**Vibrio** sp. Strain NM 10, Isolated from the Intestine of a Japanese Coastal Fish, Has an Inhibitory Effect against *Pasteurella piscicida*

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**Vibrio** sp. strain NM 10 with an inhibitory activity against *Pasteurella piscicida* K-III was isolated from the intestine of a spotnape ponyfish (*Leiognathus nuchalis*). This bacterium efficiently produced an antibacterial substance after growth at 20°C for 24 h on 1/5 PYBG agar prepared with 50% seawater at pHs of 7.5 to 9.0. The antibacterial substance was heat labile and proteinaceous, with a molecular mass of less than 5 kDa, possibly a bacteriocin or a bacteriocin-like substance.

Although fish always take a large number of bacteria into their intestines from water, sediment, and/or food, they are protected from these bacteria by the acid in gastric juice, the bile acids, and lysozyme secreted in the intestines and by immune responses (9). In addition, it is recognized that the ability to adhere to enteric mucus and wall surfaces is necessary for bacteria to become established in fish intestines (6, 7, 14). Therefore, most bacteria are temporary residents and disappear from the intestine immediately after invading; only a few bacteria can persist for a relatively long time to make up the intestinal microflora specific to host animals.

On the other hand, *Pasteurella piscicida* is a causative agent of pasteurellosis of marine fishes, which results in serious losses in aquaculture (5). Recently, Westerdahl et al. (14) isolated bacterial strains with inhibitory effects against *Vibrio anguillarum* from the intestinal tracts of turbot (*Scophthalmus maximus*). Similarly, we isolated bacteria inhibitory against pathogenic bacteria from the intestines of both marine and freshwater fishes (11, 12). These facts suggest that intestinal bacteria capable of producing antibacterial substances may prevent the establishment of invading bacteria in fish intestines. Therefore, this study was undertaken to isolate the bacterial strains which have inhibitory effects against *P. piscicida* from marine fish intestines and to examine some properties of the antibacterial substance produced.

Intestinal bacteria from eight animals, including rocky crab (*Plagusia dentipes*), barbel eel (*Plotosus lineatus*), black mullet (*Mugil cephalus*), croaker (*Agrophomus argentatus*), dragonets (*Callionymus sp.*), Japanese whiting (*Sillago japonica*), and spotnape ponyfish (*Leiognathus nuchalis*), collected in coastal regions of Enoshima Island, Kanagawa, Japan, were isolated on PYBG and 1/20 PYBG agar media (13) and identified as previously reported at the genus or family level (11).

The inhibition test was carried out by the double-layer method as reported by Dopazo et al. (2). *P. piscicida* K-III was used as the target bacterium. The tested strains were incubated for 24 h at 20°C in 1/5 PYBG broth, which consists of 500 ml of S-type artificial seawater (Jarmarin Laboratory, Osaka, Japan), 500 ml of distilled water, 2 g of Trypticase peptone, 1 g of Phytone peptone (BBL, Cockeysville, Md.), 0.4 g of Bacto yeast extract (Difco, Detroit, Mich.), 0.2 g of glucose, and 0.48 g of Lab-lemeo powder (Oxoid, Hampshire, England) adjusted to pH 7.5. Macrocolonies of the tested strains were created on 1/5 PYBG agar (1/5 PYBG broth solidified with 1.5% agar) plates by inoculating 5-μl droplets of the culture described above with a multipoint inoculator. After incubation at 25°C for 48 h under aerobic conditions, the colonies were killed with chloroform vapor (15 to 20 min). The target strain was incubated for 24 h at 25°C in 1/5 PYBG broth and diluted until the optical density at 610 nm (OD610) reached 0.2 (approximately 10⁶ CFU ml⁻¹). The culture was further diluted 100 times and suspended in 1/5 PYBG soft agar (0.1% agar), which was poured over the plates (4.5 ml per plate). After incubation at 20°C for 48 h, a bacterial strain which produced a clear inhibitory zone with a diameter more than 1 mm larger than that of the macrocolony was judged to be an antibacterial-substance producer. Although control plates without macrocolonies of the tested strains were included to evaluate the possible effect of chloroform on the growth of the target bacteria, no such effect was observed.

Of 1,335 strains of bacteria isolated, 149 (11.2%) inhibited the growth of *P. piscicida* K-III. However, activity against fish pathogens varied with taxonomic groups of the isolates. High activities (≥20-mm-diameter inhibitory zones) were seen in four isolates each of coryneforms and *Enterobacteriaceae* and one isolate each of *Pseudomonas* sp. and a *Vibrio* sp.; *Vibrio* sp. strain NM 10, isolated from the spotnape ponyfish, exhibited the highest activity against *P. piscicida* K-III. Therefore, *Vibrio* sp. strain NM 10 was chosen as an active strain and subjected to further examination.

The antibacterial spectrum of *Vibrio* sp. strain NM 10 against 363 intestinal bacteria from coastal animals was examined by the double-layer method. As shown in Table 1, 50 isolates (13.8%) belonging to *Bacillus* spp., coryneforms, *Enterobacteriaceae*, *Flavobacterium* spp., *Pseudomonas* spp., and *Vibrio* spp. were susceptible while *Acinetobacter* spp., *Micrococcus* spp., *Moraxella* spp., and *Staphylococcus* spp. were resistant to *Vibrio* sp. strain NM 10. In addition, *Vibrio* sp. strain NM 10 could produce large clear zones of inhibition against *P. piscicida* K-III (29.2-mm-diameter) and *Escherichia coli* IAM 1264 (24.7-mm-diameter) and small ones against *Vibrio vulnificus* RIMD 2219009 (14.6-mm-diameter) and *Enterococcus seriolicida* YT-3 (12.3-mm-diameter), but no clear zone was detected against *V. anguillarum* ATCC 19264. These results

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TABLE 1. Effects of *Vibrio* sp. strain NM 10 against intestinal bacteria from coastal animals

<table>
<thead>
<tr>
<th>Target bacteria</th>
<th>No. of positive isolates* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter spp. (n = 5)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Bacillus spp. (n = 24)</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>Coryneforms (n = 26)</td>
<td>4 (15.4)</td>
</tr>
<tr>
<td>Enterobacteriaceae (n = 31)</td>
<td>2 (6.5)</td>
</tr>
<tr>
<td>Flavobacterium spp. (n = 27)</td>
<td>7 (25.9)</td>
</tr>
<tr>
<td>Micrococcus spp. (n = 2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Moraxella spp. (n = 11)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Pseudomonas spp. (n = 44)</td>
<td>14 (31.8)</td>
</tr>
<tr>
<td>Staphylococcus spp. (n = 5)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><em>Vibrio</em> spp. (n = 188)</td>
<td>21 (11.2)</td>
</tr>
</tbody>
</table>

Total (n = 363) .................................................................................. 50 (13.8)

* The antibacterial effect was determined by the double-layer method with *P. piscicida* K-III.

* n, number of strains.

The antibacterial activity in the culture supernatant was measured by the disc diffusion method. Bacterial cultures of *P. piscicida* K-III with an OD<sub>610</sub> of 0.2 were cultured 100 times in 1/5 PYBG soft agar and poured over 1/5 PYBG agar plates (4.5 ml per plate). Paper discs (8 mm in diameter; Advantec Toyo, Tokyo, Japan) containing 50 μl of the culture supernatant were placed on the surface of agar plates, and kept at 25°C for 48 h. Antibacterial activity was defined as the diameter in millimeters of the clear inhibitory zone formed. The same experiment was repeated six times unless otherwise specified.

To test the effect of growth parameters on the production of the antibacterial substance, *Vibrio* sp. strain NM 10 was incubated on agar media under various conditions. Figure 1 shows the time course of antibacterial activity in the culture supernatant of *Vibrio* sp. strain NM 10 after incubation at various temperatures. For details, see the legend to Fig. 1.

As *Vibrio* sp. strain NM 10 could grow at temperatures ranging from 15 to 35°C, antibacterial activity was measured after incubation within that range of temperatures for 24 h. Activity was detected at 15 to 25°C, while no activity was observed at both 30 and 35°C, as shown in Fig. 2. The highest activity was observed in the culture grown at 20°C. The antibacterial activity in the culture supernatant of *Vibrio* sp. strain NM 10 was also examined after incubation on concentrations of PYBG agar medium ranging from 10 to 100%. However, the activity was only slightly lower in that grown in 25% seawater. Figure 4 shows the antibacterial activity in the culture supernatant of *Vibrio* sp. strain NM 10 after incubation at pH values ranging from 5.0 to 9.0. Activity was detected at pH values from 6.5 to 9.0, while no activity was observed at pH values from 5.0 to 6.0. Such differences in antibacterial activities may be attributed to a greater or lesser extent to differences in cell yields of the bacterium. Nevertheless, the above-mentioned results show that the production of the antibacterial substance by *Vibrio* sp. strain NM 10 is significantly affected by the incubation time and the temperature, concentration of seawater, and pH value of the culture medium and that the optimal conditions for production were as follows: incubation time, 24 h; temperature, 20°C; concentration of seawater, 50%; and pH value of 7.5 to 9.0.

A sample of the culture supernatant of *Vibrio* sp. strain NM 10 was analyzed for its sensitivity to nine enzymes: proteinase K (Merck, Darmstadt, Germany), trypsin, α-chymotrypsin type II, protease type XIV (Sigma, St. Louis, Mo.), lysozyme (Seikagaku Corp., Tokyo, Japan), achromopeptidase (Wako Pure containing concentrations of seawater ranging from 10 to 100% (the bacterium could not grow in 0% seawater). Antibacterial activity was detected in the cultures grown in 10 to 75% seawater; the highest activity was observed in the culture grown in 50% seawater, and only slightly lower activity was observed in that grown in 25% seawater. Figure 4 shows the antibacterial activity in the culture supernatant of *Vibrio* sp. strain NM 10 after incubation at pH values ranging from 5.0 to 9.0. Activity was detected at pH values from 6.5 to 9.0, while no activity was observed at pH values from 5.0 to 6.0. Such differences in antibacterial activities may be attributed to a greater or lesser extent to differences in cell yields of the bacterium. Nevertheless, the above-mentioned results show that the production of the antibacterial substance by *Vibrio* sp. strain NM 10 is significantly affected by the incubation time and the temperature, concentration of seawater, and pH value of the culture medium and that the optimal conditions for production were as follows: incubation time, 24 h; temperature, 20°C; concentration of seawater, 50%; and pH, 7.5 to 9.0.
Chemical, Tokyo, Japan), α-amylase, ribonuclease A type II-A, and catalase (Sigma). Each enzyme solution, prepared in 20 mM Tris HCl (pH 7.8), was added to supernatant samples to a final enzyme concentration of 0.1 mg ml⁻¹. After incubation for 1 to 3 h at 37°C, the antibacterial activity was measured by the disc diffusion method. Controls were enzymes in buffer and buffer only. Results showed that the antibacterial substance produced by *Vibrio* sp. strain NM 10 was inactivated by two serine proteases, proteinase K and trypsin, while it was resistant to the other seven enzymes. This result reveals that the antibacterial substance is proteinaceous and that its action is not due to the formation of hydrogen peroxide. Figure 1 shows that no antibacterial activity was detected after 72 h of incubation. This phenomenon may be due to activation of proteolytic enzymes produced by *Vibrio* sp. strain NM 10, as observed in lactic streptococci (3).

To assess the heat stability of the antibacterial substance, the culture supernatant of *Vibrio* sp. strain NM 10 was heated for 30 min at both 80 and 100°C and the remaining activity was measured by the disc diffusion method. The activity of the antibacterial substance produced by *Vibrio* sp. strain NM 10 decreased linearly and completely ceased at 15 and 5 min after incubation at 80 and at 100°C, respectively, indicating that the antibacterial substance is heat labile.

To estimate the molecular mass of the antibacterial substance, the culture supernatant was fractionated with a Molcut L ultrafiltration kit (nominal molecular mass cutoffs, 5 and 10 kDa; Millipore, Bedford, Mass.) and the activity of each fraction was assayed by the disc diffusion method. The same degree (30.2 to 31.4-mm) of activity was found in both <5- and <10-kDa fractions, along with the unfraccionated culture supernatant, indicating that the molecular masses of active substances are lower than 5 kDa.

Additionally, the production of siderophores was investigated by the universal chemical assay of Schwyn and Neillands (8). However, siderophore was not detected in the culture supernatant of *Vibrio* sp. strain NM 10 even at high antibacterial activities (21.8- to 25.9-mm). This result shows that the antibacterial substance produced by *Vibrio* sp. strain NM 10 has no ability to bind iron.

In general, the antibacterial effect of bacteria is due to the following factors, either singly or in combination: production of antibiotics, bacteriocins, siderophores, lysosomes, proteases, and/or hydrogen peroxide and the alteration of pH values by organic acids produced. The above-mentioned results show that the antibacterial substance produced by *Vibrio* sp. strain NM 10 is a heat-labile proteinaceous substance with a molecular mass of less than 5 kDa. These facts strongly suggest that the antibacterial substance is either a bacteriocin or a bacteriocin-like substance. If this speculation is true, then to our knowledge, this is the first demonstration that live fish can have intestinal bacteria capable of producing bacteriocin or bacteriocin-like substances. Geis et al. (3) reported that lactic streptococci preferred to produce bacteriocin on a solid rather than a liquid medium. The results of our study of *Vibrio* sp. strain NM 10 agree well with their report. Nevertheless, purification and characterization of the antibacterial substance produced by *Vibrio* sp. strain NM 10 remain to be undertaken in the near future.

At present, chemotherapeutic agents, including amoxicillin, ampicillin, florfenicol, flumequine, novobiocin, oxolinic acid, sulfamonomethoxine, and tetracycline, are used for the treatment of pasteurellosis in Japan. However, *P. piscicida* is known to easily acquire the ability to resist these drugs (4). Recently, Austin et al. (1) and Smith and Davey (10) reported the possibility that fish diseases are suppressed by treatment with probiotic strains of bacteria. Effective control of the establishment of pathogenic bacteria in fish intestines by using probiotic strains with the ability to produce bacteriocin instead of by chemotherapeutic agents may be essential in aquaculture. Further studies along this line are required in the near future.

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