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Microbes and Environments

Distribution of *Vibrio halioticoli* around an Abalone-farming Center in Japan

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Vibrio halioticoli is the dominant bacterial species in the gut of abalone, *Haliotis discus hannai*. The bacterium may contribute to the digestion of seaweed, and form a symbiotic association with the host. However, the process by which the bacterium colonizes the gut of abalone is unknown and intriguing given the non-motile nature of *V. halioticoli*. To clarify the colonization process, the distribution of the bacterium in seawater, as well as the fecal material and diet of abalone was investigated at an abalone farm. Viable bacterial counts of *V. halioticoli* were determined in each sample using a colony hybridization technique specifically designed for the bacterium. The count in seawater and culture seawater was 3 CFU/ml. The number of *V. halioticoli* in a diatom bed used as a diet for juvenile abalone was 7.9×10^2 CFU/g. Fractionation of the diatom bed sample using 0.2 and 8.0-μm filters indicated that the viable count was higher in the attached fraction (3.4×10^2 CFU/g; >8.0 μm) than in free-living fraction (76 CFU/g; 0.2 μm–8.0 μm). Furthermore, viable *V. halioticoli* was estimated to be 3.6×10^3 CFU/g in the fecal material of juvenile abalone which had ingested diatoms, as compared to 1.2×10^5 CFU/g in that of juveniles fed on artificial diet. These results suggest that *V. halioticoli* cells in seawater and/or on diatoms contribute to the colonization of the gut in abalone.

Key words: abalone, colonization, distribution, gut, *Vibrio halioticoli*

V. halioticoli is a novel alginolytic marine bacterium isolated from the gut of abalone *Haliotis discus hannai*[7]. *V. halioticoli* is the dominant bacterial species in the gut of abalone fed brown algae or an artificial diet containing alginate, accounting for more than 60% of heterotrophic bacterial isolates[6]. Therefore, the bacterium may contribute to the breakdown of seaweed ingested by abalone.

Although the microflora of marine invertebrates has been studied extensively, there have been few attempts to verify the process by which microbes colonize the gut. The colonization of the abalone by *V. halioticoli* is of special interest in that the bacterium is non-motile. We already reported that a small number of *V. halioticoli* cells were isolated from juvenile abalones fed microalgae or diatoms, and the *V. halioticoli* population in the gut of these juveniles increased when the diet was switched to *Laminaria* or an artificial diet containing seaweed powder (Submitted for publication, Tanaka *et al.*, 2001). Although these findings suggest that the main source of the bacterium is *Laminaria* fronds or artificial diets, *V. halioticoli* has never been isolated from either. The main route and timing of the gut colonization are not yet known.

The isolation of *V. halioticoli* from seawater at a farming center for abalone suggests a contribution by the environment to the microbial association in abalone gut.

Colony hybridization is a powerful and simple technique for studying bacterial ecology, often applied to the epidermiology of food-borne bacteria[1,3,5,10,11]. The method allows for the detection of bacterial species among a huge number of isolates from environmental samples. We have developed a colony hybridization method using an alkaline phosphatase-labeled genomic DNA probe (Vh-probe) highly specific for *V. halioticoli*[9]. The distribution of the bacteri-

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um in and around an abalone-farming center in Kumaishi, Hokkaido, Japan, was studied with this new method.

Materials and Methods

Sampling site and bacterial culture

The abalone-farming center is located in the town of Kumaishi, Hokkaido, Japan. Sampling sites are shown in Fig. 1. Details of the samples are also listed in Table 1. Samples for the enumeration of *V. halioticoli* were collected in July 1999 or September 2000. The supply of sea water (A), diatom bed for the feeding of juveniles (B), Water used for the culture of diatoms (C) and abalone (D, E, F), fecal material (G, M, N), seawater downstream of the farming center and head water (H, L), coastal seawater near the farming center (I), and coastal seawater 5 km north and south of the center (J, K) were collected using sterilized bottles. Each sample was serially diluted with 75% seawater, and 100 µl was spread on APY agar medium[8] and incubated at 20°C for 5 days.

A portion of samples of A, B, M, and N was fractionated using 8.0 and 0.2-µm-pore-diameter filters. Twenty milliliters of each sample was filtrated with the 8.0-µm nuclepore filter. The resultant filters (Af8, Bf8, Mf8, and Nf8) were used as the attached fraction. Each filtrate was then passed through the 0.2-µm nuclepore filter and the resultant filters (Af02, Bf02, Mf02, and Nf02) used as the free-living fraction. Each fraction was suspended in 10 ml of 75% seawater, and plated as described above.

Identification of V. halioticoli grown on the plates

An alkaline phosphatase-labeled *V. halioticoli* genomic DNA probe (Vh-probe) was used for detecting *V. halioticoli*. The probe was derived from sonicated *V. halioticoli* genomic DNA, extracted according to Marmur[4], and directly labeled with alkali phosphatase, as previously described[9]. The membrane preparation, hybridization, and probe development have also been described in a previous report[9]. The colonies positive for the probe were subjected to the 16S rDNA PCR/RFLP analysis developed by Tanaka *et al.*[8] to prevent miss-identification.

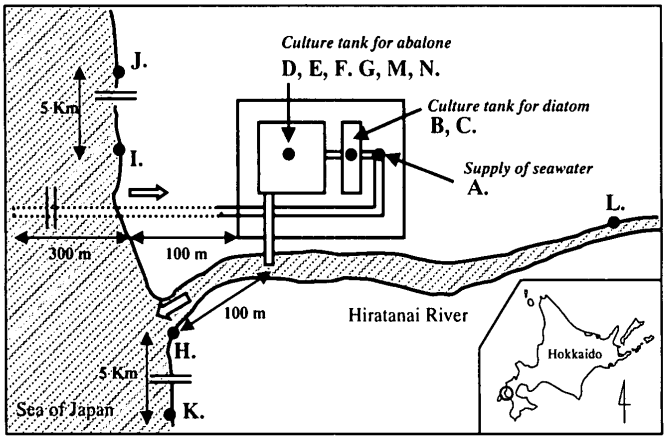

Fig. 1. Sampling sites of seawater around an abalone-farming center located in Kumaishi, Hokkaido, Japan.

Results

Isolation of V. halioticoli around the abalone-farming center

Positive colonies were detected in seawater at 9 of 12 sampling sites in and around the farming center (Table 1). Viable counts of *V. halioticoli* in the supply of seawater (A) and in culture seawater for abalone and diatoms (C, D, E, and F) ranged from 0 to 56 CFU/ml, while the count in the diatom bed (B) was 7.9×10^2 CFU/g. Fecal material from 1 year old abalone contained the highest number of positive colonies (9.9×10^6 CFU/g). Positive colonies were also found in drain water at the sea farming center (H, 35 CFU/ml) and seawater in coastal area (I, 5 CFU/ml) or 5 km from the farming center (J, and K, 0–5 CFU/ml).

Enumeration of V. halioticoli from fractionated samples

V. halioticoli in the attached fraction of diatom samples (Bf8) numbered 3.4×10^2 CFU/g, and in the free-living fraction (Bf02), 7.6×10^1 CFU/g. Almost all the positive colonies from feces of juveniles fed diatoms or the artificial diet were detected in the free-living fraction (Mf02, and Nf02), the counts being 3.6×10^3 CFU/g and 1.2×10^5 CFU/g, respectively (Table 1).

Discussion

Viable *V. halioticoli* cells were detected in the supply of sea-water (sample A), diatom bed (sample B), water used for culturing (samples C, E, and F), and feces of abalone (samples G, M, and N). The viable *V. halioticoli* count in the water supply (3 CFU/ml) was almost the same as that in the seawater used for culturing (0–5 CFU/ml), while the count in culture for the diatom bed (56 CFU/ml) was 10 times that in the water supply (Table 1). The *V. halioticoli* count was higher in diatom and fecal samples of abalone than seawater samples (Table 1). Almost all the *V. halioticoli* was detected in the diatom-attached fraction in the diatom bed, but in feces, most of the bacterium was found to be free-living (Table 1). Considering the coloniza-

Table 1. Detection of *Vibrio halioticoli* from seawater samples around the farming center for abalone.

Sampling site	Sampling date	Sample code	Average of viable count	Number of Vh-probe positive colonies[b]	Number of colonies used for the hybridization	Percentage of *V. halioticoli* (%)	*V. halioticoli* viable count
Seawater supply	July 1999	A	1.1×10^2 CFU/ml	1	44	2.3%	3 CFU/ml
Diatom	Sept 2000	B	2.8×10^4 CFU/g	11	392	2.8%	7.9×10^2 CFU/g
Culture seawater, diatom-bed	July 1999	C	2.0×10^3 CFU/ml	1	36	2.8%	56 CFU/ml
Culture seawater, 90 days[a]	July 1999	D	2.1×10^3 CFU/ml	0	242	0%	0 CFU/ml
Culture seawater, 120 days[a]	July 1999	E	8.7×10^2 CFU/ml	1	347	0.25%	2.5 CFU/ml
Culture seawater, 1 year[a]	July 1999	F	1.1×10^3 CFU/ml	4	440	9%	10 CFU/ml
Feces from abalone, 1 year	Sept 2000	G	1.9×10^8 CFU/g	2	39	5.2%	9.9×10^6 CFU/g
Drain water	July 1999	H	6.2×10^2 CFU/ml	7	123	5.7%	35 CFU/ml
Coastal area near farming center	July 1999	I	4.5×10^2 CFU/ml	2	90	2.2%	5 CFU/ml
Coastal area, 5 km west of farming center	July 1999	J	2.2×10^2 CFU/ml	0	44	0%	0 CFU/ml
Coastal area, 5 km east of farming center	July 1999	K	4.1×10^2 CFU/ml	1	82	1.2%	5 CFU/ml
Headwater	July 1999	L	3.0×10^2 CFU/ml	0	60	0%	0 CFU/ml
Filtrated samples							
Seawater supply, 8 μm	Sept 2000	Af8	2.0×10^2 CFU/ml	0	40	0%	0 CFU/ml
Seawater supply, 0.2 μm	Sept 2000	Af02	8.0×10^1 CFU/ml	0	16	0%	0 CFU/ml
Diatom, 8 μm	Sept 2000	Bf8	2.1×10^4 CFU/g	1	61	1.6%	3.4×10^2 CFU/g
Diatom, 0.2 μm	Sept 2000	Bf02	2.1×10^3 CFU/g	2	55	3.6%	76 CFU/g
Feces from abalone, 120 days[a], diatom feeding, 8 μm	July 1999	Mf8	7.4×10^3 CFU/g	1	42	2.3%	17 CFU/g
Feces from abalone, 120 days[a], diatom feeding, 0.2 μm	July 1999	Mf02	1.2×10^6 CFU/g	1	392	0.3%	3.6×10^3 CFU/g
Feces from abalone, 180 days[a], artificial diet, 8 μm	Sept 2000	Nf8	1.4×10^4 CFU/g	4	120	3.3%	4.6×10^2 CFU/g
Feces from abalone, 180 days[a], artificial diet, 0.2 μm	Sept 2000	Nf02	6.1×10^6 CFU/g	4	208	1.9%	1.2×10^5 CFU/g

[a] Day after hatching.
[b] *Vibrio halioticoli* probe for colony hybridization.

tion process in the gut of abalone, these results suggest that seawater and the diatom-bed are both sources of *V. halioticoli*.

Fig. 2 shows our hypothesis for the colonization process to the gut of abalone at the farm. After hatching, juvenile abalone settle on the diatom-bed after a 4-day swimming stage. The microalgae feeding stage is 4-months long, and juveniles are fed on the diatom-bed during this period. Then, the juveniles are fed an artificial diet containing alginate, and cultured until 2-years-old. In some cases, *Laminaria* fronds are ingested. *V. halioticoli* is not a major component of the gut microflora in juveniles fed microalgae. The gut microflora of 4-month-old abalone fed the artificial diet (in the *Laminaria*-feeding stage) comprised algal-polysaccharide-degrading or -utilizing bacteria, and the majority of the population was identified as *V. halioticoli*. As viable *V. halioticoli* cells were detected in seawater samples for culture of the abalone (Table 1), it is possible that *V. halioticoli* cells existing in seawater as a free-living form are supplied to the abalone. If *V. halioticoli* cells enter the gut of abalone in the *Laminaria*-feeding stage, the bacterium will grow and become dominant in a short period of time. However, a larger population of *V. halioticoli* was found in the diatom bed than the seawater supply and culture seawater (Table 1). This suggests that *V. halioticoli* is supplied to abalone early in their development.

Viable *V. halioticoli* (10^1–10^3 CFU/g) was detected in the fecal material of 120-day old abalone, which was still in microalgae feeding stage. Comparing the bacterial count to that of the diatom sample, the population of *V. halioticoli* did not seem to increase in microalgae-feeding juveniles

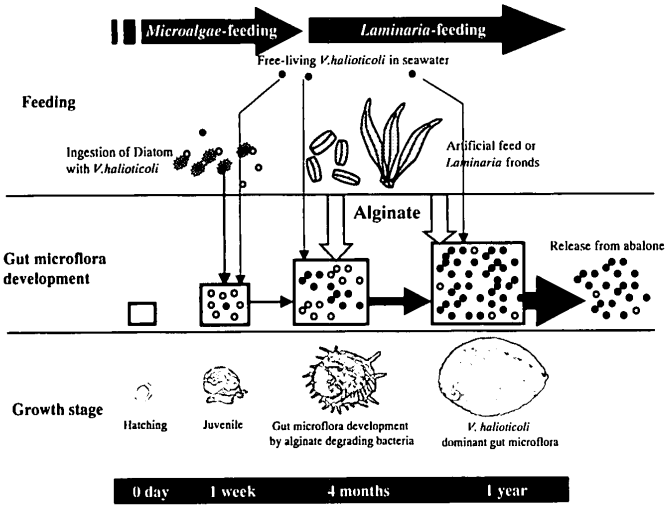

Fig. 2. Hypothetical model of the colonization process of *Vibrio halioticoli* in the gut of abalone *Haliotis discuss hannai*. ●: (*Vibrio halioticoli*.) ○: (Other intestinal bacteria.)

(Fig. 2). On the other hand, 9.9×10^6 CFU/g of *V. halioticoli* was detected in feces of 1-year old abalone (Table 1), and the bacterial count almost corresponded to that of the gut population, 10^6–10^7 CFU/g, reported previously[6]. This result suggested that *V. halioticoli* was growing actively when the host abalone started eating brown algae or the artificial diet containing alginate (Fig. 2). It also supports our finding that the gut microflora of abalone was mainly substituted by *V. halioticoli* when the host started feeding on the alginate diet (submitted for publication, Tanaka *et al.*, 2001).

Vh-probe positive colonies were also detected in drain water at the farm (Table 1). This farming center uses about 700 tons of seawater an hour, and it was estimated that 5.0×10^{11} CFU/day of *V. halioticoli* was lost. Positive colonies were also detected in the coastal area around the farm (5 CFU/ml) and in seawater 5 km from the farm (5 CFU/ml). In preliminary experiments, we observed that *V. halioticoli* did not enter a viable but non-culturable state even in a 3-month culture in sterilized seawater at 15°C (data not shown). These results indicate that *V. halioticoli* may circulate in and around the abalone farm.

It is generally considered that the resident bacteria of the gut have a mutualistic association with host-gut microbes, and transient bacteria may associate as commensalisms[2]. As *V. halioticoli* counts were the same in the fecal material and gut, *V. halioticoli* seems to be a transient microbe. Further study is needed to determine at the micro-scale, the distribution of *V. halioticoli* in the gut of abalone. This study is the first ecological report on *V. halioticoli*. The application of the colony hybridization technique with the Vh-probe in this study clearly demonstrated the distribution of *V. halioticoli* around the abalone farm. A study of the worldwide distribution of *V. halioticoli* is under way. It will improve our understanding of the ecology of this aquatic bacterial species and provide further insight into the symbiotic association between *V. halioticoli* and their host, abalone.

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