g	Tryptone
5 g	Yeast Extract
10 g	NaCl
15 g	Agar

Add the above reagents and dH_2O to a final volume of 1000 mL in a 2 L flask and autoclave the mixture. Allow to cool until the flask can be comfortably held. As desired, add an antibiotic to the cooled medium and mix well. Pour approximately 30 mL into sterile plastic petri dishes and allow agar to solidify. Let the plates sit for one to two days at room temperature until sufficiently dry. Then, store the plates at 4°C for up to 3 months. Antibiotic-containing media may have a shorter shelf-life and may be light sensitive.

LB salt (LBS) broth

10 g	Tryptone
5 g	Yeast Extract
20 g	NaCl

Add the above reagents, 975 mL dH_2O, and 25 mL of 2 M Tris pH 7.5 to a 2 L flask or beaker. Stir the mixture until solids are completely dissolved. Distribute the medium into clean bottles at the desired volume. Autoclave the bottles and store at room temperature for up to 3 months.

Some researchers include glycerol in this recipe to a final concentration of 0.3%. If added, *V. fischeri* will metabolize the glycerol and secrete acid into the medium. With prolonged growth under these conditions, *V. fischeri* will begin to die, and thus glycerol addition should be carefully considered and strain growth carefully monitored.

LB salt (LBS) agar plates

10 g	Tryptone
5 g	Yeast Extract
20 g	NaCl
15 g	Agar

Add the above reagents, 975 mL dH_2O, and 25 mL of 2 M Tris pH 7.5 to a 2 L flask. Autoclave the mixture. Allow to cool until the flask can be comfortably held. As desired, add an antibiotic to the cooled medium and mix well. Pour approximately 30 mL into sterile plastic petri dishes and allow agar to solidify. Let the plates sit for one to two days at room temperature until sufficiently dry. Then, store the plates at 4°C for up to 3 months.

When adding glucose or arabinose to LBS agar plates, allow the 1 L of LBS agar to cool. Then, add 50 mL of 20% (w/v) sterile glucose to a final concentration of 1%, or add 10 mL 20% (w/v) sterile arabinose to a final concentration of 0.2%.

Tris Minimal medium (TMM) (100 ml)

75.3 mL	dH_2O
10 mL	1 M Tris, pH 7.5
6 mL	5 M NaCl
5 mL	1 M $MgSO_4$ heptahydrate
100 µL	33 mM K_2HPO_4
100 µL	10 mM Ferrous Ammonium Sulfate ($(NH_4)_2Fe(SO_4)_2$)
500 µL	20% NH_4Cl
1 mL	20% N-acetylglucosamine
1 mL	1 M KCl
1 mL	1 M $CaCl_2$ dihydrate

Filter sterilize the 1 M Tris stock. Prepare the water and other component stocks in individual bottles and autoclave each. Combine the appropriate volumes of sterile water, Tris, and component reagents. Use the medium immediately, or store the medium at 4°C for up to two days.

The individual reagents can be made up separately, as listed here, so that the concentrations of each component may be adjusted depending on the experimental design. This strategy also prevents precipitation of calcium and phosphate. Alternatively, the sodium chloride, magnesium sulfate, potassium chloride, and calcium chloride solutions can be replaced with 35 ml of 2X ASW stock per 100 mL, with the amount of water adjusted accordingly.

If growth is poor, 1 mL of a 10% casamino acids stock can be added to enhance growth in this medium. However, this may reduce transformability.

The ferrous ammonium sulfate stock will settle to the bottom of the bottle following autoclaving and must be resuspended prior to addition to the TMM mixture.

COMMENTARY

Background Information

V. fischeri is a Gram-negative symbiont of the Hawaiian bobtail squid, *E. scolopes* (McFall-Ngai, 2014; Eric V. Stabb & Visick, 2013). In the laboratory, *V. fischeri* has been studied with respect to its phenotypes that impact this symbiosis including, but not limited to, biofilm formation, motility, and luminescence (see (Christensen & Visick, 2020) for procedures to perform these assays). Identification of the genetic determinants that shape this symbiosis has been greatly enhanced by the expansion of the genetic toolbox for this organism. These techniques have allowed researchers to generate transposon mutant libraries of *V. fischeri*, to delete DNA from the genome, and to introduce novel genetic material either into the genome directly or into the cells on a plasmid.

The genetic toolbox for *V. fischeri* includes transformation and conjugation, which together can produce many different strain derivatives. Gene replacements can introduce allelic variants or completely delete a gene. Insertion of new DNA into the genome can introduce promoters, add an additional copy of a gene, complement a gene, or introduce entirely novel DNA. Depending on the experimental question, each of these derivatives may provide unique and complementary conclusions about the relevance of the segment of DNA being studied.

Recently, the process of making mutations in *V. fischeri* was accelerated by the development of a set of helpful plasmids and strains that, with SOE PCR and subsequent transformation, can generate almost any type of mutant (Visick et al., 2018). A family of plasmids containing FRT-flanked antibiotic resistance cassettes can be used to delete a gene of interest or introduce linked DNA anywhere in the chromosome. A strain engineered to facilitate insertion of DNA at an inert site in the genome can be used for complementation, inducible gene expression, or epitope tagging (Visick et al., 2018). To further understand the capabilities of these tools and how to use them, see (Visick et al., 2018). Additionally, a set of mini-transposons have been useful in making specific or genome-wide mutations. For example, there is a single Tn*7* attachment site, located within an intergenic region of the *V. fischeri* genome, into which the mini-Tn*7* transposon integrates at a high frequency (McCann et al., 2003). By introducing DNA of interest (such as a gene for complementation) into the Tn*7* transposon on a suicide plasmid and performing a conjugation (Alternate Protocol 2), this DNA can be readily inserted into a single site in the *V. fischeri* genome (Bao et al., 1991; McCann et al., 2003). Alternatively, transposons like Tn*5* with a wider insertional range can generate a mutant library that can subsequently be screened for a phenotype of interest (Alternate Protocol 3) (Lyell et al., 2008; Stoudenmire et al., 2019).

Critical Parameters

Transformation of *V. fischeri* with linear DNA—Linear DNA introduced into the *V. fischeri* genome can come from any of several sources, including PCR amplification, sheared chromosomal DNA, digested plasmid DNA, or a synthetic construct. The efficiency with which *V. fischeri* will take up this DNA can vary, with the most efficient being chromosomal DNA.

To successfully incorporate linear DNA, there must be sufficient homology between the introduced DNA and the chromosomes and/or plasmid DNA of the parent strain. Ideally, a piece of DNA should have at least 500 bp of homology on both of its ends to ensure the DNA recombines into the appropriate location at a sufficiently high rate. The greater the homology included in the linear DNA, the better the chance will be that the recombination event will occur. However, technical issues may constrain the size of the piece being introduced: the use of PCR, even with long-range enzymes, is often inefficient in producing a DNA fragment beyond a size of about 4 kb. Thus, to introduce large, PCR-amplified fragments into the bacterium, a multi-step mutagenesis can introduce individual (selectable) pieces of DNA, or the DNA can be provided on a plasmid via conjugation. Large pieces of chromosomal DNA (> 10 kb) can be taken up and incorporated by *V. fischeri*, though an exact upper limit has not been empirically determined (Brooks, Gyllborg, Kocher, Markey, & Mandel, 2015).

In addition to chromosomal homology, the DNA fragment should also contain a selectable marker to ensure the desired mutant is obtained. If the strain is auxotrophic, introducing a DNA fragment that restores prototrophy along with the DNA of interest can allow growth of only the desired mutant. More commonly, antibiotic resistance markers are used to select for the mutant of interest on antibiotic containing plates. The strain chosen must be susceptible to the antibiotic being used, otherwise the selection will be ineffective. Rather than making these pieces *de novo*, useful component pieces of DNA such as FRT-flanked antibiotic resistance markers have been developed for rapid mutagenesis of *V. fischeri* (Visick et al., 2018).

When performing PCR reactions either to generate fragments or to confirm mutations, the design of these primers should follow common design considerations. The primers should be around 20 base pairs in length and contain between 40 – 60% GC content. However, *V. fischeri* has a relatively low GC-content (38.3%) and therefore the primers may require longer sequences to achieve proper melting temperature (Ruby et al., 2005).

Plasmid transfer into *V. fischeri* via conjugation—Introduction of a plasmid into *V. fischeri* ES114 by conjugation is very efficient. When introducing a plasmid into *V. fischeri*, three things are critical for success: ensuring the cultures are healthy, minimizing *E. coli* background, and ensuring the *V. fischeri* strain is not resistant to the antibiotic that will be used to select for the plasmid. The latter is easily checked by either streaking the strain on an LBS-antibiotic plate prior to the experiment, or readily discovered by performing the negative control as described in the protocol. To reduce the *E. coli* background, the conjugations should be performed at 24°C or 28°C, temperatures that are at/near optimal growth temperatures for *V. fischeri* but suboptimal for *E. coli* growth. As such, the *E. coli*

colonies that do develop are initially smaller than their *V. fischeri* counterparts. Prolonged growth will result in similarly sized colonies of *E. coli* and *V. fischeri*; however, these can be differentiated by the yellowish color of *V. fischeri* colonies. Furthermore, any *V. fischeri* colonies will fail to grow at 37°C, and thus the presumed identity of a given colony can be confirmed using this temperature. Additionally, utilizing *E. coli* strains that are auxotrophic and require supplementation with DAP (Δ*dapA* mutant) or thymidine (Δ*thyA* mutant) can help counterselect against the *E. coli* carrying the plasmid of interest.

Removing antibiotic resistance cassettes from the V. fischeri genome— Removal of antibiotic resistance cassettes increases the versatility of genetic manipulations by permitting the combination of two mutations initially marked by the same antibiotic resistance cassette. Flipping out a cassette necessitates that the DNA being introduced contains two FRT sites flanking the antibiotic resistance cassette. However, if multiple FRT sites are present on the genome, there is a chance for recombination to occur between distant FRT sites during a resolution. Thus, as more FRT sites are introduced into the genome, PCR assessment of both the current resolution as well as all previous FRT sites across the genome should be used to verify the integrity of the desired final strain. Alternatively, or in addition, the genome may be sequenced.

Eliminating plasmids from *V. fischeri*—Certain plasmids, such as those derived from *Vibrio* plasmid pES213 (pVSV series) (Dunn et al., 2006), are relatively stable in *V. fischeri*. However, unstable plasmids like those listed in Table 1 will be readily lost upon growth and division without selection.

Introduction of exogenous DNA via suicide plasmid—If the suicide plasmid contains the *ccdB* toxin gene under control of an arabinose-inducible P_{BAD} promoter, the donor *E. coli* harboring this plasmid must be able to replicate the plasmid and must be resistant to the CcdB toxin (*e.g.*, by mutation of *gyrA*; see Table 2) or grown in glucose conditions to repress the *ccdB* promoter. Likewise, the *V. fischeri* cells containing the integrated plasmid must be maintained in or on media containing glucose.

For all experiments that depend on delivery of a suicide plasmid (regardless of the inclusion, or not, of the *ccdB* toxin), the *E. coli* strain used must be capable of replicating the suicide plasmid. The suicide plasmids described here contain the oriR6K origin of replication that requires the π protein for replication, and thus, the *E. coli* strain must contain the *pir* gene that encodes that protein. The allele *pir-116* permits the replication of oriR6K-containing plasmids to a higher copy number; this trait can be useful when, for example, it is necessary to isolate plasmid DNA for cloning purposes.

Transposon mutagenesis via suicide plasmid—As with any mutagenesis, the *V. fischeri* recipient must be susceptible to the antibiotic to which the transposon confers resistance. To ensure that the transposon is being transferred and not the entire plasmid, the plasmid should not contain homology to the *V. fischeri* genome. Furthermore, the transposon chosen must align with the goal of the study. The use of a Tn*7*-based approach allows relatively specific integration at a single site in the chromosome with high frequency. This site, the Tn*7* attachment site, is located on the larger chromosome of *V. fischeri* between the

yeiR and *glmS* genes. Alternatively, if performing a random transposon mutagenesis, a transposon with low insertional bias like Tn*5* or Mariner can have almost genome-wide coverage. However, mutants with insertions in essential genes (*i.e.,* genes that are required for cell viability under a given condition) may not be recovered.

Troubleshooting

Transformation of *V. fischeri* with linear DNA—Transformation of linear DNA is much more efficient when using *V. fischeri* chromosomal DNA compared to when using PCR or synthetic DNA (Pollack-Berti et al., 2010). As such, transformation with chromosomal DNA is almost always successful when using TfoX-overproducing ES114 as a recipient. In cases where transformability is in question (such as when PCR DNA is used), chromosomal DNA can be used as a positive control for the reaction. Thus, once a *V. fischeri* strain has a mutation of interest, it will usually be best to prepare chromosomal DNA from that strain to move into another strain, rather than regenerate the mutation with PCR DNA. If a lawn is obtained, ensure that the parent strain is sensitive to the antibiotic. Alternatively, a highly successful transformation can also form a lawn. Plating a lesser volume or a higher dilution of the transformation mixture should produce single cells. Also, you can and should verify that the antibiotic-resistant derivative did indeed acquire the antibiotic cassette, as described below.

Selection using certain antibiotics can result in the growth of spontaneous mutants (suppressors) that do not contain the mutation of interest. Erythromycin has been shown to be a reliable antibiotic for *V. fischeri* strain ES114 (no background growth), while selection using trimethoprim or chloramphenicol often results in growth of a low number of spontaneous mutants. Thus, negative controls are always required for a transformation to enumerate the suppressors and compare the morphology to identify the mutant of interest. The negative control will also verify that the parent strain was not resistant to the antibiotic used to select the mutation. In the case that the parent strain proved resistant to the antibiotic of choice, it may be possible to remove the antibiotic cassette, if it is flanked by FRT sequences. Alternatively, another strain or another antibiotic resistance marker may be chosen.

Finally, depending on the antibiotic cassette chosen, the PCR results may be confounding. An antibiotic resistance cassette that is approximately the size of the DNA being replaced will make differentiating the mutant and parent PCR products difficult. If this is the case, a different antibiotic cassette or a different primer set can be used. For example, a primer complementary to the cassette and a primer complementary to the genome would only produce a product in the strain with the proper mutation.

Plasmid transfer into *V. fischeri* via conjugation—By using *E. coli* auxotrophs and a less optimal temperature for *E. coli* growth, the background levels of the *E. coli* is typically low. Like transformation, a negative control is required to verify that the *V. fischeri* recipient is not already resistant to the antibiotic used to select the plasmid. In the event that the experimental conjugation fails to result in resistant *V. fischeri* colonies containing the plasmid of interest, the cells from the original conjugation spot can be streaked onto a fresh

LBS-antibiotic plate. If this still does not result in colonies, *V. fischeri* resistant to the antibiotic being used can serve as a positive control to ensure the plates contain the proper antibiotic. Alternatively, if a lawn arises after plating 100 μL, dilution of the conjugation mixture allows for single colonies to be obtained.

Removing FRT-flanked antibiotic resistance cassettes from the *V. fischeri* genome—Multiple, different FRT-flanked antibiotic resistance cassettes can be eliminated in a single step using this protocol. However, having multiple FRT sites in the genome can result in undesired recombination between distant FRT sites, which can remove portions of the genome. Thus, PCR confirmation that the antibiotic resistance cassette has been eliminated should always be performed, and if multiple FRT sites are present in the genome due to serial mutageneses, each should be confirmed to ensure that these undesired recombinations did not occur.

Eliminating unstable plasmids from *V. fischeri*—In most cases, the plasmids are typically lost after a single outgrowth on an LBS plate, dropping selection for the plasmid. However, if the antibiotic resistance conferred by the plasmid remains, a second outgrowth without selection should result in loss of the plasmid. This process is facilitated by obtaining and testing multiple single colonies, as mixed colonies are more likely to contain both resistant and sensitive cells; the former will grow on the antibiotic-containing plates, obscuring the lack of growth of the sensitive cells.

Introduction of exogenous DNA via suicide plasmid—After the initial conjugation of the suicide plasmid into *V. fischeri*, colonies that arise may be antibiotic resistant from transiently harboring the plasmid or more stably resistant due to successful integration of the plasmid. To ensure that the strains contain integrated plasmids, the antibiotic-resistant strains should be passaged once or twice on a non-selective medium, followed by evaluating stability of antibiotic resistance by streaking for growth on the antibiotic containing medium.

Recovery of the *V. fischeri* with integrated plasmid may be slow and result in a high *E. coli* background or an *E. coli* lawn. To reduce the *E. coli* background, use less *E. coli* in the conjugation mixture. Alternatively, to determine whether a colony is *V. fischeri* or *E. coli*, streak a single colony to assess on two LBS plates containing the appropriate antibiotic. Place one plate at 28°C and the other at 37°C. *V. fischeri* will not grow at 37°C, while *E. coli* will grow.

When introducing a CcdB-toxin-producing plasmid into *V. fischeri*, the growth medium must contain glucose to prevent expression of the toxin gene while the plasmid is maintained in the genome. Growth of *V. fischeri* in the presence of glucose results in the production of acid, which can, in turn, kill these bacteria. Thus, care should be taken to minimize the amount of time that the strains of interest spend growing in the presence of glucose.

Transposon mutagenesis via suicide plasmid—For specific Tn*7*-based transposon mutagenesis, colonies that arise after the initial conjugation may be antibiotic resistant from transiently harboring the plasmid or more stably due to successful integration of the transposon. To ensure that the transposon has integrated into the genome, the antibiotic-

resistant strains should be passaged once or twice on a non-selective medium, followed by evaluating stability of antibiotic resistance by streaking for growth on the antibiotic containing medium.

Though the plasmid should lack homology to the chromosome, the plasmid can occasionally integrate into the genome at the Tn*7* site through an unknown mechanism: either by transposase-mediated insertion of the whole plasmid or insertion of Tn*7* followed by recombination of another copy of the delivery plasmid. The result is that some strains obtain both the plasmid-conferred and transposon-conferred antibiotic resistances. The same can occur for Tn*5;* an instance of this has been reported in (Singh et al., 2015). Thus, colonies of interest that appear to have obtained the transposon should be screened on LBS agar containing plasmid-selective antibiotic to determine whether they have also acquired the antibiotic resistance conferred by the plasmid. The insertion of the plasmid may be acceptable or may have unforeseen consequences, necessitating the construction of a new mutation or insertion.

Recovery of the *V. fischeri* with integrated transposon may be slower than the simple introduction of a replication plasmid and as a result, an increased incubation period may result in the growth of a high *E. coli* background or an *E. coli* lawn on the conjugation plate. To reduce the *E. coli* background, use less *E. coli* in the conjugation mixture. Alternatively, to determine whether a colony is *V. fischeri* or *E. coli*, streak a single colony to assess on two LBS plates containing the appropriate antibiotic. Place one plate at 28°C and the other at 37°C. *V. fischeri* will not grow at 37°C, while *E. coli* will grow.

Understanding Results

Transformation of *V. fischeri* with linear DNA—When performing a transformation, colonies of the successful transformants should arise in the presence of an antibiotic. In contrast, the parent that did not receive DNA should fail to grow on the same type of plate. If the linear DNA successfully recombined into the desired site, PCR amplification of the region should result in a shift in the size of the PCR product compared to the parent if the new DNA was inserted. Depending on the length of the recovery step, the number of transformants is expected to vary. A shorter recovery may be insufficient to express antibiotic resistance and result in few transformants. A longer recovery can cause “siblings” to arise from a single transformant that had enough time to replicate. This can artificially increase the number of individual transformants obtained after the experiment.

Plasmid transfer into *V. fischeri* via conjugation—Like transformation, successful transconjugants should arise in the presence of the antibiotic to which the plasmid confers resistance. However, when the conjugation mixture is spread onto plates (step 11b), a lawn may arise instead of single colonies if the conjugation was highly efficient. The *V. fischeri* parent that was not exposed to *E. coli* should serve as a negative control and should produce no colonies on the same type of plate. If single colonies are desired, dilutions can be made and spread onto the appropriate medium.

Removing FRT-flanked antibiotic resistance cassettes from the *V. fischeri* genome—When flipping an antibiotic cassette, the cells should become sensitive to the

antibiotic to which the parent was resistant. Thus, the strains that fail to grow on the antibiotic but are recovered on LBS are the presumed correct strains. PCR confirmation of the region where the cassette was eliminated should result in a smaller product compared to a PCR product of that region in the parent. If an unexpected recombination occurs in a strain with multiple FRT sites, the PCR reaction may produce aberrantly sized products or, more likely, a negative result because such a recombination event would result in the loss of an adjacent region of the genome, including the primer binding site.

Eliminating unstable plasmids from *V. fischeri*—Losing a plasmid of interest will result in a sensitivity to the antibiotic to which the plasmid-bearing parent strain is resistant. Thus, the correct strain will not grow on the plate containing antibiotics but will be recovered on LBS. Plasmids are lost when they fail to segregate to both daughter cells during cell division. The rate of plasmid loss may vary between different plasmids, and depend on characteristics such as the particular origin of replication and copy number of the plasmid. Thus, a single colony may contain resistant and sensitive cells (plasmid-containing and plasmid-less strains), depending on when in the formation of the colony the plasmid was lost, and loss of a plasmid may require multiple passages on non-selective medium to obtain the desired plasmid-free strain.

Introduction of exogenous DNA via suicide plasmid—If the goal is to integrate the plasmid into the genome, the correct strain should maintain the antibiotic resistance conferred by the plasmid after outgrowth in a non-selective medium. The correct insertion can be confirmed by checking the size of the PCR product generated with primers flanking the region into which the plasmid should recombine. However, insertion of a large plasmid may require a plasmid-specific primer and a genome-specific primer to successfully identify an integration.

If the goal is to remove/insert a piece of DNA, the strains should not maintain the resistance conferred by the plasmid by the end of the protocol. However, the final outcome after selection for loss of the integrated plasmid may be either the starting parent strain or the desired mutant. In the absence of a visual phenotype for the mutation, its presence must be verified by PCR amplification, with the expectation that successful generation of a mutation will result in a PCR product with an altered size (typically smaller for a deletion) relative to the parent.

Transposon mutagenesis via suicide plasmid—For any transposon integration, the final strain should obtain stable antibiotic resistance conferred by the transposon but should not obtain the resistance conferred by the plasmid. If using the Tn*7* transposon, a strain that successfully integrated the transposon will yield a larger PCR product than the parent strain when using PCR primers flanking the Tn*7* attachment site.

If performing a random transposon mutagenesis, the antibiotic resistant strains can be screened in the desired phenotypic assay. Then, any mutants of interest can be assessed to determine the site of transposition using arbitrarily primed PCR reaction (Saavedra, Schwartzman, & Gilmore, 2017). To estimate the depth of coverage of the mutant library, count the number of colonies obtained and compare that to the size of the *V. fischeri*

genome. Since the genome is ~4.3 × 10^6 base pairs long and a gene is 1000 bp long on average, if 1 × 10^5 colonies are obtained, then there is an insertion every 45 base pairs on average. This would mean that a given gene could be mutated as many as 22 times. However, the success of mutating a given gene will also depend on other factors, such as its requirement for growth under the conditions used.

Time Considerations

Transformation of *V. fischeri* with linear DNA—Transformation of *V. fischeri* is a protocol that takes a total of four days. The first day requires growth of an overnight culture at the end of the day and for the best results, fresh preparation of enough TMM for the overnight culture and the subculture. The second day, the entire procedure takes around 7 hours, although most of that time is spent during incubation periods. Subculturing will take 5 hours for a sufficient optical density, although this time may be extended depending on the health of the overnight culture. This step should not be overextended as higher optical densities (OD_{600}>1) may reduce transformation frequency. Once a sufficiently high cell density is reached, the incubation with linear DNA takes 30 minutes, and the recovery period takes another 90 minutes. The recovery period can be shortened or extended. A shorter recovery period may not be sufficient for cells to express antibiotic resistance and may result in the recovery of fewer transformants. A longer recovery may result in “siblings” that arise after replication of a single transformant. Plating the cells should be quick and should be proportional to the number of transformations being performed. Though the consequence has not been tested directly, the DNA incubation period can be extended up to one hour. The recovery period can be extended overnight, and the transformation mixture plated the next day. If colonies arise, representative colonies can be streaked for purity. On the following day, the colonies can be confirmed by PCR for the transformed DNA and, if the cells are to be prepared as a frozen stock, an overnight culture can be prepared. Apart from the overnight and subculture, this protocol is resilient to extensions of time and can be performed in parallel with dozens of transformations.

Plasmid transfer into *V. fischeri* via conjugation—Conjugation should take approximately four days with minimal hands-on work. On the first day, overnight cultures of the *V. fischeri* recipient and *E. coli* are grown. The following day, the cultures are subcultured into fresh medium for approximately three hours. However, conjugation is very efficient, and the subculture can be shorter if need be; if the culture is slightly turbid, the conjugation may proceed although it may yield a reduced recovery of transconjugants. Cells can be quickly concentrated by spinning the early culture in a microcentrifuge and resuspending in a smaller volume. Once the cells are grown and mixed, they are plated on an LBS plate and incubated for a period between three hours and overnight. For applications such as transposon mutagenesis, the optimum incubation period may be around eight hours but should be empirically determined. The conjugation spot is either resuspended in LBS or simply restreaked onto an LBS plate containing the selective antibiotic. On the third day (or fourth day if performing an overnight incubation), any successful conjugants are restreaked for purity onto a fresh LBS plate with the selective antibiotic. On the fourth day, the cells carrying the plasmid of interest are ready for use or for preparation of a glycerol stock.

Removing FRT-flanked antibiotic resistance cassettes from the *V. fischeri* genome—The first three days are identical to a regular conjugation, though a fifth day is required. On the fourth day, the transconjugant is patched onto an LBS plate containing an antibiotic for the cassette being eliminated and an LBS plate to assess sensitivity and thus loss of the cassette. On the fifth day, the sensitive strains can be restreaked for purity, then used or frozen into glycerol stocks.

Eliminating unstable plasmids from *V. fischeri*—This process should take approximately three days. However, it may take longer if the plasmid is not lost in the colonies chosen for evaluation.

Introduction of exogenous DNA via suicide plasmid—To integrate the plasmid, the process will take seven or eight days. The hands-on time is comparable to the conjugation protocol, while the remaining time is mostly hands-off during multiple rounds of restreaking. To mutagenize after plasmid excision, this entire process can take 10–11 days total. Again, much of the time lies in waiting for the colonies to arise after restreaking.

Transposon mutagenesis via suicide plasmid—For Tn*7*-based transposon mutagenesis, because of the similarities to Campbell mutagenesis, this process will also take between seven or eight days depending on how long it takes for initial conjugants to arise. Most of the time is hands-off while the cells are streaked to identify the initial transconjugants, loss of the plasmid, and stable maintenance of the antibiotic resistance.

For random transposon mutagenesis, the process is akin to a normal conjugation and will take three to four days depending on how long the conjugation is allowed to proceed. However, the second day may be approximately 11 hours if performing an 8 hour conjugation. If performing a saturating mutagenesis, this procedure may take multiple days until the desired number of mutants are obtained.

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Literature Cited

Allen RD, & Baumann P (1971). Structure and arrangement of flagella in species of the genus *Beneckea* and *Photobacterium fischeri*. J Bacteriol, 107(1), 295–302.

Bao Y, Lies DP, Fu H, & Roberts GP (1991). An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. Gene, 109(1), 167–168. doi:10.1016/0378-1119(91)90604-a

Boettcher KJ, & Ruby EG (1990). Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. J Bacteriol, 172(7), 3701–3706. doi:10.1128/jb.172.7.3701-3706.1990

Bongrand C, & Ruby EG (2019). Achieving a multi-strain symbiosis: strain behavior and infection dynamics. ISME J, 13(3), 698–706. doi:10.1038/s41396-018-0305-8

Brooks JF, Gyllborg MC, Cronin DC, Quillin SJ, Mallama CA, Foxall R, … Mandel MJ (2014). Global discovery of colonization determinants in the squid symbiont *Vibrio fischeri*. Proc Natl Acad Sci U S A, 111(48), 17284–17289. doi:10.1073/pnas.1415957111

Brooks JF, Gyllborg MC, Kocher AA, Markey LE, & Mandel MJ (2015). TfoX-based genetic mapping identifies *Vibrio fischeri* strain-level differences and reveals a common lineage of laboratory strains. J Bacteriol, 197(6), 1065–1074. doi:10.1128/JB.02347-14

Christensen DG, & Visick KL (2020). *Vibrio fischeri:* Laboratory Cultivation, Storage, and Common Phenotypic Assays. Curr Protoc Microbiol, 57(1), e103. doi:10.1002/cpmc.103

Datsenko KA, & Wanner BL (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A, 97(12), 6640–6645. doi:10.1073/pnas.120163297

Dunn AK, Martin MO, & Stabb EV (2005). Characterization of pES213, a small mobilizable plasmid from *Vibrio fischeri*. Plasmid, 54(2), 114–134. doi:10.1016/j.plasmid.2005.01.003

Dunn AK, Millikan DS, Adin DM, Bose JL, & Stabb EV (2006). New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression *in situ*. Appl Environ Microbiol, 72(1), 802–810. doi:10.1128/AEM.72.1.802-810.2006

Goodman AL, Wu M, & Gordon JI (2011). Identifying microbial fitness determinants by insertion sequencing using genome-wide transposon mutant libraries. Nat Protoc, 6(12), 1969–1980. doi:10.1038/nprot.2011.417

Graf J, & Ruby EG (1998). Host-derived amino acids support the proliferation of symbiotic bacteria. Proc Natl Acad Sci U S A, 95(4), 1818–1822. doi:10.1073/pnas.95.4.1818

Graf J, & Ruby EG (2000). Novel effects of a transposon insertion in the *Vibrio fischeri glnD* gene: defects in iron uptake and symbiotic persistence in addition to nitrogen utilization. Mol Microbiol, 37(1), 168–179. doi:10.1046/j.1365-2958.2000.01984.x

Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, & Schweizer HP (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene, 212(1), 77–86. doi:10.1016/s0378-1119(98)00130-9

Jones BW, & Nishiguchi MK (2004). Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). Marine Biology, 144(6), 1151–1155. doi:10.1007/s00227-003-1285-3

Koehler S, Gaedeke R, Thompson C, Bongrand C, Visick KL, Ruby E, & McFall-Ngai M (2018). The model squid-vibrio symbiosis provides a window into the impact of strain- and species-level differences during the initial stages of symbiont engagement. Environ Microbiol. doi:10.1111/1462-2920.14392

Le Roux F, Binesse J, Saulnier D, & Mazel D (2007). Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. Appl Environ Microbiol, 73(3), 777–784. doi:10.1128/AEM.02147-06

Levisohn R, Moreland J, & Nealson KH (1987). Isolation and characterization of a generalized transducing phage for the marine luminous bacterium *Vibrio fischeri* MJ-1. Microbiology, 133(6), 1577–1582. doi:https://doi.org/10.1099/00221287-133-6-1577

Lyell NL, Dunn AK, Bose JL, & Stabb EV (2010). Bright mutants of *Vibrio fischeri* ES114 reveal conditions and regulators that control bioluminescence and expression of the *lux* operon. J Bacteriol, 192(19), 5103–5114. doi:10.1128/JB.00524-10

Lyell NL, Dunn AK, Bose JL, Vescovi SL, & Stabb EV (2008). Effective mutagenesis of *Vibrio fischeri* by using hyperactive mini-Tn5 derivatives. Appl Environ Microbiol, 74(22), 7059–7063. doi:10.1128/AEM.01330-08

Lyell NL, Septer AN, Dunn AK, Duckett D, Stoudenmire JL, & Stabb EV (2017). An expanded transposon mutant library reveals that *Vibrio fischeri* δ-aminolevulinate auxotrophs can colonize *Euprymna scolopes*. Appl Environ Microbiol, 83(5). doi:10.1128/AEM.02470-16

Mandel MJ, & Dunn AK (2016). Impact and influence of the natural *Vibrio*-Squid symbiosis in understanding bacterial-animal interactions. Front Microbiol, 7, 1982. doi:10.3389/fmicb.2016.01982

Mandel MJ, Wollenberg MS, Stabb EV, Visick KL, & Ruby EG (2009). A single regulatory gene is sufficient to alter bacterial host range. Nature, 458(7235), 215–218. doi:10.1038/nature07660

McCann J, Stabb EV, Millikan DS, & Ruby EG (2003). Population dynamics of *Vibrio fischeri* during infection of *Euprymna scolopes*. Appl Environ Microbiol, 69(10), 5928–5934. doi:10.1128/aem.69.10.5928-5934.2003

McFall-Ngai MJ (2014). The importance of microbes in animal development: lessons from the squid-vibrio symbiosis. Annu Rev Microbiol, 68, 177–194. doi:10.1146/annurev-micro-091313-103654

Meibom KL, Blokesch M, Dolganov NA, Wu CY, & Schoolnik GK (2005). Chitin induces natural competence in *Vibrio cholerae*. Science, 310(5755), 1824–1827. doi:10.1126/science.1120096

O'Shea TM, Klein AH, Geszvain K, Wolfe AJ, & Visick KL (2006). Diguanylate cyclases control magnesium-dependent motility of *Vibrio fischeri*. J Bacteriol, 188(23), 8196–8205. doi:10.1128/JB.00728-06

Ondrey JM, & Visick KL (2014). Engineering *Vibrio fischeri* for inducible gene expression. Open Microbiol J, 8, 122–129. doi:10.2174/1874285801408010122

Pollack-Berti A, Wollenberg MS, & Ruby EG (2010). Natural transformation of *Vibrio fischeri* requires *tfoX* and *tfoY*. Environ Microbiol, 12(8), 2302–2311. doi:10.1111/j.1462-2920.2010.02250.x

Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, … Greenberg EP (2005). Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. Proc Natl Acad Sci U S A, 102(8), 3004–3009. doi:10.1073/pnas.0409900102

Saavedra JT, Schwartzman JA, & Gilmore MS (2017). Mapping transposon insertions in bacterial genomes by arbitrarily primed PCR. Curr Protoc Mol Biol, 118, 15.15.11–15.15.15. doi:10.1002/cpmb.38

Schlake T, & Bode J (1994). Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. Biochemistry, 33(43), 12746–12751. doi:10.1021/bi00209a003

Shibata S, Yip ES, Quirke KP, Ondrey JM, & Visick KL (2012). Roles of the structural symbiosis polysaccharide (*syp*) genes in host colonization, biofilm formation, and polysaccharide biosynthesis in *Vibrio fischeri*. J Bacteriol, 194(24), 6736–6747. doi:10.1128/JB.00707-12

Singh P, Brooks JF, Ray VA, Mandel MJ, & Visick KL (2015). CysK plays a role in biofilm formation and colonization by *Vibrio fischeri*. Appl Environ Microbiol, 81(15), 5223–5234. doi:10.1128/AEM.00157-15

Stabb EV, & Ruby EG (2002). RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. Methods Enzymol, 358, 413–426. doi:10.1016/s0076-6879(02)58106-4

Stabb EV, & Visick KL (2013). *Vibrio fischeri:* A bioluminescent light-organ symbiont of the bobtail squid Euprymna scolopes. In D. E. F. Rosenberg E, Lory S, Stackebrandt E, Thompson F (Ed.), The Prokaryotes (pp. 497–532). Berlin, Heidelberg: Springer.

Stoudenmire JL, Black M, Fidopiastis PM, & Stabb EV (2019). Mutagenesis of *Vibrio fischeri* and other marine bacteria using hyperactive mini-Tn5 derivatives. Methods Mol Biol, 2016, 87–104. doi:10.1007/978-1-4939-9570-7_9

Sun Y, LaSota ED, Cecere AG, LaPenna KB, Larios-Valencia J, Wollenberg MS, & Miyashiro T (2016). Intraspecific competition impacts *Vibrio fischeri* strain diversity during initial colonization of the squid light organ. Appl Environ Microbiol, 82(10), 3082–3091. doi:10.1128/AEM.04143-15

Tischler AH, Hodge-Hanson KM, & Visick KL (2019). *Vibrio fischeri* – squid symbiosis. eLS, 1–9.

Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, & Mazel D (2012). Genome engineering in Vibrio cholerae: a feasible approach to address biological issues. PLoS Genet, 8(1), e1002472. doi:10.1371/journal.pgen.1002472

Visick KL, Hodge-Hanson KM, Tischler AH, Bennett AK, & Mastrodomenico V (2018). Tools for rapid genetic engineering of *Vibrio fischeri*. Appl Environ Microbiol, 84(14), e00850–00818. doi:10.1128/AEM.00850-18

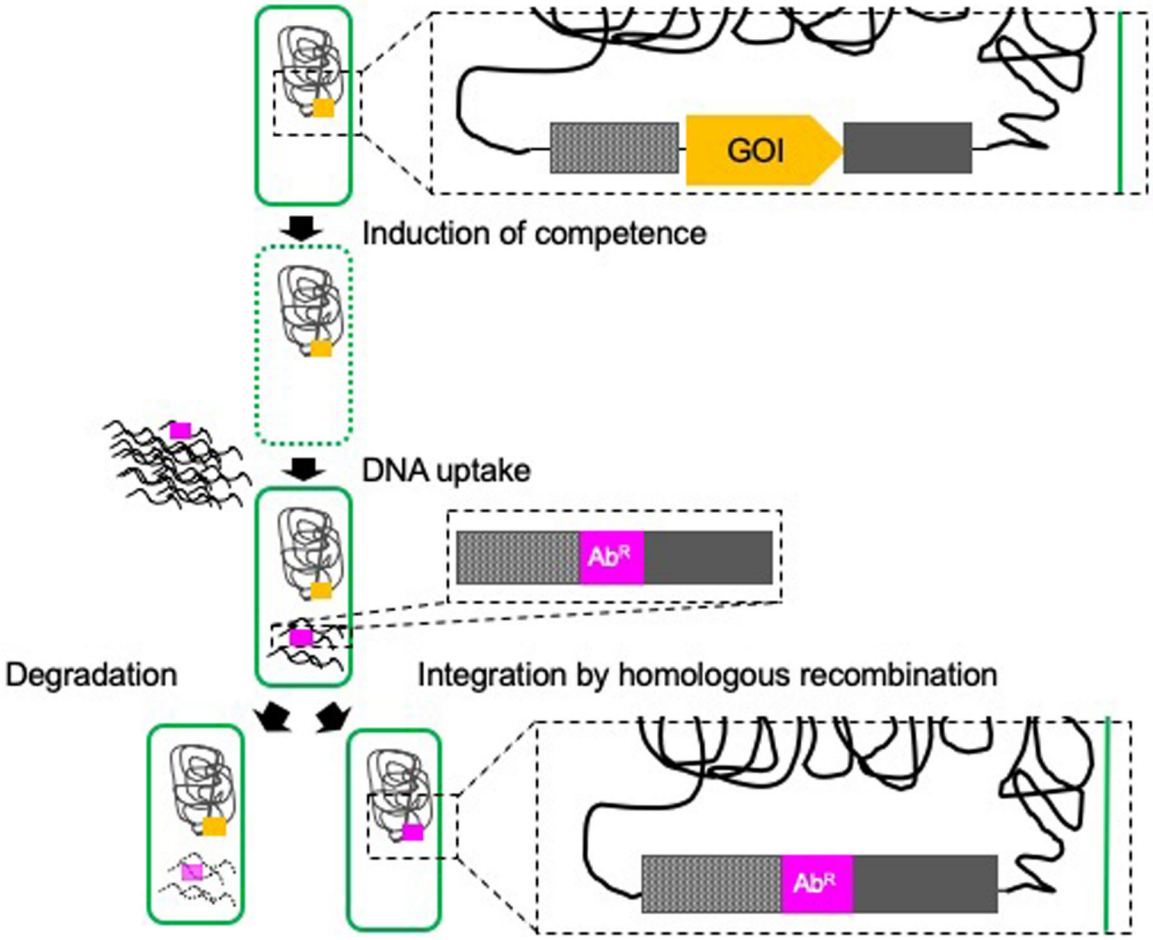


Figure 1.

Transformation in *V. fischeri*. Uptake of DNA by *V. fischeri* is typically achieved by overproduction of competence regulator TfoX (not shown), which induces competence (indicated by the dotted line of the cell envelope). Bacteria can take up linear DNA in the form of PCR fragments or genomic fragments. In the case of a successful transformation (bottom right), the introduced DNA will be integrated into the genome of the recipient via homologous recombination, possibly resulting in the replacement of a gene of interest (GOI) in the recipient (gold square) with the allele of the gene on the newly acquired DNA (pink square); selection for the antibiotic resistance (Ab^R) will permit isolation of cells that have undergone this recombination event. Alternatively, the introduced DNA may be degraded (bottom left, indicated by dotted lines), which will prevent transformation.

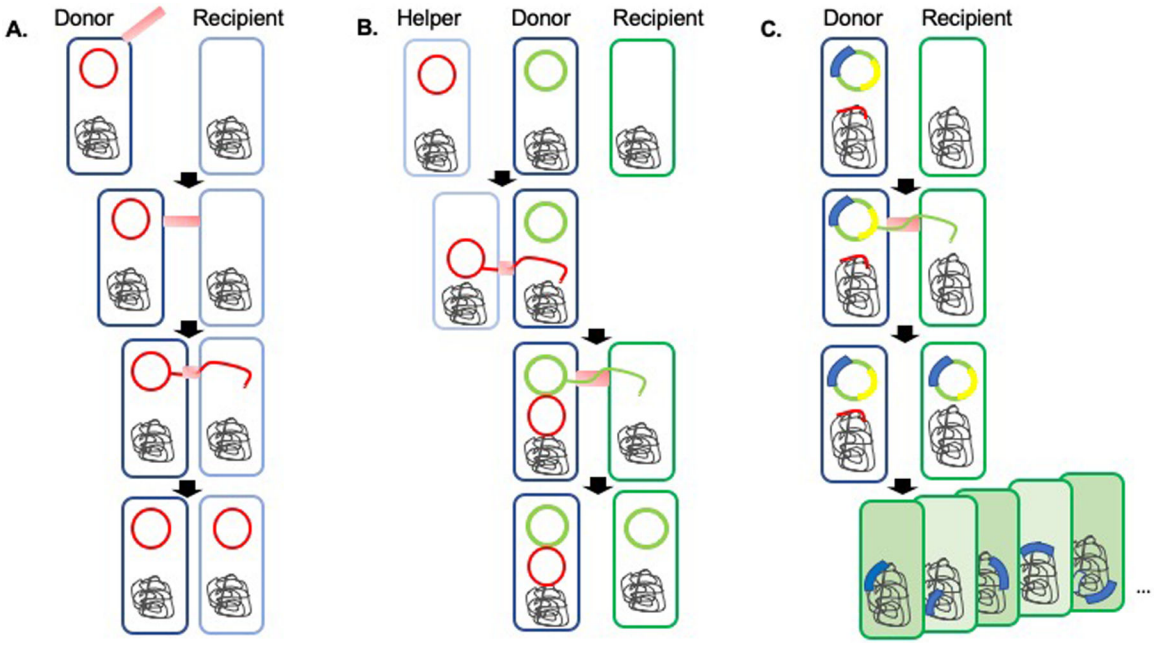
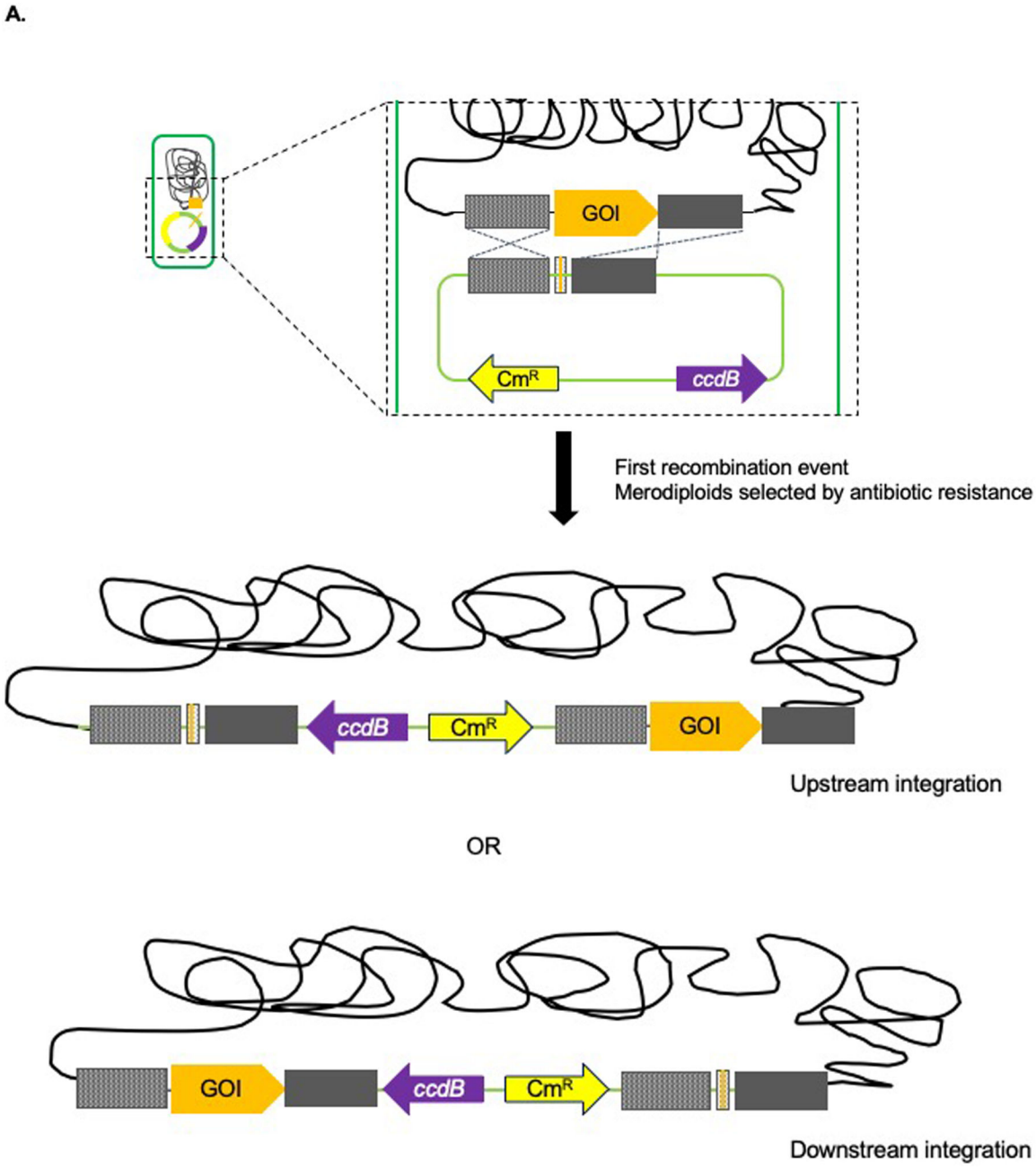


Figure 2.

Overview of bacterial conjugation. A) A typical mating between a donor and a recipient strain. The donor bacteria produce a conjugative pilus (red rectangle) that can transfer the plasmid of interest (red circle) to the recipient cell. B) A triparental mating between a donor *E. coli*, helper *E. coli*, and *V. fischeri* recipient. The donor *E. coli* is unable to transfer its plasmid until it receives the plasmid from the helper *E. coli* that encodes the conjugative machinery (green circle), at which point it is able to interact with and transfer DNA into the recipient. C) A biparental mating between a donor *E. coli* strain, in which the conjugative machinery is encoded on the chromosome and the plasmid to be transferred carries a transposon (blue), and a *V. fischeri* recipient. This conjugation results in movement of the transposon-encoding plasmid into *V. fischeri* cell, insertion of the transposon into the *V. fischeri* genome and generation of a library of mutant clones each with a transposon at a distinct location.



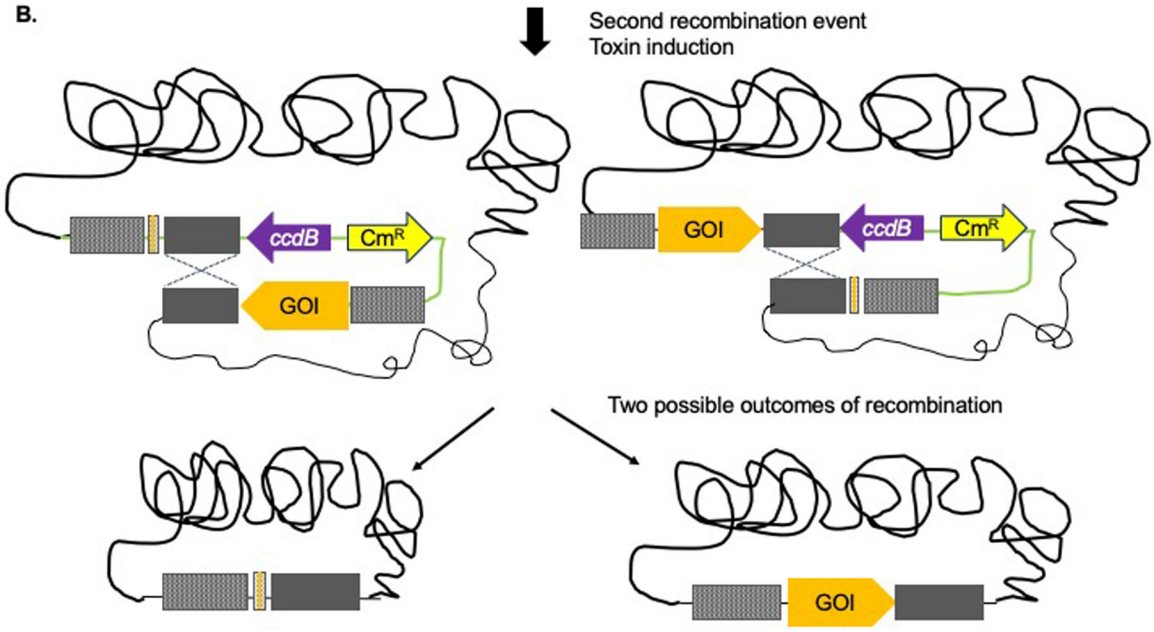


Figure 3.

Gene replacement using a suicide plasmid. (A) A plasmid encoding a piece of DNA flanked by the upstream and downstream regions around a gene of interest (GOI) to be replaced is introduced into *V. fischeri* by conjugation. Recombination at either of these flanking regions (first recombination event) will lead to integration of the entire plasmid into the chromosome. Recombination at the upstream or downstream region of homology leads to similar but distinct merodiploids containing the integrated plasmid. (B) A second recombination event results in removal of the integrated plasmid from the genome, leaving behind the interrupted region for the gene of interest (bottom left) or restoring the original genome structure (bottom right). Although only a single recombination outcome is depicted for each starting merodiploid, both final outcomes are possible. Providing an inducer (i.e., arabinose) will lead to expression of a toxin-encoding gene (here *ccdB*), killing cells that retain the integrated plasmid and enriching for cells that have undergone the second recombination event.

Table 1.

Common plasmids used for *V. fischeri* genetic manipulation[a]

Plasmid	Resistance Marker[b,c]	Antibiotic concentration for *V. fischeri*[d]	Uses	Source
Stable plasmids				
pVSV102	Kn^R (50 μg/mL)	100 μg/mL	Stable gene expression	(Dunn, Millikan, Adin, Bose, & Stabb, 2006)
pVSV105	Cm^R (12.5 μg/mL)	5 μg/mL	Stable gene expression	(Dunn et al., 2006)
Unstable plasmids				
pEVS104	Kan^R (50 μg/mL)	100 μg/mL	Helper plasmid for conjugation	(E. V. Stabb & Ruby, 2002)
pKV496	Kan^R (50 μg/mL)	100 μg/mL	Flippase-expressing plasmid	(Visick et al., 2018)
pLostfoX	Cm^R (12.5 μg/mL)	5 μg/mL	Induce competence for transformation	(Pollack-Berti et al., 2010)
pLostfoX-Kan	Kan^R (50 μg/mL)	100 μg/mL	Induce competence for transformation	(Brooks et al., 2014)
Suicide plasmids[e]				
pEVS122	Erm^R (150 μg/mL)[f]	2.5 μg/mL	Disrupt a gene or insert DNA by Campbell mutagenesis	(Dunn, Martin, & Stabb, 2005)
pESY20	Erm^R (150 μg/mL)[f]	2.5 μg/mL	Disrupt a gene or insert DNA by Campbell mutagenesis	(O'Shea, Klein, Geszvain, Wolfe, & Visick, 2006)
pKV363	Cm^R (12.5 μg/mL)	1 μg/mL	For gene deletion/allelic replacement	(Shibata, Yip, Quirke, Ondrey, & Visick, 2012)
pSW7848	Cm^R (12.5 μg/mL)	1 μg/mL	For gene deletion/allelic replacement	(Val, Skovgaard, Ducos-Galand, Bland, & Mazel, 2012)
pSW8197	Kan^R (50 μg/mL)	100 μg/mL	For gene deletion/allelic replacement	(Le Roux et al., 2007)
Suicide plasmids for transposon mutagenesis[e]				
pUX-BF13	Amp^R (100 μg/mL)	N/A	Helper plasmid with Tn*7* transposition genes	(Bao et al., 1991)
pEVS107	Kan^R (50 μg/mL)	100 μg/mL	Carries site-specific Tn*7* transposon (Erm^R)	(McCann et al., 2003)
pEVS170	Kan^R (50 μg/mL)	100 μg/mL	Carries Tn*5* transposon (Erm^R)	(Lyell et al., 2008)
pJMO10	Kan^R (50 μg/mL)	100 μg/mL	Carries Tn*5* transposon (Erm^R) with outward facing *lac* promoter	(Ondrey & Visick, 2014)
pMarVF1 (Addgene 99579)	Amp^R (100 μg/mL)	N/A	Carries mariner transposon (Erm^R)	(Brooks et al., 2014)

[a] Plasmids listed here can be requested from the authors of the publication listed.

[b] Kan^R, kanamycin; Cm^R, chloramphenicol; Erm^R, erythromycin

[c] The concentrations in parentheses are those concentrations required for plasmid maintenance in *E. coli*.

[d] The concentration of antibiotics for *V. fischeri* may vary for different strains. These numbers are for the laboratory strain ES114. ES114 is highly resistant to Amp.

[e] Replication of each suicide plasmid indicated here requires a strain expressing the π protein, encoded by the *pir* gene.

[f] *E. coli* must be grown on BHI medium.

Table 2.

Common *E. coli* strains used for conjugation

Strain name	Genotype [Antibiotic resistances[a]]	Use[b]	Source
TAM1 λ*pir*	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL endA*1 *nupG attl*::*pir*+	Replicate suicide plasmids	Active Motif Inc #11097
β3914	RP4–2-Tc::Mu Δ*dapA*::(*erm-pir*) *gyrA462 zei-298*::Tn*10* [Kn^R, Erm^R, Tc^R]	Replicate suicide plasmids, DAP auxotroph	(Le Roux et al., 2007)
π3813	*lacI*^{q}*thi-1 supE44 endA1 recA1 hsdR17 gyrA462 zei-298*::Tn*10* Δ*thyA*::(*erm-pir-116*) [Tc^R, Erm^R]	Replicate suicide plasmids to high copy number, Thymidine auxotroph	(Le Roux et al., 2007)
DH5α	*supE44* Δ*lacU169* (Φ80*lacZ*ΔM15) Δ*argF hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Plasmid storage, facilitate cloning	Thermofisher Scientific, Inc #18265017
GT115	*mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ80*lacZ*Δ*M15* Δ*lacX74 recA1 endA1* Δ*dcm uidA(*Δ*MluI)*::*pir-116* Δ*sbcC-sbcD*	Plasmid storage	Invivogen #gt115-11

[a] Kan^R, kanamycin; Erm^R, erythromycin; Tc^R, tetracycline

[b] The suicide plasmids described here require the π protein, encoded by the *pir* gene, for growth. The indicated strains may not replicate all suicide plasmids, but will replicate π-dependent plasmids.