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Simple conjugation and outgrowth procedures for tagging vibrios with GFP, and factors affecting the stable expression of the *gfp* tag

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Abbreviations: GFP, green fluorescent protein; DAPI, 4', 6-diamidino-2-phenylindole; CCD, charged coupled devise.

Abstract

Aim: Our goal was to develop a simple system for tagging wild-type marine bacteria with *gfp*.

Methods and Results: *Escherichia coli* strain CC118λ*pir* carrying the conjugative helper plasmid pEVS104 and the *gfp*-containing plasmid pKV111 was used to transfer *gfp* to *Vibrio* recipients. Four different media were tested for their ability to support the growth of recipients, but not the *E. coli* donor, to allow powerful enrichment of *gfp*-tagged wild-type vibrios from mating mixes. Forty-three vibrio strains, representing thirty-nine different species, were successfully tagged with *gfp* using conjugative transfer from *E. coli* followed by selective outgrowth at 15°C on ZoBell 2216E agar containing 0.5% sodium alginate. Using this outgrowth medium, colonies of GFP-expressing vibrio clones were detectable within 4 days. The percentage of visibly fluorescent cells in three representative GFP-tagged vibrios was higher at 15°C than at 20- or 25°C (ca 50% vs. 45% or 40%, respectively), and was also higher during aerobic rather than anaerobic culturing (ca 50% vs. 35%, respectively).

Conclusions: We found a simple selective outgrowth technique that enabled us to isolate a wide variety of GFP-tagged marine vibrios following conjugative transfer of *gfp* from *E. coli*.

Significance and Impact of Study: Tagging cells with GFP and related fluorescent proteins is a powerful approach for investigating bacteria *in situ*, particularly during colonization of hosts. The simple and cost-effective outgrowth condition described in this study could be applied to construct a wide variety *gfp*-tagged marine bacteria.

Introduction

Fluorescent molecules that are stable and resistant to photobleaching make ideal tags to monitor microorganisms in complex multi-organism settings, and can be used to monitor gene expression or to localize particular cells or proteins (Valdivia and Falkow 1997; Unge *et al*. 1998; Southward and Surette 2002; Bloemberg *et al*. 2004). Green fluorescent protein (GFP) and its derivatives or relatives are widely used as fluorescent tags in such studies (Prasher *et al*. 1992; Chalfile *et al*. 1994; Heim *et al*. 1994; Bloemberg *et al*. 2004; Dunn *et al*. 2006). Tagging microorganisms with GFP has been a useful experimental approach, allowing researchers to view the behavior of particular microbes *in situ*, particularly during interactions with host tissues (Gage *et al.* 1996; Unge *et al*. 1998; Stabb *et al*. 2000; Nyholm *et al*. 2000; Ling *et al*. 2001; Dunn *et al*. 2006).

Despite the power of this approach, most bacterial species have not been tagged with GFP (Unge *et al*. 1998). In most instances where GFP has been used as a marker, bacteria have been cultured and transformed with the *gfp* gene. This methodology is necessarily limited to culturable bacteria, and even then there may be obstacles to transformation. For example, direct transformation with plasmid DNA is often severely limited by restriction/modification systems. Conjugation of *gfp*-containing plasmids into a strain of choice from *Escherichia coli* bypasses this limitation, because the single stranded DNA intermediate transferred is not a substrate for most restriction enzymes. This approach raises the technical problem that after conjugative transfer the recipient cells must be separated from the *E. coli* donor, which is often accomplished using spontaneous antibiotic-resistant mutants as recipients and counterselecting against *E. coli* with antibiotics after conjugative transfer. However, these antibiotic-resistant recipients are not true wild-type strains,

and often have unexpected pleiotropic phenotypes.

Such challenges are illustrated in the isolation of *gfp*-tagged *Vibrio fischeri*. Electroporation of plasmid DNA into *V. fischeri* was inefficient and relied on an undefined mutant receptive to exogenous DNA (Visick and Ruby 1996). Similarly, conjugative transfer of plasmids from *E. coli* to *V. fischeri* was first accomplished using rifampicin-resistant mutant ESR1 as the recipient, allowing selection against the *E. coli* donor on rifampicin-containing media (Graf *et al*. 1994). However, rifampicin-resistant mutants are affected in multiple unpredictable phenotypes (Jin and Gross 1989), and ESR1 did not colonize its symbiotic host, the Hawaiian squid *Euprymna scolopes*, as well as its parent ES114 did (McCann *et al*. 2003). Recently a procedure was developed to separate ES114 from *E. coli* following conjugation (Stabb and Ruby 2002), and once this wild-type *V. fischeri* was labeled with *gfp* the events leading to infection of *E. scolopes* were directly visualized (Nyholm *et al*. 2000; Dunn *et al*. 2006).

Given this success with *V. fischeri*, expanding the use of *gfp* to more vibrios appears worthwhile. Vibrios are ubiquitous and important marine bacteria, encompassing eighty recognized species of γ-proteobacteria belonging to the *Vibrionaceae*, *Enterovibrionaceae*, *Photobacteriaceae*, and *Salinivibrionaceae* families (Farmer and Hickman-Brenner 1999; Thompson *et al*. 2004). Like *V. fischeri*, many vibrios associate with animals (McFall-Ngai and Ruby 1991; Haygood 1993; Farmer and Hickman-Brenner 1999; Austin and Austin 1999; Thompson *et al*. 2004; Sawabe 2006). For example, *V. cholerae* causes cholera in humans and also colonizes planktonic crustaceans (Colwell 1996). Other human pathogens such as *V. parahaemolyticus* and *V. vulnificus* have diverse mechanisms for associating with hosts, and probably have as yet

unknown associations with marine animals (Farmer and Hickman-Brenner 1999; Makino *et al*. 2003, Park *et al*. 2004). Similarly, the broad host range pathogen *V. harveyi* causes mass mortalities in a wide variety of fish and shellfish (Grimes *et al*. 1984; Austin and Austin 1999; Nicholas *et al*. 2002). On the other hand, several bioluminescent vibrios enter specific mutualistic symbioses (McFall-Ngai and Ruby 1991; Haygood 1993; Ruby and Lee 1998; Visick and McFall-Ngai 2000; Nyholm *et al*. 2002), and *V. halioticoli* appears to be a beneficial gut symbiont that has a coevolved partnership with in abalone (Sawabe *et al*. 1998a; Sawabe 2006). Fluorescence-based techniques for visualizing these bacteria *in vivo* would help elucidate such varied bacteria-host associations; however, GFP has not been effectively used in most of these vibrios.

One obstacle to the use of GFP in studying vibrio-host interactions is the difficulty of selecting GFP-tagged wild-type vibrios apart from conjugative donor cells of *E. coli*, which grows quickly and is a γ-proteobacterium similar to the vibrios. Although plasmid transfer to *V. fischeri* has been accomplished, the methodology used is not directly applicable to all vibrios, particularly slow-growing species such as *V. halioticoli.* Here, we show a simple outgrowth procedure that effectively separates a wide range of *gfp*-tagged vibrios from donor *E. coli* cells following a conjugative transfer protocol modified from that established for *V. fischeri* (Stabb et al. 2000; Stabb and Ruby 2002).

Materials and Methods

Bacterial strains

Escherichia coli CC118λ*pir* carrying pEVS104 (*tra trb* Kn^r) and also carrying either pKV111

(*gfp* Cm^r) or pKV112 (*gfp* Cm^r Er^r) was used as a conjugative donor of *gfp* (Stabb *et al.* 2000; Stabb and Ruby 2002). These *E. coli* donor strains were cultured on LB agar, or in LB broth, containing 20 μg ml^{-1} chloramphenicol and 40 μg ml^{-1} kanamycin at 37°C. Forty-three vibrio strains belonging to the genera *Vibrio*, *Photobacterium*, *Enterovibrio*, or *Grimontia* (Table 1), as well as *Pseudoalteromomas elyakovii* IAM14594 and *P. haloplanktis* IAM12915^T were obtained from the IAM (Institute of Applied Microbiology, Tokyo University) or LMG (Laboratory Microbiology, Gent University) culture collections and were streak plated to confirm their purity. *Flexibacter* sp. and *Sphingomonas* sp. were also used as representatives of diverse phylogenetic groups other than *r*-proteobacteria. These marine bacteria were cultured on ZoBell2216E medium (0.5% polypeptone, 0.1% yeast extract, 1.5% agar, 75% seawater, pH 7.5) at 20°C with the exception of *V. furnisii* (37°C), *V. vulnificus* (30°C), *V. aerogenes* (30°C), and *V. logei* (15°C). All bacteria were stored at -80°C in 20% glycerol.

Testing the selectivity of outgrowth media

Our preliminary data indicated that *E. coli* grew poorly at 15°C on AI2 agar, which contains seawater and alginate (Sawabe *et al.* 1997), and we therefore tested the relative growth of *E. coli* and potential recipient strains on AI2 medium and three other seawater-based media: ZoBell 2216E agar, ZoBell2216E agar containing 0.5% sodium alginate (ZoBell+Alg), and CSY agar (Sawabe *et al.* 1998b). Fresh cultures of representative marine bacteria, *V. halioticoli* IAM14596^T and *P. elyakovii* IAM14594, and the *gfp*-donor *E. coli* were streak plated on the test media and incubated at 15°C and 25°C, respectively. *V. halioticoli* was selected as a representative of slow growing vibrios, and the potential seaweed pathogen *P. elyakovii* was chosen as a representative non-vibrio aerobic marine bacterium (Sawabe *et al*. 2000). Growth of

the bacterial strains on the four media was recorded one, two, and four days after inoculation.

Conjugative transfer and isolation of GFP-tagged vibrios

The *E. coli* donor and vibrio recipients were grown in broth as described above, with *E. coli* incubated overnight and vibrios incubated for 24 hours. 100-μl aliquots of donor *E. coli* and recipient vibrios cultures were mixed in a 1.5 ml tube and centrifuged. The pelleted cells were washed with fresh broth to remove antibiotics, centrifuged again, and the pellet was resuspended in 10 μl of ZoBell broth and dotted onto a ZoBell+Alg agar plate. This mating mix was incubated at 20°C for 1-2 days, the bacterial cells were then recovered, suspended in 700 μl ZoBell broth, and subsamples were plated onto ZoBell+Alg supplemented with chloramphenicol (5-20 $\mu g\ ml^{-1}$) for pKV111-recipients or with erythromycin (20-50 $\mu g\ ml^{-1}$) for pKV112-recipients. Minimum inhibitory concentrations of chloramphenicol or erythromycin were determined for each vibrio recipient, and appropriate concentrations of antibiotics were added to achieve selection for each recipient. Following the mating procedure, these selective plates were incubated at 15°C, and fluorescence of bacterial cells on this medium was observed under an epifluorescense microscope through a No. 10 filter (excitation 450-490 nm, emission 515-565 nm) (Axioscope, Zweiss, Germany) or with a Light Capture system (Model 6960, ATTO, Japan) equipped with a 470 nm light source and YA3 orange filter. GFP expressing clones were isolated to purity using multiple rounds of streak plating for isolated colonies on ZoBell+Alg at 15°C or TCBS agar (Nissui Seiyaku, Japan) at 20-37°C. The identity of the purified GFP-tagged vibrios was confirmed and distinguished from *E. coli* using XM-G medium (Nissui Seiyaku, Tokyo, Japan) and TCBS medium, which are used to selectively identify coliforms and vibrios, respectively.

Determining the ratio of fluorescent and non-fluorescent cells in culture

The effects of temperature and oxygenation on retention and expression of GFP tags was determined for three representative GFP-tagged marine vibrios: *V. halioticoli* IAM14596^T, *V. alginolyticus* LMG4409^T and *V. pelagius* ATCC25916^T. Each GFP-tagged strain was cultured aerobically on ZoBell2216E agar containing 5-20 μg mL^{-1} chloramphenicol at 15, 20, and 25°C for 3-5 days. These three test strains were also grown at 15°C in anaerobic jars (Becton Dickinson, USA). Bacterial cells grown under each condition were suspended in 75% seawater and fixed by the addition of 10% glutaraldehyde (Chalfile *et al*. 2000). These cells were counterstained with DAPI, and collected on 0.2 μm Nuclepore filters. GFP-expressing cells and DAPI-stained cells were enumerated by epifluorescence microscopy, and the ratio of GFP-expressing cells to total (DAPI-stained) cells was determined for each culture.

The ratio of green-fluorescent to non-green-fluorescent cells was also determined under starvation conditions in autoclaved seawater at 15-25°C for the same three representative GFP-tagged vibrios. These strains were grown on ZoBell2216E agar plates containing chloramphenicol at 15-25°C, and one loopful of the GFP-expressing cells was suspended in 10 ml of seawater with and without chloramphenicol. The bacterial suspensions (ca. 10^8 CFU ml^{-1}) were incubated at 15, 20, and 25°C for a week. Samples of the bacterial suspension were taken daily and the decrease in the relative number of visibly GFP-expressing cells was measured as described above.

Results

Simple selective culture to isolate GFP-tagged marine bacteria from *E. coli* donor cells

To find conditions where we could isolate marine vibrios apart from conjugative *E. coli* donor cells following matings, we tested four media frequently used for culture of marine bacteria belonging to the genera *Vibrio* and *Pseudoalteromonas* (Table 2). At 25°C both the *E. coli* donor and the vibrio recipients grew on each medium, and we therefore lowered the incubation temperature to 15°C, which we predicted would provide a relative growth advantage for the vibrios. At 15°C the donor *E. coli* strain formed colonies on CSY plates after two days (Table 2), almost as quickly as the vibrio strains grew, rendering this medium unsuitable for counterselection against *E. coli*. However, *E. coli* did not form colonies on AI2, ZoBell, or ZoBell+Alg media even when incubated for up to four days at 15°C (Table 2). Of these media, faster and more robust growth of the *V. halioticoli* and *P. elyakovii* test strains was observed on ZoBell+Alg and AI2 media (Table 2). Based on these results, we chose outgrowth at 15°C on ZoBell+Alg, supplemented with an antibiotic appropriate to the plasmid being transferred, as an effective way to isolate vibrio transconjugants away from *E. coli* donors after matings.

GFP-tagging a wide variety of vibrios

By outgrowing mating mixes selectively on ZoBell+Alg, we successfully GFP-tagged forty-three vibrios, including thirty species of *Vibrio*, three of *Photobacterium*, one of *Enterovibrio*, and one of *Grimontia* (Table 1). These vibrios included pathogens of both humans and marine animals, as well as mutualistic animal symbionts. We were even able to GFP tag slow-growing psychrophilic vibrios, as well as mesophilic vibrios that are frequently found in association with warm-blood animals, as these strains also rapidly outgrew the *gfp*-donor *E. coli* on ZoBell+Alg plates at 15°C. GFP fluorescence derived from GFP-tagged *V. halioticoli* IAM14596^{T} was bright green under epifluorescence microscope (Fig. 1A), and the fluorescence from the colonies was

also recorded by cooled-CCD camera system (Fig. 1B). Unfortunately, *P. elyakovii* and *P. haloplanktis* were not tagged with GFP using this conjugative system (Table 1).

Effect of culture conditions and starvation on the percentage of cells visibly expressing GFP

The percentage of cells visibly expressing GFP, determined microscopically based on their green fluorescence, was higher at lower culture temperature in three representative marine vibrios: *V. halioticoli* IAM14596^T, *V. alginolyticus* LMG4409^T and *V. pelagius* ATCC25916^T. The average percentages of green fluorescent cells were 49-54%, 44-47%, and 40-43% in cultures grown at 15°C, 20°C, and 25°C, respectively (Fig. 2). The average percentage of green fluorescent cells in these three GFP-tagged vibrio strains was also 16% lower when GFP-tagged cells were grown at 15°C in anaerobic jars (Fig. 3). Finally, we found that the number of visibly green fluorescent cells decreased over six days in nutrient poor conditions (e.g. in suspended in seawater) either with or without chloramphenicol present to maintain selective pressure for the *gfp*-containing plasmid pKV111 (Fig. 4). However, 30-40% of the cells were still fluorescent even after 4 days under these starvation conditions (Fig. 4).

Discussion

A critical step for constructing GFP-tagged marine bacteria by conjugative transfer of *gfp*-containing plasmids is the selective outgrowth following matings, wherein the *gfp*-transformed recipients must be significantly enriched to separate them from *gfp*-donor *E. coli* cells. We found that low temperature and high-osmolarity marine-based media are suitable for selective culture of vibrio recipients apart from donor *E. coli*. Among four media tested, we found that ZoBell+Alg agar was a suitable, simple selective culture medium for constructing GFP-tagged vibrios at 15°C

(Tables 1 and 2). The mechanism of selection against the *gfp*-donor *E. coli* on alginate-containing seawater medium is unknown; however, the osmolarity, nutritional balance, and low temperature may all contribute to this condition supporting much better growth of marine bacteria than of *E. coli*. Temperature may be especially important, because *E. coli* grew on ZoBell+Alg at 25°C well enough to support conjugative transfer to vibrio recipients. Only after mating mixes were suspended and plated selectively at 15°C was the most striking difference between the growth of vibrios and *E. coli* observed.

We applied this selective outgrowth technique to isolate transconjugants of thirty-four species from the *Vibrionaceae* family (Table 1). The species successfully labeled with GFP included the recently described coral pathogen *V. coralliilyticus* (Ben-Haim *et al*. 2003), important human opportunistic human pathogens like *V. parahaemolyticus* and *V. vulnificus*, as wells as commensal or mutualistic symbiotic species such as *V. logei* and *V. halioticoli* (Haygood 1993; Ruby and Lee 1998; Sawabe 2006). Thus, the use of GFP to visualize a variety of vibrio-animal interactions can now be examined using the GFP-tagged strains constructed in this study. The labeling of *V. halioticoli* with GFP is particularly significant, because this relatively slow-growing vibrio presented a greater challenge for selective enrichment than faster growing symbionts like *V. fischeri*.

Once cells were tagged with *gfp*-containing plasmids, the ratio of visibly fluorescent cells was affected by temperature and oxygen in the cultures (Figures 2 and 3). The rather low percentage (50%) of GFP-expressing cells observed in three representative vibrio species was unexpected. Loss of the plasmid in a subpopulation may partially explain this phenomenon; however, it is

worth noting that number of non-fluorescent cells was largely unaffected by the presence of chloramphenicol, which should select against plasmid-free derivatives (e.g. Fig. 4). Lack of fluorescence in a subpopulation may also be due to plasmid rearrangements that remove the *gfp* gene while maintaining chloramphenicol resistance or by asynchronous GFP expression on a plate culture. It is also possible that some GFP is destroyed by the glutaraldehyde fixation process, although it is not clear why this would occur in some cells and not others.

Whatever the reason for variable green fluorescence, to optimize the use pKV111 as a GFP tag in these vibrios it may be necessary to use 15°C conditions to obtain an abundant population of traceable GFP-expressing cells. It is worth noting, however, that 15°C is lower than the optimum growth temperatures of *V. halioticoli* (25°C), *V. pelagius* (30°C) and *V. alginolyticus* (35°C). Recently, Dunn *et al*. (2006) reported that plasmids based on pES213, which was originally isolated from *V. fischeri*, were more stable in vibrio cells without antibiotic selection than vectors that, like pKV111, are based on the plasmid p15A replication origin. More stable expression of *gfp* in a wide range of vibrios may be accomplished using this new pES213-based plasmid system, combined with the methods described in this paper for conjugative transfer and selective outgrowth.

Vibrios are facultative anaerobes, and some species are present in the normal gut microbiota of marine animals (Farmer and Hickman-Brenner 1999; Thompson *et al*. 2004; Sawabe 2006). To better understand vibrio-animal interactions, GFP-tagged vibrios should be useful in gut environments, which are naturally anaerobic or microaerobic conditions. Although GFP-expressing cell were observed in *V. halioticoli*, *V. alginolyticus*, and *V. pelagius* in anaerobic

cultures, the percentage of fluorescent cells was as low as 33-38% (Fig. 3). Again, this problem may be fixed with a more stable vector system, but it may also be due to the fact that oxidation of the GFP fluorophore (Ser-Tyr-Gly sequence at position 65-67) is required for full maturation, and therefore GFP might lose some fluorescence under anaerobic culture condition (Heim *et al*. 1994; Hansen *et al*. 2001). GFP fluorescence expressed in GFP-tagged *Streptococcus gordonii* under oxygen-limited biofilms is recovered within 20 min after shifting from anaerobic to aerobic conditions (Hansen *et al*. 2001), and such a shift may be useful in studies of gut bacteria. It is noteworthy though that many (over a third) of the vibrio cells were fluorescent in anaerobic jars, suggesting that GFP-tagged vibrios may be directly used for studies of vibrio-animal associations in oxygen-limited conditions, even if fluorescence is not maximal in all cells.

Under nutrient-depleted conditions, three representative marine vibrios gradually lost the green fluorescence of their GFP tags over four days, and rapidly lost green fluorescence in their populations thereafter (Fig. 4). This occurred even if chloramphenicol was added to seawater to select against plasmid-free cells (Fig. 4). Thus, our results suggest that the GFP tag from pKV111 is best used for up to four days under starvation conditions. In symbiotic experiments, the GFP-tagged vibrio strain(s) may receive host-derived nutrient support when coexisting with live host animals. Therefore, we speculate that the GFP-tagged vibrios could remain detectable for longer periods in closed aquaria under the selection pressure of antibiotics during growth in a host.

Recently, a bright red fluorescent protein (RFP) was developed from DsRed, and an RFP-tagging system for *V. fischeri* was established (Dunn *et al*. 2006). That study used essentially the same conjugative transfer system as that described here, with slight differences in temperature and

media used. Thus, we expect that the simple selective culture system presented here can be adapted for use in the construction of a variety of RFP-tagged vibrios. The combination of GFP- and RFP-tagged vibrio strains may provide new approaches for dual-strain visualization and thereby reveal unexpected new findings in *Vibrio*-animal associations.

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Figure Legends

Fig. 1. Images of GFP-tagged *Vibrio halioticoli* IAM 14596^T taken (A) microscopically of individual cells, or (B) of colonies using a CCD camera.

Fig. 2. Effect of temperature on the percentage of green fluorescent cells in culture. The percentage was expressed as the average, and error bars indicate standard deviation (n=3). Close bar: 15 °C, shaded bar: 20 °C, open bar: 25 °C.

Fig. 3. Effect of oxygenation on the percentage of green fluorescent cells in culture. The percentage of fluorescent cells was expressed as the average, and error bars indicate standard deviation (n=3). Closed bar: Aerobic culture, open bar: Anaerobic culture.

Fig. 4. Decreasing ratio of GFP-expressing vibrio cells over time following dilution into seawater. The percentage of fluorescent cells was expressed as the average, and error bars indicate standard deviation (n=3). (A) *Vibrio halioticoli*, (B) *Vibrio pelagius*, and (C) *Vibrio alginolyticus*. Cells were suspended in chloramphenicol-containing seawater at 15°C (closed circle), 20°C (closed triangle), and 25°C (closed square), or in seawater without antibiotics at 15°C (open circle), 20°C (open triangle), and 25°C (open square).

Table 1. Bacterial strains used for GFP tag

Species	Strain	Concentration of antibiotics used for selection (ug ml^{-1})*	Common niche or isolation location	GFP tag
Enterovibrio norvegicus	LMG19840	Cm 10	Gut of healthy Norwegian turbot	+
Grimontia hollisae	LMG17719^T	Cm 5	Stool, seawater, and digestive tracts	+
Photobacterium angustum	LMG8455^T	Cm 5	Seawater	+
Photobacterium damselae	LMG7892^T	Cm 5	Diseased fish and wound of human	+
Photobacterium leiognathi	LMG4228^T	Cm 10	Light organs and seawater	+
Vibrio aerogenes	LMG19650^T	Cm 5	Seagrass bed	+
Vibrio aestuarianus	LMG7909^T	Cm 10	Seawater	+
Vibrio alginolyticus	LMG4409^T	Cm 20; Er 50	Seawater and digestive tracts	+
Vibrio 'algosus'	LMG7905	Cm 5	Seaweed	+
Vibrio campbellii	LMG11216^T	Cm 5	Seawater and digestive tracts	+
Vibrio cincinnatiensis	LMG7891^T	Cm 5	Seawater	+
Vibrio corallylitica	LMG20984^T	Cm 5	Diseasd corals	+
Vibrio cyclitrophicus	LMG21359^T	Cm 10	Brackish water, seawater and mud	+
Vibrio diazotrophicus	LMG7893^T	Cm 5	Seawater	+
Vibrio fischeri	LMG4414^T	Cm 5; Er 50	Light organs, digestive tracts, and seawater	+
Vibrio fluvialis	LMG7894^T	Cm 5	Seawater	+
Vibrio furnissii	LMG7910^T	Cm 50	Seawater	+
Vibrio gallicus	HT1-26	Cm 10	Gut of French abalone	+
Vibrio halioticoli	IAM14596^T	Cm 5; Er 10	Gut of Japanese abalone	+
Vibrio harveyi	LMG4044^T	Cm 20	Dead decapods, digestive tracts and seawater	+
Vibrio harveyi	LMG7890	Cm 10	Diseased shark	+
Vibrio harveyi	S35	Cm 5	Diseased abalone	+
Vibrio harveyi	S20	Cm 5	Diseased abalone	+
Vibrio logei	LMG14011	Cm 5	Light organs and seawater	+
Vibrio mediterranei	LMG11258^T	Cm 5; Er 50	Seawater and diseased coral	+
Vibrio metschnikovii	LMG11664^T	Cm 5	Seawater and brackish water	+
Vibrio mytili	LMG19157^T	Cm 5	Mussels	+
Vibrio mytilli	LMG16865	Cm 20	Mussels	+
Vibrio natriegens	LMG10935^T	Cm 5	Seawater	+
Vibrio navarrensis	LMG15976^T	Cm 5	Sewage	+
Vibrio nereis	LMG3895^T	Cm 20	Seawater	+
Vibrio nigripulchritudo	LMG3896^T	Cm 5; Er 10	Seawater	+
Vibrio ordalii	LMG13544^T	Cm 5	Diseased fish and seawater	+
Vibrio orientalis	LMG7897^T	Cm 5	Seawater	+
Vibrio parahaemolyticus	LMG2850^T	Cm 5	Bivalves, digestive tracts, mud, seawater and stools	+
Vibrio pelagius	ATCC25916^T	Cm 10	Seawater	+
Vibrio proteolyticus	LMG3772^T	Cm 10	Seawater	+
Vibrio rumoiensis	S-1-6^T	Cm 5	Waste water	+
Vibrio splendidus	LMG19031^T	Cm 5	Seawater	+
Vibrio superstes	B2-3	Cm 10	Gut of Australian abalone	+
Vibrio tapetis	LMG19706^T	Cm 5	Diseased clam	+
Vibrio tubiashii	LMG10936^T	Cm 5	Diseased clam and oyster	+
Vibrio vulnificus	LMG13545^T	Cm 5; Er 50	Brackish water and mud	+
Flexibacter sp.		Cm 10	Diseased marine animals	-
Pseudoalteromonas elyakovii	IAM14594	Er 50	Seawater and seaweed	-
Pseudoalteromonas haloplanktis	IAM12915^T	Er 50	Seawater	-
Sphingomonas sp.		Cm 5	Seawater	-

* Cm: Chloramphenicol; Er: Erythromycin. Some strains were GFP tagged with pKV112 under erythromycin selection.

Table 2 Outgrowth conditions of *gfp* -recipient marine bacteria on various selective agar plates using *gfp* -donor *Escherichia coli*

Media	Incubation period (Days)	Growth[+]		
		E. coli	*V. halioticoli*	*P. elyakovii*
ZoBell+Alg[*]	1	-	-	-
	2	-	-	+
	4	-	+	+
ZoBell	1	-	-	+
	2	-	-	+
	4	-	-	+
CSY	1	-	+	+
	2	+	+	+
	4	+	+	+
AI2	1	-	-	-
	2	-	-	+
	4	-	+	+

* ZoBell+Alg: ZoBell agar containing 0.5% sodium alginate

+ Growth is reported at 15℃: -, no growth; +, growth.

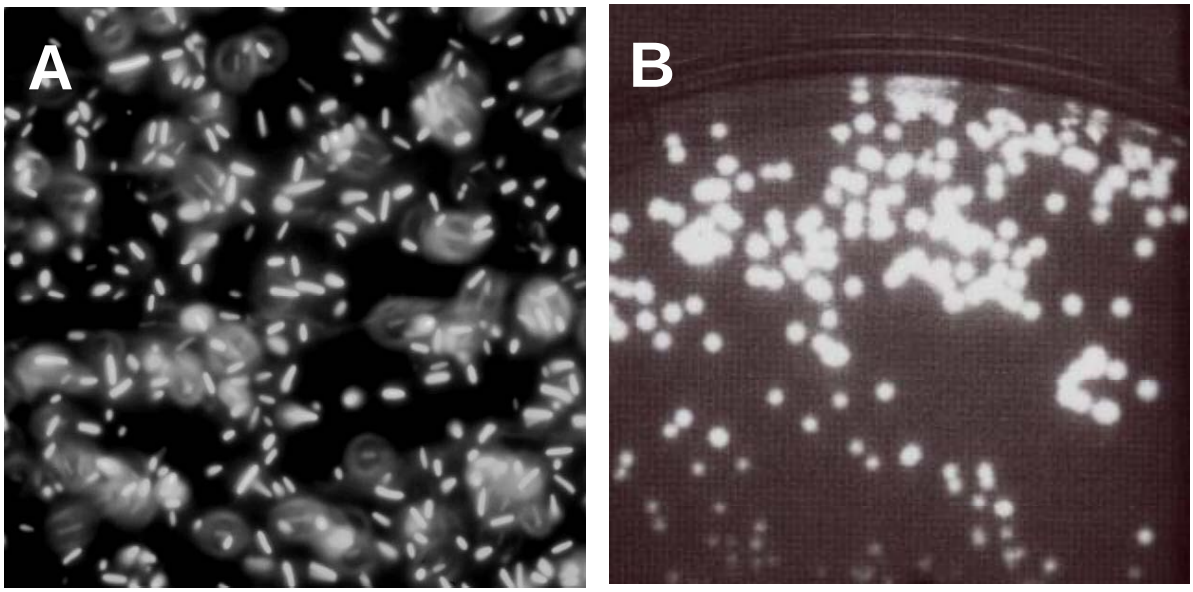

Fig. 1. Sawabe et al.

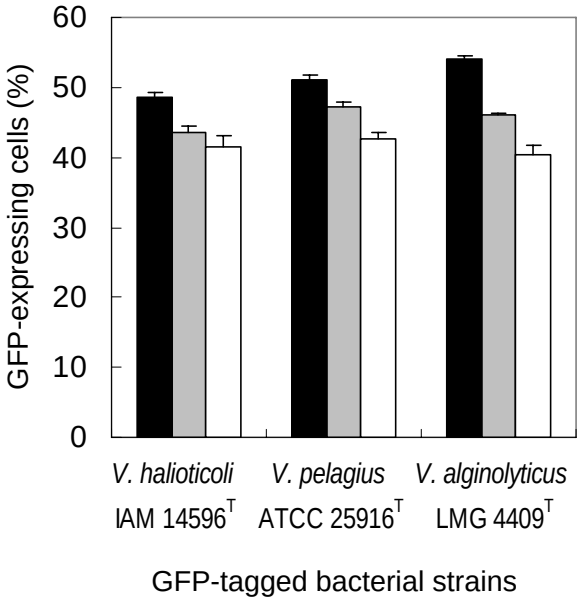

Fig. 2. Sawabe et al.

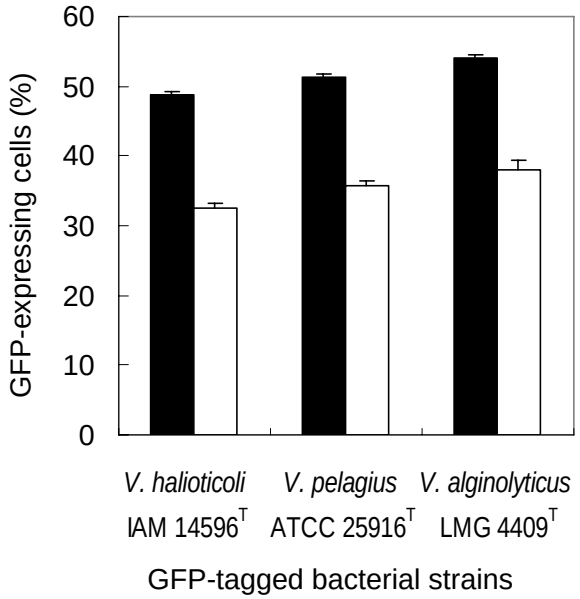

Fig. 3. Sawabe et al.

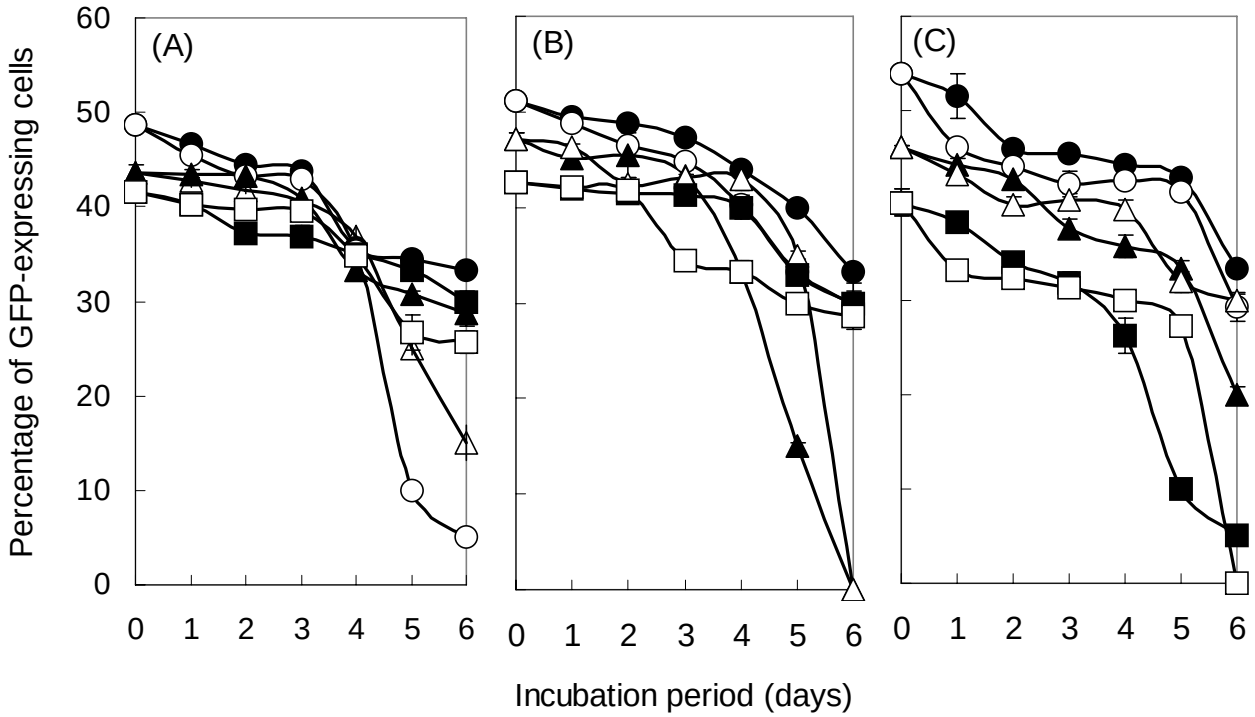

Fig. 4. Sawabe et al.