Rapid and efficient protocol to introduce exogenous DNA in *Vibrio harveyi* and *Pseudoalteromonas* sp.

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Abstract :

Vibrio campbellii BAA-1116 is renowned for its bioluminescence properties, and genetic tools are available to genetically track this strain. However, many other ecologically important *V. harveyi* strains exist, for which only few genetic tools are available. In this study, a rapid electroporation protocol was developed to transform replicative plasmids in various environmental *V. harveyi* and *Pseudoalteromonas* strains. Moreover, a mini-*Tn*7 delivery system was modified to site-specifically integrate mini-transposons in the genome of *V. harveyi* ORM4. As a proof-of-principle, replicative plasmids carrying bioreporters were introduced by electroporation in *V. harveyi* ORM4 cells, and gene expression was followed at the single cell level. We could demonstrate that a flagellar gene is subjected to bimodal gene expression in *V. harveyi* ORM4, being highly expressed in 10% of the population in stationary phase. This study extends the possibilities to study environmental *Vibrio* strains and uncovers the occurrence of phenotypic heterogeneity in flagellar expression in *Vibrio*.

Keywords : Electroporation, Mini-transposon, *Vibrio harveyi*, *Pseudoalteromonas*, phenotypic heterogeneity, Bioreporter, Flagellum expression

1. Introduction

Bacteria from the genus *Vibrio* are commonly found in marine environments, either free living in the water column or associated with planktonic particles and animals. Many *Vibrio* strains are human pathogens, including *Vibrio cholerae* responsible for the deadly cholera disease, or *Vibrio parahaemolyticus*. However, other pathogenic *Vibrio* strains exist, including some infecting a wide range of marine organisms, from fish to marine invertebrates (shrimp, molluscs). Among them, one can cite *Vibrio alginolyticus*, *Vibrio aestuarianus* or *Vibrio tapetis*. Finally, even though the *Vibrio harveyi* type strain BAA-1116 (recently reclassified as *Vibrio campbellii* (Lin *et al.*, 2010)) has been extensively studied for its quorum sensing and bioluminescence systems (Plener *et al.*, 2015), other strains belonging to this species are known to be pathogens of fish, shrimps and molluscs (Austin & Zhang, 2006). Interestingly, while the host spectrum of this species is very broad, colonization is host-specific, with a single strain of *V. harveyi* being able to infect a limited group of hosts. This is the case for *V. harveyi* ORM4 which specifically infects the European abalone *Haliotis tuberculata* (Nicolas *et al.*, 2002). *V. harveyi* ORM4 was isolated in 2002 after a severe outbreak which resulted in the mortality of 50% to 90% of the natural European abalone stock in Brittany and Normandy (France) in the late 90's (Nicolas *et al.*, 2002). *V. harveyi* ORM4 is also able to provoke abalone mortality in laboratory experiments, with 70% to 90% of death occurring within 3 days post-infection (Travers *et al.*, 2009, Cardinaud *et al.*, 2014).

Unfortunately, despite its pathogenicity, the lack of genetic tools for environmental *Vibrio* strains like *V. harveyi* ORM4 hampers a better understanding of the infection mechanisms. Moreover, genetic tools to introduce exogenous DNA (either as replicative plasmids or as mini-transposons) are available for the type strain *V. campbellii* BAA-1116, but they almost exclusively rely on conjugation, using plasmid-bearing *Escherichia coli* strains. Conjugation is a powerful mean to genetically manipulate bacteria, but this system is time consuming, requiring typically 4-5 days including the inoculation of strains, the mating on plates and the selection of potential transformants. Moreover, the counter-selection of the *E. coli* donor cells can also be challenging. Therefore, alternatives to conjugations are necessary to introduce foreign DNA in *V. harveyi* strains.

In this study, we developed an electroporation protocol to introduce replicative plasmids and mini-transposons in *V. harveyi* ORM4. As a proof, we introduced a bioreporter in this strain to follow the expression of a flagellum promoter. Finally, we extended this

protocol to *V. harveyi* strain LMG7890 *and V. campbellii* BAA-1116, as well as to bacterial strains outside the *Vibrionales* order and belonging to the genus *Pseudoaltermonas*.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Unless otherwise stated, *E. coli* strains were routinely grown at 37 °C in LB, *V. harveyi* strains at 28 °C in LB supplemented with 20 g/l (final concentration, f.c.) NaCl (LBS) and *Pseudoalteromonas* strains at 20 °C in MLB (15 g/l sea salt, 10 g/l peptone and 5 g/l yeast extract). If necessary, the following antibiotics and components were used: ampicillin (100 µg/ml), kanamycin (100 µg/ml for *E. coli*, 250 µg/ml for *V. harveyi*), chloramphenicol (5 µg/ml), trimethoprim (10 µg/ml), diaminopimelic acid (DAP, 0.3 mM) and agar 15 g/l. The list of strains and plasmids used in this study can be found in Table 1.

2.2. Construction of the pTNS3-derivative plasmids

The minimal origin of replication of pES213 (Dunn *et al.*, 2005), amplified using primers $5'$TTTTTTGCATGCACGCGTATCGATCTCGAGAACTATCAATAGAACGTGTATTTA$3'$ and $5'$TTTTTTGTCGACGCAGCTCTTAATATTTTCTTTTT$3'$, was cloned into pTNS3 (Choi *et al.*, 2008) between the *Sph*I and *Sal*I sites, creating pFD051. The chloramphenicol resistance gene was amplified from pVSV208 (Dunn *et al.*, 2006) using $5'$TTTTTTGCATGCGTGTTATGAGCCATATTCAAC$3'$ and $5'$TTTTTTCTCGAGCAATAACTGCCTTAAAAAAATTAC$3'$, cloned in pFD051 using *Sph*I + *Xho*I, creating pFD052. The kanamycin resistance gene was amplified from pVSV102 (Dunn *et al.*, 2006) using $5'$TTTTTTGCATGCGGGGTGCCTAATGAGTGAG$3'$ and $5'$TTTTTACGCGTGCTCTGCCAGTGTTACAAC$3'$, cloned in pFD051 using *Sph*I + *Mlu*I, creating pFD061.

2.3. Construction of the bioreporters

The P*flgFGH* promoter of the *flgFGH* operon of *V. harveyi* ORM4 (unpublished genome) was amplified using $5'$TTTTTTCTAGAGCTACAACAAAATATCCTGC$3'$ and $5'$TTTTTGTCGACGCTCATTGCGAGAACAGTG$3'$ and cloned in pJLS199 using *Xba*I +

*Sal*I, creating pFD058. The P*lac* promoter was digested from pVSV102 (Dunn *et al.*, 2006) using *Xho*I + *Nhe*I and cloned into pJLS199 using the same enzymes, creating pFD063.

2.4. Electroporation protocol

V. harveyi and *Pseudoalteromonas* strains were grown overnight at 37 °C and 24 °C, respectively. Overnight cultures were placed on ice and 2 ml aliquots were washed 3 times with 1 ml of an ice-cold modified sucrose buffer (Visick & Ruby, 1996), containing 272 mM sucrose, 1 mM MgCl$_2$, 7 mM K$_2$HPO$_4$ adjusted to pH 7.5 and sterilized by autoclaving. Immediately before use, 15% glycerol (f.c.) was added to the buffer to avoid cell lysis. After 3 washing steps, electrocompetent cells were concentrated in 50 µl with the same buffer and placed in an ice-cold 1 mm electroporation cuvette, mixed with plasmid DNA and directly electroporated with a Gene Pulser II + Pulse Controller Plus (Biorad) set at 1.5 kV, 200 Ω resistance and 25 µF capacitance. Immediately after electroporation, 1 ml LBS for *V. harveyi* or MLB for *Pseudoalteromonas* was added, and cells were allowed to recover for 4 h at 37°C or 24°C for *V. harveyi* and *Pseudoalteromonas*, respectively. Cell suspensions were then plated on selective plates and incubated overnight at 28°C for *V. harveyi* and for 2-3 days at 20 °C for *Pseudoalteromonas*.

2.5. Microscopy experiments

One colony of pFD058- or pFD063-bearing strains of *V. harveyi* ORM4 was inoculated in 5 ml LBS+Trim. 100 µl of each overnight suspension were used to inoculate 5 ml LBS+Trim. Microscopy was performed after overnight growth and after 2 h, 4 h and 6 h of growth, in triplicates. Images were acquired using the Zeiss Axio Imager.M2 microscope equipped with a 63X Apochromat oil objective, at 200 ms exposure time for GFP (with 10% and 100% LED intensity, for pFD063 and pFD058, respectively). Images were analysed using an in-house written Matlab script, with at least 1000 cells per replicate (Delavat *et al.*, 2016).

3. Results and discussion

3.1 An electroporation method to transform V. harveyi and Pseudoalteromonas strains

A one-day-long electroporation protocol was sufficient to obtain several hundreds of trimethoprim-resistant colonies of *V. harveyi* ORM4 when transformed with pFD063.

Efficient electroporation was obtained when using overnight cultures of *V. harveyi* ORM4 grown at 37°C, while no transformant was obtained when cells were grown at 28°C or when using exponentially-growing cells at 37°C (Table 2). Presence of the plasmid in all tested colonies of *V. harveyi* ORM4 was confirmed by colony-PCR verification (at least 8 colonies tested per electroporation, at least 5 independent experiments performed) and by a bright green colour of the colony (Figure 1A). This temperature-dependent electroporation efficiency might be surprising, but it is supposed that cells grown under supra-optimal temperature (37°C instead of 28°C) have an altered restriction-modification system, making them more prone to accept foreign DNA, as demonstrated for *Pseudomonas aeruginosa* (Rolfe & Holloway, 1969).

Typically, 200 ng of plasmid DNA were sufficient to obtain at least 300-400 colonies, *i.e.* to get a transformation efficiency of at least 1.5×10^3 CFU/µg DNA. Moreover, the number of CFUs obtained was in the same range when using as low as 50 ng of plasmid DNA (transformation efficiency of 9×10^2 s.d 2.5×10^2 CFU/µg DNA), giving the possibility to transform low-copy number plasmids. pFD063 is a rather small plasmid (4.8 kb) but successful electroporation was also obtained with the 6.5 kb-long pVSV208 (Dunn *et al.*, 2006), albeit with a lower efficiency (around $2-3 \times 10^2$ CFU/µg DNA).

In addition, the washing buffer used to prepare competent cells contains 15% glycerol (f.c.), allowing competent-cells to be flash-frozen in liquid nitrogen and stored at -80°C until further use. Cells retain full transformability after having been stored for at least one month at -80°C, with several hundreds of CFUs obtained with 200 ng of pFD063 (transformation efficiency of at least 6×10^3 CFU/µg DNA).

Using the exact same protocol as for *V. harveyi* ORM4, we could also transform by electroporation *V. harveyi* LMG 7890, isolated from a dead brown shark kidney, with 200 ng of the pFD063 plasmid DNA. This resulted in hundreds of pFD063-bearing colonies of *V. harveyi* LMG 7890 obtained within one single day (electroporation efficiency between 1.5 and 6×10^3 CFU/µg DNA). In addition, the type strain *V. campbellii* BAA-1116 could also be transformed using the exact same protocol, with an electroporation efficiency reaching 2.7×10^2 CFU/µg DNA s.d. 1.3×10^2). Finally, we applied our protocol on four *Pseudoalteromonas* sp. strains, namely *Pseudoalteromonas* sp. PV1, *Pseudoalteromonas* sp. MV21, *Pseudoalteromonas* sp. 3J3 and *Pseudoalteromonas* sp. 3J6, using the 6.8 kb-long pOriT-4Em (Yu *et al.*, 2014) plasmid. Under these conditions, between 50 and 60 ampicillin-resistant and pOriT-4Em-bearing colonies of *Pseudoalteromonas* sp. MV21 and 3J6 were

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obtained (at least 1.25 x10^2 CFU/µg DNA), while no transformant was obtained for *Pseudoalteromonas* sp. PV1 and 3J3.

Thus, we developed an efficient electroporation method to obtain transformants of *V. harveyi* and *Pseudoalteromonas* strains within one day, with as low as 50 ng of plasmid DNA and with the possibility to store competent cells for future use. We expect this protocol to be easily adapted to other marine environmental strains with a few modifications, presumably at the growing and recovering temperatures used.

3.2. Phenotypic heterogeneity associated with gene expression in V. harveyi ORM4

Replicative plasmids are useful tools for different purposes, *e.g.* in genetics for gene complementation and overexpression or in molecular and synthetic biology to construct bioreporters. Bioreporters are molecular systems engineered to follow and monitor gene expression at the single-cell or population-wide levels. Typically, a bioreporter is a circuit composed of two parts, *i.e.* a reporter gene (*e.g. gfp* or *β-gal*), itself being transcriptionally fused to a promoter whose expression is studied. Fluorescent bioreporters are of particular interest, because their activity can be quantified in real time and at the single-cell level, without the need of external substrates.

A bacterial bioreporter held on a plasmid was constructed, by cloning in *E. coli* the promoter of the *flgFGH* operon of *V. harveyi* ORM4 (unpublished genome) into the pJLS199 plasmid, upstream of the promoterless *gfp* gene. The resulting plasmid pFD058 was transformed back into *V. harveyi* ORM4 by electroporation, and successful introduction of pFD058 in *V. harveyi* ORM4 was confirmed by colony-PCR. Expression of the P$_{flgFGH}$-*gfp* bioreporter was measured in single cells of *V. harveyi* ORM4-pFD058 when grown from exponential phase to stationary phase. Interestingly, while expression from the P$_{flgFGH}$ promoter in *V. harveyi* ORM4 is rather homogeneous during exponential phase, only 9.9% (s.d. 1.6%) of the cells from this isogenic population strongly express this promoter during stationary phase (Figure 1B, white arrow). This bimodal distribution of gene expression is specific to P$_{flgFGH}$, since cells of *V. harveyi* ORM4 carrying a similar bioreporter in which the *gfp* gene is under the control of the constitutive P$_{lac}$ promoter behave generally similarly, whatever the condition (between 95 and 99.7% of the population, Figure 1B). In order to rule out a possible loss of pFD058, *V. harveyi* ORM4-pFD058 grown to stationary phase in LBS+Trim were plated on LBS without antibiotic and then restreaked both on LBS and LBS containing trimethoprim. All of the tested colonies (3 replicates, n=106) were still

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trimethoprim-resistant and contained the pFD058 plasmid. Moreover, the stability of pJLS199-derived plasmids in the absence of selective pressure was quantified by measuring the proportion of cells still bearing pFD058 after circa 40 generations. Under this non-selective condition, 94% (s.d. 10%) still carried the plasmid, highlighting its good stability and confirming the bimodal gene expression from the P_{flgFGH} promoter in *V. harveyi* ORM4 grown to stationary phase.

Phenotypic heterogeneity in bacterial gene expression has been observed in many bacterial processes, *e.g.* for spore formation or competence in *Bacillus subtilis*, during horizontal gene transfer of integrative and conjugative elements (Delavat *et al.*, 2017) or in genes controlled by the quorum sensing system of *V. campbellii* BAA-1116 (Anetzberger *et al.*, 2012). In addition, flagellum expression is known to be prone to phenotypic noise, as discovered in *Salmonella enterica* ssp. I serovar Typhimurium (Freed *et al.*, 2008). However, to our knowledge, nothing is known about the phenotypic heterogeneity associated with flagellum expression in any *V. harveyi* strain nor in any other strain from the genus *Vibrio*.

3.3. Chromosomal insertion of a mini-transposon

While replicative plasmids are useful tools to rapidly introduce foreign DNA, it is sometimes important to stably introduce foreign DNA in chromosomes and express it from a single copy. This is typically achieved by the insertion of mini-transposons. Mini-*Tn*7 transposons insert site-specifically at the 3' end of the *glmS* gene of many Gram-negative bacteria, ruling out insertions in important genes biasing the phenotype of the resulting mutant (Choi *et al.*, 2005). In a first attempt, we sought to use the existing mini-*Tn*7 delivery system, composed of a strain carrying the transposase-encoding pTNS3 plasmid (Choi *et al.*, 2008) and a strain carrying the pUC-mini*Tn*7T-Km plasmid. Tri-parental mating was performed by mixing these strains with *V. harveyi* ORM4. Kanamycin-resistant clones of *V. harveyi* ORM4 containing the mini-Tn7T were obtained under this condition. However, none of them carried a mini-Tn7T integrated at the 3' of *glmS*. Moreover, colony-PCR on the obtained clones demonstrated the presence of the pUC backbone plasmid (data not shown). As pUC-derivative plasmids cannot be stably maintained as episomes in *Vibrio* species, it is likely that the entire pUC-mini-*Tn*7T-Km plasmid has inserted somewhere in the genome by homologous recombination.

We hypothesized that the limiting factor of transposition was the transient expression of the transposase from pTNS3. To test this hypothesis, the smallest fragment containing the

origin of replication from the pES213 (Dunn *et al.*, 2005) and a chloramphenicol-resistance gene were cloned into pTNS3. The resulting plasmid (pFD052) was introduced in *V. harveyi* ORM4 by tri-parental mating, and chloramphenicol-resistant colonies of *V. harveyi* ORM4 were obtained. Presence of the pFD052 plasmid was confirmed by colony-PCR. Bi-parental mating between *V. harveyi* ORM4 and *E. coli* carrying the pUC-mini-*Tn*7T-Km plasmid was performed, and all *V. harveyi* ORM4 transconjugants obtained on LBS+kanamycin contained the mini-*Tn*7T-Km transposon. Moreover, the mini-*Tn*7T-Km transposon was inserted in 7 out of the 8 tested clones at the 3' end of the *glmS* gene. 5 of those clones were randomly chosen for a southern blotting experiment, which confirmed that insertion occurred at a single location within the entire genome of *V. harveyi* ORM4 (data not shown). Finally, an overnight growth of one positive clone in LBS+Km without chloramphenicol was sufficient to cure out the pFD052 plasmid in all of the tested colonies (n=64).

We next replaced the chloramphenicol-resistance gene in pFD052 by the kanamycin-resistance gene, and the resulting plasmid (pFD061) was again introduced by tri-parental mating in *V. harveyi* ORM4. Electrocompetent cells of the resulting strain were then prepared and transformed with the pUC-mini-*Tn*7T-Cm plasmid. Cm-resistant clones were obtained, and most of them had the mini-*Tn*7T-Cm transposon inserted at the 3' of the *glmS* site. By contrast, clones obtained by electroporation of *V. harveyi* ORM4 with both pTNS3 and pUC-mini-*Tn*7T-Cm did not give any transformant on LBS+Cm plates.

We were therefore able to improve the mini-*Tn7* delivery system by the creation of an unstable transposase-producing replicative plasmid for *Vibrio* species, allowing introduction of mini-*Tn*7T transposons, both by conjugation and electroporation.

To conclude, we developed an electroporation protocol enabling the introduction of replicative plasmids (and GFP-labelling) in various *V. harveyi* and *Pseudoalteromonas* strains, and improved the mini-Tn7 delivery system to allow mini-transposon insertions in single copy in the genome of *V. harveyi* ORM4. Moreover, we demonstrated for the first time in *Vibrio* that gene expression from a flagellum promoter is restricted to a subpopulation of the isogenic cell population, highlighting the occurrence of phenotypic heterogeneity in this pathogenic strain for this particular trait. It remains to be discovered whether this phenotypic heterogeneity plays a role in the virulence of this economically important bacterium.

Acknowledgments

Legend to Figure 1.

Images resulting from electroporations of plasmids in *V. harveyi* ORM4. (A), Plate obtained after electroporation of *V. harveyi* ORM4 with pFD063. Note the green colour of the colonies. (B), microscopy images (x630 magnification) of the bioreporters. On the left, bioreporter expression from the P$_{flgFGH}$ promoter. On the right, bioreporter expression from the P$_{lac}$ promoter. Note that only a fraction (white arrow) of the population is expressing GFP from the P$_{flgFGH}$ promoter, while expression from P$_{lac}$ is more homogeneous.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α-λpir	*supE44* D*lacu169 (f80 lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab strain
V. harveyi ORM4	Virulent strain infecting the European abalone *Haliotis tuberculata*	(Nicolas *et al.*, 2002)
V. harveyi LMG 7890	Avirulent strain of *V. harveyi* isolated from the kidney of a dead brown shark	(Grimes *et al.*, 1984)
V. campbellii BAA-1116	Type strain of *V. harveyi*, recently reclassified as *Vibrio campbellii*	(Bassler *et al.*, 1997)
Pseudoalteromonas sp. PV1	Strain of *Pseudoalteromonas* isolated from the indian ocean	Gift from Dr. Alexis Bazire
Pseudoalteromonas sp. MV21	Strain of *Pseudoalteromonas* isolated from the indian ocean	Gift from Dr. Alexis Bazire
Pseudoalteromonas sp. 3J3	Strain isolated from a glass slide immersed in seawater	(Grasland *et al.*, 2003)
Pseudoalteromonas sp. 3J6	Strain isolated from a glass slide immersed in seawater	(Grasland *et al.*, 2003)
Plasmids		
pJLS199	Replicative plasmid for *Vibrio (ori pES213)*. Similar to pJLS198 (Stoudenmire *et al.*, 2018) except the *gfp* gene encodes the original stable GFP variant used in that study. Trim^R	Gift from Pr. E. Stabb
pTNS3	Replicative plasmid for *E. coli*. Expresses *tnsABCD* from *PI* and *lac* promoters. Allows Tn7 insertions at the *glmS* site. Ap^R	(Choi *et al.*, 2008)
pFD051	Derivative of pTNS3 carrying the minimal origin of replication from pEVS213. Replicates in *Vibrio* species. Ap^R	This study
pFD052	Derivative of pFD051 carrying a chloramphenicol resistance gene. Ap^R, Cm^R	This study
pFD058	Derivative of pJLS199 carrying the P*flgFGH* promoter of *V. harveyi* ORM4 cloned upstream of the *gfp* gene. Trim^R	This study
pFD061	Derivative of pFD051 carrying a kanamycin resistance gene. Ap^R, Km^R	This study
pFD063	Derivative of pJLS199 carrying the P*lac* promoter from pVSV102 cloned upstream of the *gfp* gene. Trim^R	This study
pFD066	Derivative of pUC18-mini*Tn7*T-Gm (AY599231) containing the Chloramphenicol resistance gene. Ap^R, Cm^R	This study

pUC18-mini*Tn7*T-Km	Derivative of pUC18-mini*Tn7*T-Gm (AY599231) containing the kanamycin resistance gene. Ap^R, Km^R	Gift from the Masataka Tsuda lab
pOriT-4Em	Replicative plasmid for *Pseudoalteromonas* species. Ap^R, Em^R	(Yu *et al.*, 2014)

Table 2. Electroporation conditions used in this study

Strain	Plasmid used for electroporation	Condition of Electroporation	Electroporation efficiency (CFU/µg DNA)
V. harveyi ORM4	pFD063 (4.8 kb)	Stationary-phase cells grown at 28°C	No transformant
V. harveyi ORM4	pFD063 (4.8 kb)	Exponentially-growing cells at 37°C	No transformant
V. harveyi ORM4	pFD063 (4.8 kb)	Stationary-phase cells grown at 37°C	1.5×10^3 to 6×10^3
V. harveyi ORM4	pVSV208 (6.5 kb)	Stationary-phase cells grown at 37°C	$2-3 \times 10^2$
V. harveyi LMG 7890	pFD063 (4.8 kb)	Stationary-phase cells grown at 37°C	1.5×10^3 to 6×10^3
V. campbellii BAA-1116	pFD063 (4.8 kb)	Stationary-phase cells grown at 37°C	2.7×10^2
Pseudoalteromonas sp. PV1	pOriT-4Em (6.8 kb)	Stationary-phase cells grown at 24°C	No transformant
Pseudoalteromonas sp. MV21	pOriT-4Em (6.8 kb)	Stationary-phase cells grown at 24°C	1.25×10^2
Pseudoalteromonas sp. 3J3	pOriT-4Em (6.8 kb)	Stationary-phase cells grown at 24°C	No transformant
Pseudoalteromonas sp. 3J6	pOriT-4Em (6.8 kb)	Stationary-phase cells grown at 24°C	1.25×10^2

Conflict of interest

The authors declare that they have no competing interests.

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- Development of an electroporation protocol for *Vibrio harveyi* and *Pseudoalteromonas*
- New genetic tool for the insertion of mini-*Tn*7 transposons in *V. harveyi*
- A flagellum promoter of *V. harveyi* is subjected to phenotypic heterogeneity