

# Antibiotic Production by Marine Pigmented Bacteria-I

## Antibacterial Effect of *Alteromonas luteo-violaceus*

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### Abstract

Marine purple bacteria belonging to the species *Alteromonas luteo-violaceus* were isolated from coastal water of Kinko Bay in southern Japan and examined for antibacterial action.

These isolates produce antibacterial substance(s) which inhibits the growth of various kinds of test organisms including marine purple bacteria themselves.

The antibacterial activity was found in the crude extract obtained from the cells by sonication, which contained heat-labile macromolecular substance(s).

On addition of the crude extract to natural sea water samples, colony forming abilities in sea water were inhibited completely.

Some chromogenic marine bacteria have recently reported to produce different types of antibiotic substances. For example, ANDERSEN et al<sup>1)</sup> showed that a marine *Chromobacterium* sp produces a series of highly brominated metabolites which are responsible for antibiotic action as well as autoinhibition. GAUTHIER et al<sup>2,3,4,5)</sup> also described that four species of marine *Alteromonas* synthesize polyanionic polysaccharide antibiotics which act on bacterial respiration and that some strains of *A. luteo-violaceus* concomitantly produced brominated antibiotics which are small molecules but not diffusible due to association with cells.

As described in previous paper<sup>6,7)</sup>, we isolated *Alteromonas luteo-violaceus* strains from coastal water of Kinko Bay in Japan, which showed antibacterial effects against various kinds of bacteria. These isolates usually die out within one week unless subcultured in fresh media, suggesting their autotoxication.

In this paper, we examined the inhibitory effect of antibacterial substance(s) produced by marine isolates belonging to the species *Alteromonas luteo-violaceus* against various kinds of bacteria including *A. luteo-violaceus*.

### Materials and Methods

**Organisms** Ten strains of marine purple bacteria, belonging to *A. luteo-violaceus*, isolated from Kinko Bay, Kagoshima, Japan in 1979 as shown in Fig.5. One strain 9K-V9 among ten isolates, which formed the strongest purple color colony, was used exclusively in this study.

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All strains were grown on modified ZoBell 2216E slants (pH 7.6) at 25 C and subcultured every four days.

The following organisms were used for growth inhibition test: 1) *Alteromonas luteo-violaceus* 9K-V9, 2) *Vibrio parahaemolyticus* Vp H-K15 (serotype 05:K15 isolated from patient by Dr. K. TAKAGI at Hokkaido University), 3) *V. alginolyticus* Va F-1 (NIH 154-78), 4) *A. haloplanktis* 1055-1 (by Dr. M. SAKAI), 5) *Pseudomonas* I-6 (isolated from sea water), 6) *Micrococcus lysodeikticus* (laboratory strain), 7) *Staphylococcus citreus*, 8) *Bacillus subtilis* Marburg strain 6051, 9) *Pseudomonas aeruginosa*, 10) *Escherichia coli* B strain, 11) *Aeromonas hydrophila* AHH (IFO 12981), 12) *A. salmonicida* ASS (IFO 12718).

**Extraction of antibacterial substances from the cells** Purple bacterium 9K-V9 was incubated statically in 4 flasks containing 250 ml modified ZoBell broth at 25 C for 3 days. Bacterial cells were harvested with centrifugation at 8,000 g for 20 min and then the bacterial suspension in deionized water (30 ml) was submitted to sonic disintegration for 3 min at 2A (Kaijo Denki type 4280). The supernatant solution after centrifuged at 17,000 g for 20 min was dialyzed against deionized water for one day and filtrated with Millipore filter (HAWP 0.45  $\mu$ m) to sterilize contaminated bacterial cells.

**Antibacterial activity** Growth inhibition of test organisms was examined by two methods. One is the double agar method, in which test organisms were seeded in ZoBell agar (pH 6.3 for marine bacteria) and TSA (pH 6.3 for terrestrial bacteria and *Vibrio* strains) plates. The paper disks impregnated with the culture filtrate or cell extract or a loopful of bacterial cells were placed on the bacterial lawns of test organisms. Inoculated plates were incubated at 25 C for 48 hr, after which time zones of growth inhibition were measured. The other is the broth culture method, in which 0.1 ml culture of test organisms were inoculated into L-form test tubes containing 9.5 ml ZoBell broth (pH 7.6 for marine bacteria) or Z-A (pH 7.2 for terrestrial bacteria) and the cell extract (0.3 ml), and then incubated at 30C for 72 hr on a shaking incubator. The turbidity of culture was determined by using a spectrophotometer (Fuji Kogyo, 660 nm filter).

**Effect of the cell extract on colony forming ability of natural sea water** Soft agar containing half volume of the cell extract and 0.1 ml natural sea water was overlaid on basal agar plate and incubated at 25C for 72 hr. After incubation period, bacterial colonies formed were counted, and compared with those of control plate without the addition of the cell extract.

## Results

**Changes with cultivation time in antibacterial activity** Figs. 1 and 2 show the zone sizes of growth inhibition against test organisms caused by the cells or culture filtrate of purple bacteria during culture period.

Antibacterial activity appeared in the cells but not released into culture medium at one day with shaking culture of purple bacteria (70 strokes per min). Some inhibition by the culture

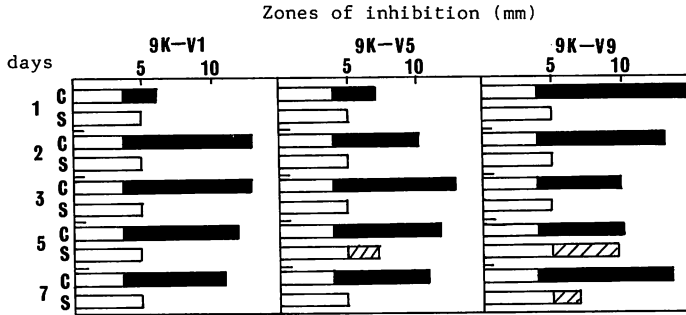


Fig. 1. Growth inhibition of *Vibrio parahaemolyticus* H-K15 by strains of *Alteromonas luteo-violaceus*. The cells (C) or paper disks impregnated with the supernatant (S) from *Alteromonas* strains were placed on the surface of a double layer agar (TSA pH 6.3) seeded with *Vibrio parahaemolyticus* H-K15 and incubated for one day at 25°C. Blank columns indicate diameters of the colonies or paper disks

filtrate was occasionally detected at 5 or 7 days (Fig.1). If large amount of active substances would be released from the cells into medium due to autolysis, a paper disk impregnated with the filtrate has activity enough to inhibit the growth of test organisms on agar plates.

The production of antibacterial substances by a strain 9K-V9 varied depending on culture methods (Fig.2). Growth inhibition against Vp H-K15 was detected at one day with static

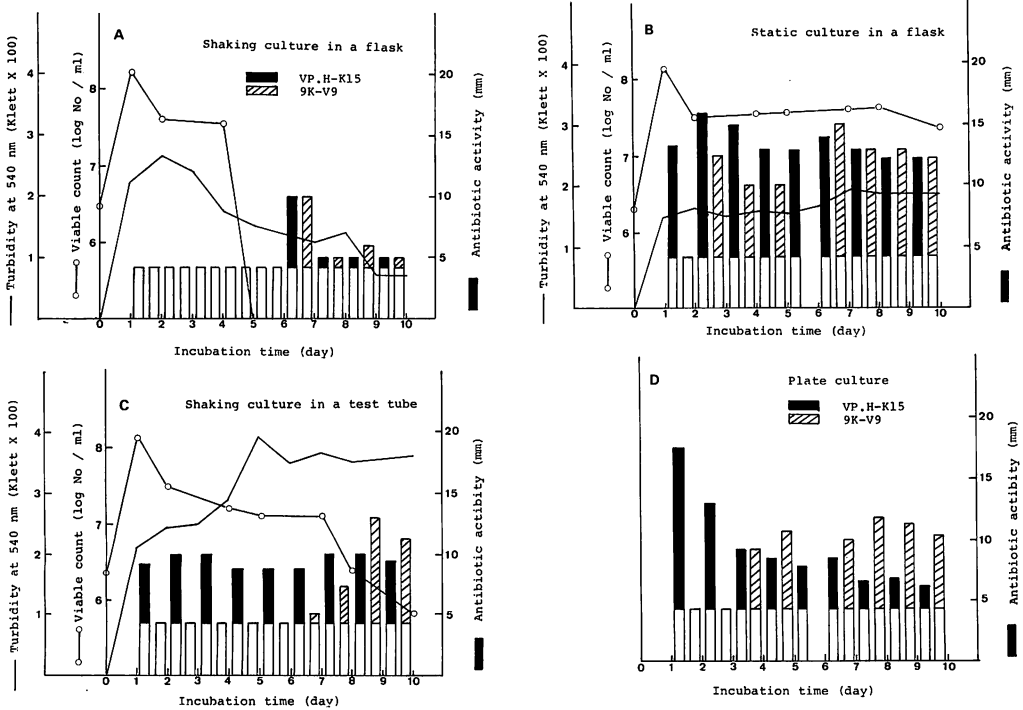


Fig. 2. Time course of growth and antibiotic production of *Alteromonas* 9K-V9.

culture in a flask, shaking culture in a test tube and on an agar plate culture of 9K-V9 but no or slight inhibition occurred with shaking culture in a flask (100 st/min) during early period of culture. On the other hand, self inhibition against 9K-V9 appeared at 2 days with static culture in a flask and at some days later with plate culture and test tube culture. In the case of shaking culture in a flask (100 st/min), the appearance of antibacterial activity was retarded after 5-6 days, just before which time bacterial viability had decreased rapidly. The greater and earlier production of antibacterial substance(s) and longer viability occurred concomitantly with static culture than with shaking culture of 9K-V9.

**Treatment for the release of antibacterial substance(s) from the cells** As shown in Table 1, antibacterial substance(s) was associated with the cells not so tightly and considerable amount of the substance(s) was released from the cells into suspending solution (deionized water). Furthermore, sonic treatment (5 min at 2A) attained the release of the substance(s) from the cells almost completely. Turrax treatment (Ultra-Turrax, Ikawerk TP 18-10 type, 2 min) gave no or slight effect on the release of activity compared with sonication.

Table 1. Comparison among treatments for the release of antibacterial substances from the cells.

Test strains	Zones of inhibition (mm)			
	Suspended in D.W.	Turrax	Sonication	
9K-V1	Cell <sup>a</sup>	14 (4) <sup>c</sup>	13 (4)	6 (4)
	Sup. <sup>b</sup>	13 (5)	13 (5)	16 (5)
9K-V5	Cell	14 (4)	13 (4)	6 (4)
	Sup.	14 (5)	14 (5)	16 (5)
9K-V9	Cell	13 (4)	13 (4)	6 (4)
	Sup.	13 (5)	13 (5)	16 (5)

<sup>a</sup> The cells of 9K-V9 obtained after each treatment.

<sup>b</sup> The supernatant solution obtained after each treatment.

<sup>c</sup> Figure in parenthesis indicates a diameter of cell pellets or a paper disk soaked in the supernatant.

**Heat treatment of antibacterial substance(s)** The crude extract obtained after sonic treatment was heated at each temperature for 10 min as shown in Fig.3. The inhibition activity against VP H-K15 was lost at 60°C by half and at 70°C completely.

**Precipitation of antibacterial substance(s)** Table 2 demonstrates that antibacterial substance(s) was precipitated with 80% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, ethanol (50% v/v) and acetone (50% v/v). The substance(s) was not dialyzable and not inactivated in deionized water. These results indicate that the antibacterial substance(s) has high molecular weight.

**Growth inhibition of various kinds of bacteria** Growth inhibition of various test organisms by the crude extract obtained from the cells of 9K-V9 is demonstrated in Fig.4. On addition of the crude extract to L-form test tubes inoculated with test organisms, all the test

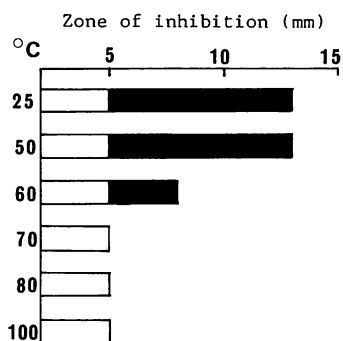


Fig. 3. Effect of heat treatment on antibacterial activity of the cell extract from 9K-V9 against *V. parahaemolyticus* H-K15.

The cell extract was heated at each temperature for 10 min.

Table 2. Effect of precipitation and dialysis of the cell extract from *Alteromonas* 9K-V9 on its antibacterial activity.

Treatment	Zones of inhibition (mm)	
	9K-V9	Vp H-K15
Dialysis <sup>a</sup>	17 (5) <sup>c</sup>	10 (5)
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ppt. <sup>b</sup>	14 (5)	8 (5)
Ethanol ppt.	12 (5)	7 (5)
Acetone ppt.	14 (5)	10 (5)

<sup>a</sup> The crude extract from 9K-V9 dialyzed directly without precipitation.

<sup>b</sup> The crude extract dialyzed after precipitation.

<sup>c</sup> Figure in parenthesis indicates a diameter of a paper disk.

organisms were inhibited, even if to different extent. Most of test organisms were not able to grow at all but some terrestrial strains grew after long lag period under existence of the crude extract.

Antibacterial activity of the crude extract of 9K-V9 was examined against natural sea water by using double agar method (Fig.6). It is very interesting that the crude extract inhibited colony forming ability of natural sea water completely, in contrast with 600-1,000 c.f.u. per plate for natural sea water samples without the crude extract. Same results were obtained with the culture filtrate, which was separated from the cells after 3 days culture was treated directly with sonication.

### Discussion

Marine purple bacteria, *Chromobacterium* spp and *Alteromonas* spp, have been reported to produce antimicrobial substances. Chemical structure of these substances were demonstrated

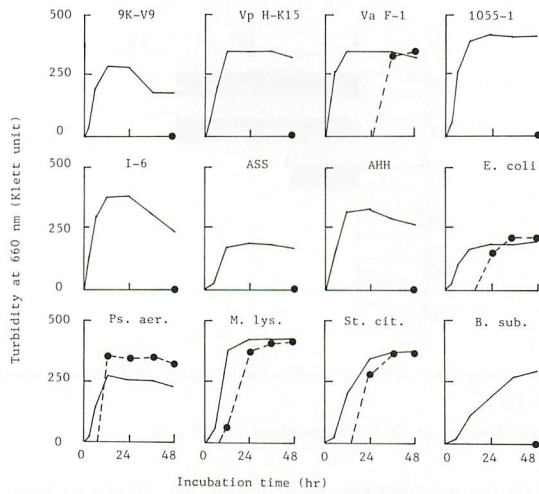


Fig. 4. Effect of the addition of crude extract from the cells of 9K-V9 on the growth of test organisms.

—: without crude extract, ●---●: with crude extract.

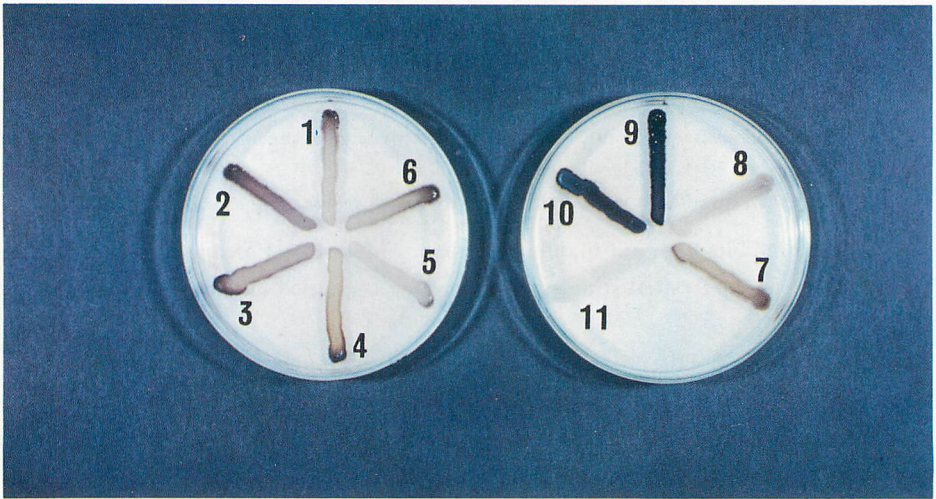


Fig. 5. Plate cultures of 11 strains of *Alteromonas luteo-violaceus* isolated from Kinko Bay.

1: 9K-V1, 2: 9K-V2, 3: 9K-V3, 4: 9K-V4, 5: 9K-V5, 6: 9K-V6, 7: 9K-V7, 8: 9K-V8, 9: 9K-V9, 10: 9K-V10, 11: 8K-V2.

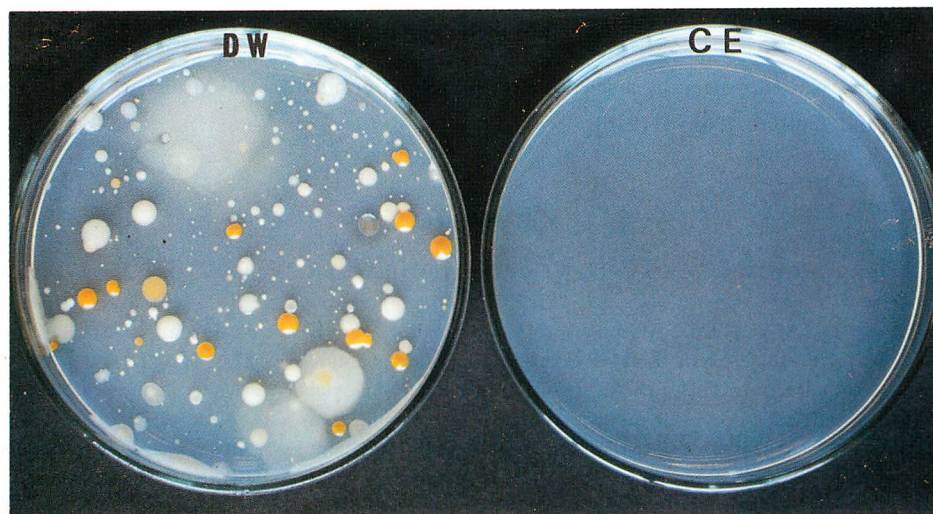


Fig. 6. Antibacterial activity of the crude extract from 9K-V9 against natural sea water. left: without the crude extract, right: with the crude extract.

to be brominated pyrrole or benzene derivatives<sup>1)</sup> or polyanionic polysaccharide compounds<sup>4)</sup>. They are also responsible for autoinhibition as well as for antibiotic action against other kinds of bacteria. Especially, GAUGHIER<sup>5)</sup> presented that an antibacterial polysaccharide could act at the level of the respiratory chain by setting up a flavinic respiration.

The crude extract obtained from the cells of our strain *A. luteo-violaceus* 9K-V9, which isolated from sea water of Kinko Bny, was demonstrated to include heat-labile, non-dialyzable macromolecular substance(s). Antibacterial substance(s) was shown to comprise different kinds of activities, for example, against VP H-K15 and 9K-V9 itself, judging from difference in production time of the substance(s). The characteristics of antibacterial substance(s) extracted from the cells of 9K-V9 suggest the possibility that it is composed of proteinous compound(s), such as bacteriolytic or autolytic enzyme(s).

Time course experiments on production of antibacterial substance(s) indicate that static culture gave rise to earlier and greater production than shaking culture did. The cells of purple bacteria grew well in surface layer of broth medium with static culture and sank into bottom layer, followed with newly growing cells in surface layer. In surface layer of static culture or on agar plate, O<sub>2</sub> supply is considered to be sufficient for bacterial cells to synthesize antibacterial culture to be homogenous and supply O<sub>2</sub> in lower concentration as a whole culture.

Further experiments will be studied with purified substance to elucidate the structure and mechanism of antibacterial substance(s) produced by *Alteromonas luteo-violaceus*.

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