植物プランクトンNannochloropsis oculataと共存する細菌Sulfitobacter sp. RO3株による病原性の高い魚病細菌Vibrio anguillarum株の増殖阻害

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Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat
Mixed Cultures of the Phytoplankton *Nannochloropsis oculata* and the Marine Bacterium *Sulfitobacter* sp. R03 Inhibit the Growth of Virulent Strains of the Major Fish Pathogen *Vibrio anguillarum*

Emilia Noor Sharifah and Mitsuru Eguchi*

Abstract: High- and low-virulence serotypes of the fish pathogen *Vibrio anguillarum* were challenged with a beneficial bacterium (*Sulfitobacter* sp. R03) in the presence of the phytoplankton *Nannochloropsis oculata*. The inhibitory activity of *Sulfitobacter* sp. against these *V. anguillarum* serotypes were observed in (1) phytoplankton culturing control medium (ESM) and (2) NCF, a filtrate prepared from *N. oculata* cultured in ESM. The presence of *Sulfitobacter* sp. in ESM significantly reduced the number of viable cells of all *V. anguillarum* serotypes tested (02, 03, 04, 09 and 010). In NCF, *Sulfitobacter* sp. totally eradicated the 02 and 03 serotypes. The viabilities of the low-virulence serotypes 04, 09 and 010 when cultured in NCF in the presence of *Sulfitobacter* sp. were not significantly different from those cultured in ESM. We conclude, therefore, that phytoplankton cultured in the presence of naturally occurring *Sulfitobacter* sp. provide an essential tool for inhibiting the growth of the economically significant high-virulence fish pathogen *V. anguillarum*.

Key words: *Nannochloropsis oculata; Vibrio anguillarum; Roseobacter; Growth inhibition*

Vibriosis is a serious bacterial disease caused by *Vibrio* spp. that adversely affects the aquaculture industry (Colwell and Grimes 1984; Sørensen and Larsen 1986; Defoirdt et al. 2007). *Vibrio anguillarum* causes major losses of over 42 species of fish, crustaceans and mollusks (Colwell and Grimes 1984; Defoirdt et al. 2007). *Vibrio anguillarum* is divided into 10 different serotypes (01–010); serotypes 01–3 are much more virulent than 04–010 (Sørensen and Larsen 1986). Serological studies elucidated the epizootiological and ecological characteristics of strains isolated from vibriosis outbreaks in aquaculture animals (Sørensen and Larsen 1986).

Controlling bacterial infections with antibiotics is a prerequisite for maintaining a sustainable aquaculture industry (Watson et al. 2008). Known problems associated with antibiotic treatment include selection of drug-resistant bacteria (Akinbowale et al. 2006) and creating the potential for zoonotic transfer of antibiotic resistance genes (Watson et al. 2008). Thus, the use of antibiotics has been banned in some countries (Watson et al. 2008) for use in aquaculture, leading to the use of probiotics in larviculture for controlling pathogens.

A probiotic is defined by FAO/WHO (2001) as “live microorganisms which when administered in adequate amounts, confer a health benefit on the host”. *Roseobacter* clade bacteria produce antibacterial compounds, making them candidate probiotics for aquaculture (Buchan et al. 2005; Planas et al. 2006; Porsby et al. 2008; D’Alvise et al. 2010). *Sulfitobacter* sp. R03, belongs to the *Roseobacter* clade was isolated from *Nannochloropsis oculata* culture eradicates *V. anguillarum* (serotype J-O-1) only in the presence of phytoplankton (Sharifah and Eguchi, 2011). Besides that, a species of *Sulfitobacter*, showed positive effect towards cod larviculture
(McIntosh et al. 2008). Thus, it provides a good model system for investigating interactions among phytoplankton, beneficial bacteria and fish pathogens. Furthermore, *Roseobacter* clade abundances correlate closely to those of the phytoplankton microbiota (Biddanda and Benner 1997; González et al. 2000).

Phytoplankton such as *N. oculata* are utilized widely in the aquaculture industry and are required for larval rearing either for direct consumption (mollusks) or via zooplankton to feed the fish larvae (Brown et al. 1997; Muller-Feuga 2000). The introduction of phytoplankton into fish larvae tanks, which is called the "green water technique," improves fish larval survival and growth (Nakase and Eguchi 2007; Nakase et al. 2007). The reasons for this are unknown, but may be due to the modification of either the beneficial or pathogenic bacterial populations (Defoirdt et al. 2007; Nakase and Eguchi 2007; Nakase et al. 2007).

Therefore, our objective was to investigate the influence of *N. oculata* on the inhibitory activity of *Sulfitobacter* sp. RO3 against high- and low-virulence serotypes of the major fish pathogen *V. anguillarum*.

**Materials and Methods**

**Phytoplankton culture conditions**

*Nannochloropsis oculata* was originally obtained from the Susami Fish Nursery Center, Kinki University, Japan, and was cultured continuously in freshly prepared phytoplankton culturing medium (ESM) for 7 days at 15°C. ESM medium consists of 12 mg NaN03, 0.5 mg K2HP04, 0.1 μg vitamin B12, 0.1 μg biotin, 10 μg thiamine HCl, 25.9 μg Fe-EDTA, 33.2 μg Mn-EDTA, 100 mg Tris (hydroxymethyl) aminomethane and 2.5 ml of soil extract (Provasoli et al. 1957) in 1 l of artificial seawater (nine salt solution, NSS) (Eguchi et al. 1996). When the density of phytoplankton (estimated daily using a hemocytometer, Hirschmann Techcolor) reached 10^8 cells/ml in the late log-phase, the cultures were filtered as described below.

**Bacterial culture conditions**

Five different *V. anguillarum* serotypes (high virulence: 02 and 03; low virulence: 04, 09 and 010) were used (Table 1). *Sulfitobacter* sp. RO3 (accession no: AB607863), a lineage of the *Roseobacter* clade (Table 1), was used as a potential beneficial bacterium. Bacteria were cultured for at least 16 h with shaking (120 rpm) in 1/2 ZoBell 2216E (ZoBell 1941) liquid medium. After incubation, each bacterial culture was centrifuged at 8,000 × g for 5 min and washed twice with sterile 3% NaCl.

**Nannochloropsis oculata culture filtrate and media preparation**

Two types of media were prepared to observe the growth and/or survival of *Sulfitobacter* sp. RO3 and *V. anguillarum* strains. ESM, a phytoplankton culturing medium (artificial seawater base), was used as a control medium without phytoplankton. NCF, a filtrate containing substances excreted by *N. oculata*, was prepared by culturing *N. oculata* for 1 week in ESM until the late log phase. After 1 week, *N. oculata* culture was centrifuged at 5,000 × g for 15 min. The supernatant was filter sterilized (Sterivex, 0.22 μm, Millipore). ESM medium was sterilized by autoclaving at 121°C for 15 min. Thirty milliliter aliquots of all media were prepared in quadruplicate.

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<td><strong>Species</strong></td>
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<td><em>Sulfitobacter</em> sp.</td>
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*In the present serotyping system, ATCC 43306 (02a) (Grisez and Ollevier 1995). A, Sharifah and Eguchi (2011) (accession #AB607863); B, Sorensen and Larsen (1986).
**Microbial interactions**

*Sulfitobacter* sp. RO3 was inoculated into ESM and NCF together with each of the five *V. anguillarum* strains at a final concentration of $10^5$ cells/ml. For individual control cultures, *Sulfitobacter* sp. RO3 and these *V. anguillarum* strains were also inoculated individually into both media. Aliquots (1 ml) were taken from each culture daily from days 0–6 and every 2 days from days 8 to 14. Ten microliters of 10-fold serially diluted samples were dropped (5 drops per dilution) onto 1/2 ZoBell 2216E agar plates by using the drop plate method (Herigstad et al. 2001), incubated in the dark at 20°C for 3 days and then colonies were counted using a stereomicroscope (Nikon model: 232063, 2 × objective). *Sulfitobacter* and *V. anguillarum* colonies could be easily differentiated due to the difference in the color of their colonies—cream-brown and white-gray, respectively. Experiments were carried out in quadruplicate.

**Statistical analysis**

Normal population distributions were determined by the Shapiro-Wilk test. Significant differences between samples of individual (control) and mixed cultures in each medium were analyzed independently using the Student’s t-test. For data that were abnormally distributed, the Mann-Whitney U test was used. Statistical analyses were performed using the StatPlus:mac 2009 (AnalystSoft Inc., USA) program.

**Results**

In ESM, the cell densities of *V. anguillarum* serotypes were significantly ($P < 0.05$) inhibited by *Sulfitobacter* sp. RO3 (open squares in Fig. 1) compared to the controls, *V. anguillarum* only (closed squares in Fig. 1). Inhibition by *Sulfitobacter* sp. RO3 was not significantly different among the 5 different serotypes (Fig. 1). In NCF, containing substances excreted by *N. oculata*, the viable cell densities of high-virulence *V. anguillarum* O2 and O3 were eradicated in the presence of *Sulfitobacter* sp. RO3 (open circles in Figs. 2a and 2b) compared to the controls, *V. anguillarum* only (closed circles in Figs. 2a and 2b). The colony counts of both high-virulence serotypes were not detectable at days 14 and 2, respectively (open circles in Figs. 2a and 2b). This inhibitory activity

![Fig. 1. Vibrio anguillarum cell densities in ESM medium. High-virulence V. anguillarum: a, serotype O2 (ATCC43306); b, serotype O3 (ATCC43307). Low-virulence V. anguillarum: c, serotype O4 (ATCC43308); d, serotype O9 (ATCC43313); e, serotype O10 (ATCC43314). Closed squares, V. anguillarum only (control); open squares, V. anguillarum in mixed culture with Sulfitobacter sp. RO3. Error bars = standard deviation. n = 4.](image-url)
was different from that against low virulence serotypes 04, 09 and 010 (open circles in Figs. 2c-2e). Serotypes 04, 09 and 010 colony counts showed no statistically significant differences when cultured in NCF (open circles in Figs. 2c-2e) compared with culture in ESM (open squares in Figs. 1c-1e) in the presence of *Sulfitobacter* sp. RO3.

*Sulfitobacter* sp. RO3 cell densities were 10-fold higher in NCF (Fig. 3b) than in ESM (Fig. 3a). There were no significant differences between control, *Sulfitobacter* sp. RO3 alone (closed squares in Fig. 3) and *Sulfitobacter* sp. RO3 in the presence of high-virulence *V. anguillarum* O2 (open circles in Fig. 3) or low-virulence *V. anguillarum* O4 (open triangles in Fig. 3). Other high- and low-virulence *V. anguillarum* strains grew similarly in both ESM and NCF (data not shown).

**Discussion**

*Vibrio anguillarum* serotypes are characterized by the presence of the O-antigen (Sørensen and Larsen 1986; Samuel and Reeves 2003; Wang et al. 2010). The initial 10 serotypes identified (Sørensen and Larsen 1986) were later extended to include 23 serotypes in total (Grisez and Ollevier 1995). Of these, O1, O2 and O3 are important causative agents of disease and mortality in

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**Fig. 2.** *Vibrio anguillarum* cell densities in NCF medium. High-virulence *V. anguillarum*: a, serotype O2 (ATCC43306); b, serotype O3 (ATCC43307). Low-virulence *V. anguillarum*: c, serotype O4 (ATCC43308); d, serotype O9 (ATCC43313); e, serotype O10 (ATCC43314). Closed circles, *V. anguillarum* only (control); open circles, *V. anguillarum* in mixed culture with *Sulfitobacter* sp. RO3. Error bars = standard deviation. n = 4.

**Fig. 3.** *Sulfitobacter* sp. RO3 cell densities in ESM (a) and NCF (b). Closed squares, *Sulfitobacter* sp. RO3 only (control); open circles, *Sulfitobacter* sp. RO3 in mixed culture with high virulence *V. anguillarum* serotype O2 (ATCC43306); open triangles, *Sulfitobacter* sp. RO3 in mixed culture with low-virulence *V. anguillarum* serotype O4 (ATCC43308). Error bars = standard deviation. n = 4.
aquaculture animals (Sørensen and Larsen 1986; Silva-Rubio et al. 2008). Serotype O1 mainly affects salmonids and turbot, serotype O2 affects both salmonids and marine fish species, and O3 affects various marine fish species (Sørensen and Larsen 1986; Mikkelsen et al. 2007). Here, we used the high-virulence O2 and O3 serotypes and the low-virulence O4, O9 and O10 serotypes.

The *Roseobacter* clade is one of the most common marine bactérioplatkton groups (Buchan et al. 2005). *Roseobacter* clade bacteria inhibit *V. anguillarum* O2a and O2b (Fjellheim et al. 2010) and also *V. anguillarum* O1 (Hjelm et al. 2004; Gram et al. 2010). The studies cited in the above sentence showed the inhibitory activity of *Roseobacter* clade bacteria against O1, O2a and O2b. We showed that *Sulfitobacter* sp. RO3 significantly inhibited high-virulence *V. anguillarum* strains. *Vibrio anguillarum* serotypes O2 and O3 were eradicated in the presence of substances excreted by phytoplankton (Fig. 2).

*Roseobacter* clade abundances correlate significantly with the presence of phytoplankton microbiota (Biddanda and Benner 1997; González et al. 2000). Dimethylsulfoniopropionate (DMSP) released from natural phytoplankton blooms (González et al. 2000; Zubkov et al. 2001) is believed to play an important role in oceanic sulfur and carbon cycling (Cole et al. 1982; González et al. 2000). Thus, *N. oculata* may also have excreted some substances, such as DMSP, that were favorable to *Roseobacter* clade bacteria such as *Sulfitobacter* sp. Such a mechanism is supported by our observations that viable *Sulfitobacter* sp. cell numbers were 10-fold higher when cultured in the presence of *N. oculata* filtrate (NCF) compared with culture in ESM medium without phytoplankton (Fig. 3).

*Roseobacter* clade bacteria produce the antibacterial tropodithietic acid (TDA) (Geng et al. 2008). *Sulfitobacter* sp. RO3 might produce antibacterial compounds such as TDA and could inhibit various serotypes of *V. anguillarum* (Fig. 1). Furthermore, the observation that high-virulence *V. anguillarum* (Fig. 2) was eradicated indicated that the excreted substances produced by *N. oculata* had more specific influence on the inhibitory activity of *Sulfitobacter* sp. RO3 against high-virulence *V. anguillarum*.

Beside TDA production, *Roseobacter* clade bacteria isolated from marine snow produce small signaling molecules similar to acyl-homoserine lactones (AHLs), which are produced by bacteria as components of an intercellular communication system that regulates gene expression (Gram et al. 2002). Among the AHLs, N-hexanoyl-l-homoserine lactone (HHL) and N-(3-oxohexanoyl)-l-homoserine lactone (OHLH) are produced by *Roseobacter* strains (Gram et al. 2002). Phytoplankton *N. oculata* produces analogues of OHL and HHL (Natrah et al. 2011). Similar molecules present in the *N. oculata* extract may be responsible for transmitting specific signals to *Sulfitobacter* sp., resulting in the inhibition of growth and survival of *V. anguillarum* serotypes (Fig. 2). *Vibrio anguillarum* also produces AHLs (Buch et al. 2003; Buchholtz et al. 2006) and most isolates (serotypes O1–O10 and nontypable) (148 out of 150 strains) produce N-(3-hydroxy-hexanoyl)-l-homoserine lactone (3-hydroxy-C6-HSL) and N-(3-oxodecanoyl)-l-homoserine lactone (3-oxo-C10-HSL) as the dominant signaling molecules (Buch et al. 2003; Buchholtz et al. 2006). However, these AHLs are not key factors in differentiating *V. anguillarum* by its level of virulence. Milton et al. (2001) also reported that *V. anguillarum* strain NB10 (serotype O1) produces HHL infrequently. Thus, inhibitory factors such as TDA or signaling molecules such as OHLH and HHL analogues from phytoplankton, which influence *Roseobacter* gene expression, might have been responsible for the inhibitory activity exhibited by the *Roseobacter* species here.

We found that *Sulfitobacter* sp. RO3 variably inhibited the growth of all 5 tested serotypes of the fish pathogen *V. anguillarum*. 

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The high-virulence *V. anguillarum* serotypes were more sensitive to the beneficial bacterium, *Sulfitobacter* sp. RO3, especially in the presence of substances excreted by phytoplankton. Thus, phytoplankton naturally associated with *Sulfitobacter* sp. RO3 may be an essential tool for the inhibition of high-virulence fish pathogens. Our research thus provides a better understanding of the advantages of using phytoplankton in the aquaculture industry.

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植物プランクトン Nannochloropsis oculata と共存する細菌
Sulfitobacter sp. RO3株による病原性の高い
魚病細菌 Vibrio anguillarum 株の増殖阻害

Emilia Noor Sharifah · 江口 充

Nannochloropsis oculata の培養ろ液中で Sulfitobacter sp. RO3株は、病原性の高い Vibrio anguillarum 株（血清型O2とO3）に対してより高い増殖抑制効果を示し、いずれの血清型も1週間以内に生菌数が検出限界以下まで低下した。植物プランクトン培養ろ液中の Sulfitobacter sp. のような細菌が、病原性の高い V. anguillarum の増殖を抑制しており、これは飼育水の中の細菌群集のバイオコントロールに有効な手段となる。