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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*.

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 and 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*.

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Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α-λpir	*supE44* Dlac*u169 (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. 3 J3	Strain isolated from a glass slide immersed in seawater	(Grasland et al., 2003)
Pseudoalteromonas sp. 3 J6	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*Tn*7T-Gm (AY599231) containing the Chloramphenicol resistance gene. Ap^R, Cm^R	This study
pUC18-mini*Tn*7T-Km	Derivative of pUC18-mini*Tn*7T-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)

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′TTTTTTGCATGCACGCGTATCGATCTCGAGAACTATCAATAGAACGTGTATTTA3′ and 5′TTTTTTGTCGACGCAGCTCTTAATATTTTCTTTTT3′, 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′TTTTTTGCATGCGTGTTATGAGCCATATTCAAC3′ and 5′TTTTTTCTCGAGCAATAACTGCCTTAAAAAAATTAC3′, cloned in pFD051 using *Sph*I + *Xho*I, creating pFD052. The kanamycin resistance gene was amplified from pVSV102 (Dunn et al., 2006) using 5′TTTTTTGCATGCGGGGTGCCTAATGAGTGAG3′ and 5′TTTTTACGCGTGCTCTGCCAGTGTTACAAC3′, 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′TTTTTTCTAGAGCTACAACAAAATATCCTGC3′ and 5′TTTTTGTCGACGCTCATTGCGAGAAACAGTG3′ 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 and Ruby, 1996), containing 272 mM sucrose, 1 mM $MgCl_2$, 7 mM K_2HPO_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 63× 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