# Achieving antibiotic-free shrimp farming using diverse antagonistic bacteria


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**Research objective**

Penaeid shrimp aquaculture is continuously expanding worldwide. However, fish are frequently exposed to diseases caused by pathogenic bacteria and viruses, and the application of adaptive immunity-based vaccines to invertebrates such as penaeid shrimp is difficult. Therefore, biological control methods that use antagonistic bacteria to suppress the growth of pathogens are attracting attention as a strategy against fish diseases in marine invertebrate aquaculture. Bacterial culture methods using agar plates are commonly used to isolate new useful bacteria from the natural environment. However, the large amounts of space, materials, and labor required by such methods become a bottleneck in the response to constantly emerging infectious diseases. In recent years, it has become possible to efficiently cultivate microorganisms using microdroplets with a diameter of 30–100 μm as culture compartments, created using microfluidic channels and fluorine oil.[1)] Therefore, the aim of this study was to use microdroplets as culture compartments for the high-throughput screening and isolation of antagonistic bacteria from penaeid shrimp aquaculture for practical use.

**Methods**

In this study, the kuruma shrimp (*Penaeus japonicus*) was selected as the target organism for infection tests, whereas *Vibrio harveyi*[2)] isolated from a kuruma shrimp farm in Japan was used as a model pathogenic bacterium for screening antagonistic bacteria.

**Generation of a *V. harveyi* strain constitutively expressing green fluorescent protein**

For gene introduction into *Vibrio* species, which are gram-negative non-model bacteria, we used *Escherichia coli* conjugation as a homologous recombination system. A composite sequence consisting of the *V. harveyi*-derived 16S rRNA gene and a gene sequence for the constitutive expression of enhanced green fluorescent protein (eGFP) was inserted into the suicide vector pRE112, which was then introduced into *E. coli* SM10 cells via electroporation. Subsequently, the vector-carrying SM10 strain and *V. harveyi* were co-cultured on an HI agar plate with a 0.02 μm membrane filter to promote conjugal transfer of the vector. *V. harveyi* cells that had successfully undergone homologous recombination were selected on the basis

of the chloramphenicol resistance gene incorporated in pRE112. Subsequently, the suicide vector was excluded by culturing the bacterial cells in a sucrose-containing medium, and *V. harveyi* with eGFP integrated at the target position was prepared. The resulting fluorescent strain was subjected to whole-genome resequencing using the long-read sequencer GridION (Oxford Nanopore Technologies, UK) to confirm the successful introduction of the target gene into the *V. harveyi* chromosome.

**Microdroplet screening of antagonistic bacteria using fluorescent *V. harveyi***

The bacterial flora in kuruma shrimp rearing water was selected as a source for screening antagonistic bacteria. The rearing water was passed through a 0.22 μm membrane filter, after which the filter with the collected bacterial flora was immersed in Marine Broth (Becton, Dickinson and Company, USA) for pre-culture of the microorganisms. This culture sample and the eGFP-expressing *V. harveyi* were co-encapsulated in 30-μm-diameter microdroplets using an On-chip droplet generator (On-chip Biotechnologies, Japan) and cultured statically at 25°C. After cultivation, microdroplets with low fluorescence intensity were sorted into a 96-well plate using an On-chip droplet selector (On-chip Biotechnologies). The isolated bacteria were re-cultured in Marine Broth and stored at –80°C in a glycerol stock with a final concentration of 15%.

**Analysis of isolated candidate antagonistic bacterial strains**

The full-length 16S rRNA gene sequences of the isolated strains were sequenced using Sanger sequencing. Phylogenetic analysis based on the obtained sequences was performed using MAFFT software. Additionally, VSEARCH was used to cluster operational taxonomic units with 99% sequence similarity. To investigate the ability of the candidate antagonistic bacterial strains to control infectious pathogens, kuruma shrimp were co-infected with *V. harveyi* and the candidate strains via intramuscular injection, and changes in the shrimp mortality rate were determined.

**Results**

**Generation of a green fluorescent protein-expressing *V. harveyi* strain**

To enable high-throughput screening, we generated a *V. harveyi* strain that expresses eGFP constitutively. The successful integration of the eGFP expression cassette into the 16S rRNA gene locus on the bacterial chromosome was confirmed. The resulting strain exhibited stable green fluorescence under a microscope (Figure 1A), and whole-genome resequencing confirmed the correct insertion of the gene cassette (Figure 1B).

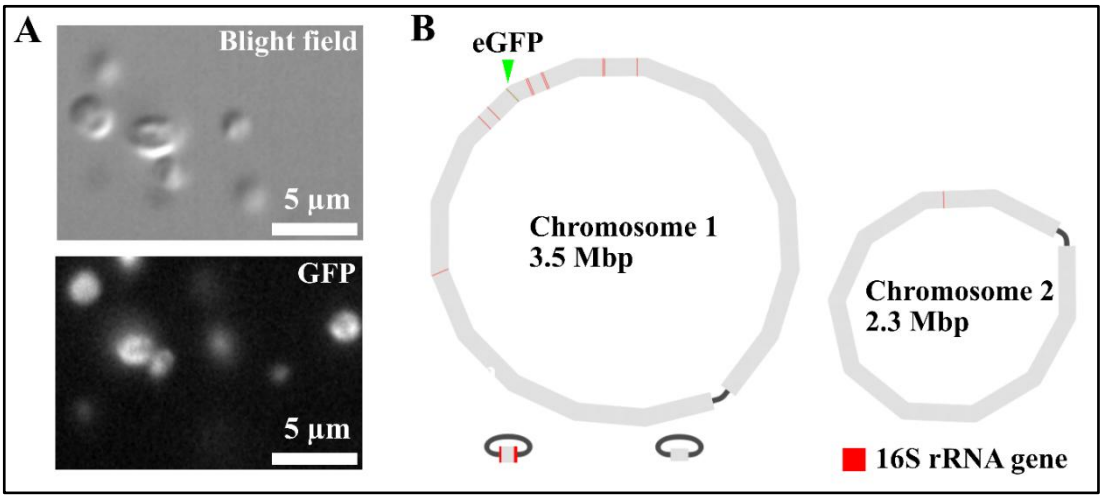


**Figure 1.** Generated *Vibrio harveyi* strain constitutively expressing green fluorescent protein (GFP). (A) GFP-expressing *V. harveyi* cells in bright- and fluorescent-field views. (B) Whole-genome assembly map of GFP-expressing *V. harveyi* visualized using Bandage.

## Isolation of candidate antagonistic bacterial strains

Using the fluorescent *V. harveyi* as a reporter, we screened for antagonistic bacteria in kuruma shrimp rearing water. After microdroplet-based co-culturing of the environmental bacteria and fluorescent *V. harveyi*, droplets with reduced or no green fluorescence were sorted (Figure 2A). This high-throughput screening led to the successful isolation of 20 candidate antagonistic bacterial strains (Figure 2B).

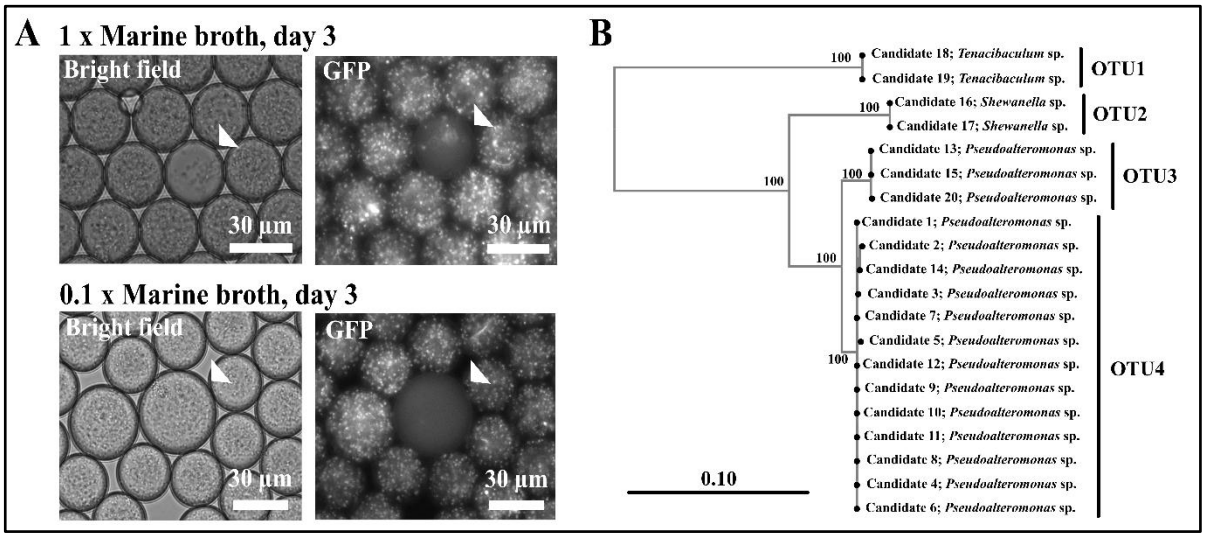


**Figure 2.** Screening of antagonistic bacteria using microdroplets. (A) Microdroplets with low green fluorescence in bright- and fluorescent-field views. (B) In total, 20 candidate antagonistic bacterial strains were isolated by microdroplet screening.

## Protective effect of candidate strains against *V. harveyi* infection

The disease-suppressing abilities of the isolated candidates were evaluated using an *in vivo* challenge test. When the candidate antagonistic bacterial strains and *V. harveyi* were co-injected intramuscularly into kuruma shrimp, a notable decrease in the shrimp mortality rate—compared with that of the control group infected with *V. harveyi* alone—was confirmed for several candidate strains (Figure 3).

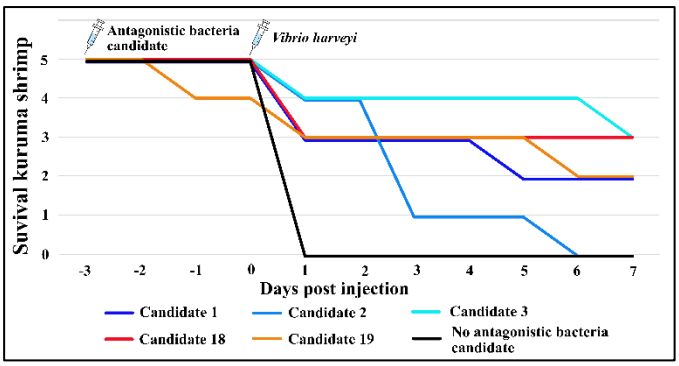


**Figure 3.** Survival of shrimp co-infected with antagonistic bacteria and *Vibrio harveyi* via intramuscular injection.

## Conclusion

To the best of our knowledge, this study is the first to screen for antagonistic bacteria against aquatic pathogenic bacteria using microdroplet technology. We used a homologous recombination system to introduce the eGFP sequence into *V. harveyi* and succeeded in producing a fluorescent strain. Through microdroplet screening, we isolated 20 candidate antagonistic bacterial strains belonging to three genera. The results of a challenge test suggest that some of these bacterial strains can suppress infectious diseases in kuruma shrimp.

## References

1) Ota, Y. et al. (2019) Fluorescent nucleic acid probe in droplets for bacterial sorting (FNAP-sort) as a high-throughput screening method for environmental bacteria with various growth rates. *PLoS One* **14:** e0214533.

2) Yuasa, H. et al. (2025) Genome sequence of *Vibrio harveyi* strain TUMSAT-2019, isolated from kuruma shrimp, *Penaeus japonicus*. *Microbiol. Resour. Announc.* **14:** e0074624.