The Biology of Vibrios
Edited by F. L. Thompson et al.

Chapter 15

The Mutual Partnership between *Vibrio halioticoli* and Abalones

TOMOO SAWABE

INTRODUCTION

Intensive investigations of the microflora associated with zooplankton, shrimp, mollusks, and other marine invertebrates have been conducted for the last 50 years. The pathogenic potential of vibrios is commonly recognized in connection with serious outbreaks of food- or waterborne infections, especially those resulting from the consumption of seafood (Colwell and Liston, 1960, 1962; Kaneko and Colwell, 1973, 1974, 1975a,b; Colwell, 1984; Colwell et al., 1977). Consequently, there has been increasing attention given to understanding the roles of the gut microflora of aquatic animals (Harris, 1993; Sawabe et al., 1995). In particular, vibrios are among the most commonly reported groups of gut bacteria in marine vertebrates and invertebrates (Colwell and Liston, 1960, 1962; Harris, 1993; Thompson et al., 2004). However, there has been little conclusive evidence about positive (beneficial) relationships between gut microbes and marine animals (Harris, 1993). Even the taxonomy of the nonpathogenic vibrios has yet to be fully resolved; often, they are given informal names, such as "gut group *Vibrio*" (Colwell and Liston, 1960, 1962).

Most of the gut microbial ecosystems are improperly understood. Commonly, gut microbial ecosystems comprise hundreds of microbial species in dense populations (Hungate, 1966; Breznak, 1982; Stewart and Bryant, 1988; Russell and Rychlik, 2001; Hooper et al., 2002; Xu et al., 2003, 2004; Hooper, 2004; Bäckhed et al., 2004). The variety and complexity of the indigenous microflora have been determined for each animal species and population (Breznak, 1982; Harris, 1993). However, it is clear that the vast and complex consortium of gut microorganisms forms dynamic ecosystems coexisting with the animal hosts from birth to death and is affected by postnatal development of the digestive tract (Hooper and Gordon, 2001). Eukaryotic microbes are also major components of gut microbial communities, especially of ruminants (Hungate, 1966; Stewart and Bryant, 1988) and termites (Breznak, 1982; Dolan, 2001), being involved in the supply of nutrients for the host or as symbionts with prokaryotic partners. However, recent progress in the study of gut microbe–host interactions of herbivorous animals, xylophagous insects, and omnivorous humans has revealed the presence of so-called uncultured microbes (Ohkuma and Kudo, 1996; Whitford et al., 1998; Suau et al., 1999). These have been studied with gnotobiotic animal models (Falk et al., 1998). It is noteworthy that *Bacterioides thetaiotaomicron*, which has been considered a human commensal bacterium, has a number of important physiological roles in mammalian development, including glycan production, angiogenesis, and nutrient uptake (Stappenbeck et al., 2002; Xu et al., 2003, 2004; Hooper, 2004; Bäckhed et al., 2004). The findings on human symbionts are the second paradigm of microbe-induced morphogenesis of host animals, followed by *Vibrio fischeri*-induced squid light organ morphogenesis (MacFall-Ngai and Ruby, 1991; Hooper, 2004). Production of volatile short-chained fatty acids is also recognized as being of major importance in *B. thetaiotaomicron* and in rumen and termite gut microbes (Russell and Rychlik, 2001; Hooper et al., 2002).

Gut microbes make continuous and intimate contact through mucus cells in the epithelial tissues of animal digestive tracts from birth (Breznak, 1982; Stewart and Bryant, 1988; Russell and Rychlik, 2001; Hooper et al., 2002, 2004). Vibrios have important functions in balancing host physiology, specifically involving the gut ecosystem of marine vertebrates and invertebrates (Harris, 1993). Ruminants, termites, and herbivorous marine animals consume plant materials,

Tomoo Sawabe • Laboratory of Microbiology, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan.

which are rich in carbohydrates. However, the majority of seaweed carbohydrates are not digestible by terrestrial herbivorous animals. Therefore, it is believed that the gut microbial ecosystem of marine herbivorous animals must be a good model for studying the symbiotic association of gut vibrios. Among the huge number of marine herbivorous animals, abalone has been selected as a model because of (i) the availability of specimens from cultured and wild-caught populations, (ii) the possession of a ruminant-like digestive tract, (iii) the long life span, and (iv) its economic importance. In fact, *Vibrio* cells occupy 40% of the total gut bacterial community of abalone, as determined by whole-cell hybridization techniques (Tanaka et al., 2004). The abundance and diversity of *Vibrio halioticoli* and related species in the gut of abalones, and the possible mutual partnership of vibrios in the abalone gut microbial ecosystem, have now been clearly demonstrated.

AN OVERVIEW OF ABALONE BIOLOGY

Abalone is the common name for a member of the family *Haliotidae*, which is grouped in the class *Gastropoda* and the phylum *Mollusca*, and is a well-known marine invertebrate of considerable economic value (Oakes and Ponte, 1996). More than 100 abalone species are recognized, and they inhabit rocky shores along the Red Sea, Indian Ocean, Madagascar, South Africa, West Africa, Mediterranean, Northeastern Atlantic, Caribbean Sea, South America, Panamaic Province, Northeastern Pacific, temperate Northwestern Pacific, Indo-Malayan Archipelago, Central Pacific Australia, and New Zealand (ABMAP project by D. L. Gieger; http://www.vetigastropoda.com/ABMAP/text/index.html). Because of the hard shell of abalones, the oldest fossils of California and Caribbean species have been found from the Maastrichian age of the Upper Cretaceous period (65 to 73 million years ago) (Ino, 1952; Geiger and Groves, 1999).

Abalone taxonomy is based on "shell" morphology. Fossils and molecular phylogenetic techniques provide important clues to determining the evolutionary history (Lee and Vacquier, 1995; Coleman and Vacquier, 2002). The Cretaceous fossils found in California and the Caribbean share similarities to modern species of *Haliotis iris* (a modern New Zealand species) and *Haliotis cyclobates* (a modern Australian species), respectively (Geiger and Groves, 1999).

The major habitat of the abalones is the rocky shore with kelp forests >50 m depth. The feeding behavior of abalones is herbivorous; and the animals bite off bits of algae using a tongue-like buccal mass and radula, which is the major feeding apparatus of the *Gastropoda* (Ino, 1952; Kohn, 1983). Wild abalones show selective preferences for brown algae, including a variety of *Laminariales* (*Laminaria*, *Undaria*, *Eisenia*, *Ecklonia*, *Alaria*, *Egregia*, *Macrocystis*, and *Nereocystis*) and *Fucales* (*Desmarestia*), and red algae, including *Chondrus*, *Pterocladis*, *Gigaritina*, and *Asparagopsis* (Ino, 1952; Leighton and Booloottian, 1963). The choice of seaweed is restricted to the indigenous fauna for each species and/or each population of abalones. For example, Japanese abalones *Haliotis discus hannai* prefer *Laminaria*; the South African abalones *Haliotis midae* like another brown alga, *Ecklonia*; but Australian abalones seek out and grow better on indigenous red algae (Fleming and Hone, 1996).

Development and Aquaculture

Abalones spawn at different times in different habitats. The variation in this process is largely dependent on water temperature (Ino, 1952; Bevelander, 1988). Along the Japanese coastline, abalones spawn during late August to December, when the seawater temperature falls to ~20°C (Ino, 1952). Sperms and eggs are released from respiratory pores on the shell through the right renal organ of matured abalones. The tiny (0.2 mm in diameter) green or green-brownish eggs are fertilized in seawater. Within a day, these fertilized eggs develop into veliger larvae. The veliger has a swimming organ, a thick shell, and a lobed velum, mainly consisting of an upper and a lower epithelium (Kohn, 1983). After the short swimming stage, the veligers metamorphose and settle on the rocky sea floor, where they start eating diatom on substrata. The metamorphosed snail-like juveniles develop the feeding apparatus (buccal mass and radula) and behave as adult abalones (Kohn, 1983; Bevelander, 1988). Commonly, juveniles of >5 mm in size start ingesting seaweed in accordance with the development of the digestive system. Sensory cells, called taste buds, are observed in the tissue, which means that the animals are capable of selective feeding for "tasty" food (Ino, 1952). The most notable feature of the anatomy of abalone is the bending (V-shaped) stomach, which consists of four histologically differentiated parts (Ino, 1952; Bevelander, 1988) (Fig. 1). The first and second parts of the bending stomach are apparent in all abalone species (Ino, 1952; Bevelander, 1988; Erasmus et al., 1997). These are referred to as the crop and stomach, or simply as the first and second stomach, respectively (Fig. 1). Mucus cells are not observed inside the crop, which may be involved with food storage. Several ducts pass from the hepatopancreas in the upper part of the stomach. The inside of the (second) stomach is cov-

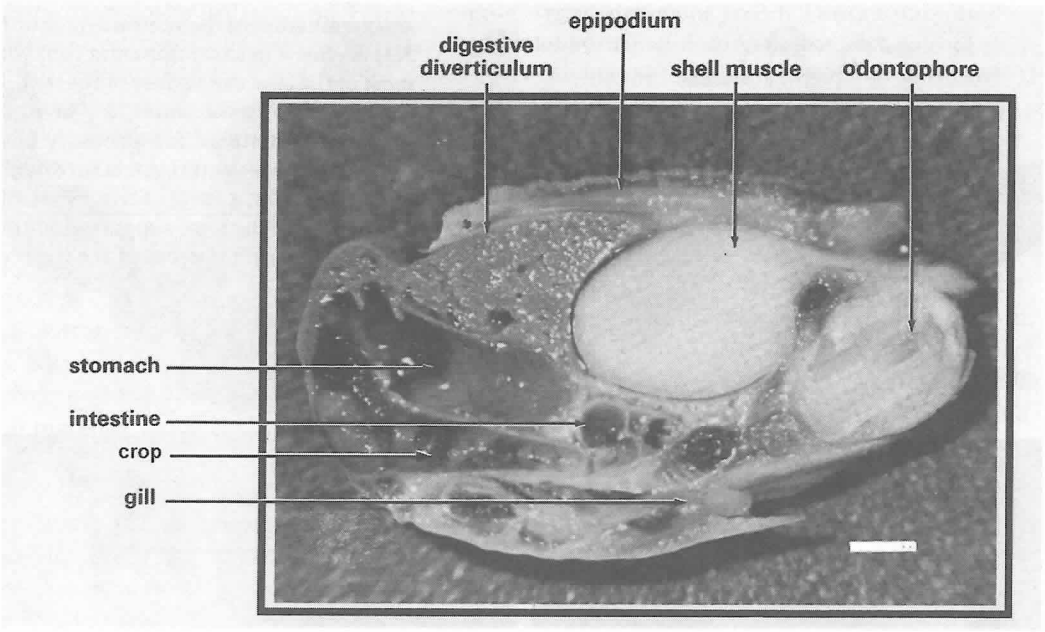

Figure 1. Sectioned view of *Haliotis* abalone. The bending stomach consists of the crop and stomach. Bar, 3 mm. The picture is reproduced from Bevelander (1988) with permission of the publisher.

ered by epithelium (Ino, 1952). In the Japanese abalone *H. discus*, third and fourth stomachs have also been observed. Of interest, the third stomach is covered with long epithelial cells (Ino, 1952). The digestive system seems to be a miniaturized version of that which exists in ruminant animals (Erasmus et al., 1997). The recorded levels of <0.38 mg of dissolved oxygen per liter in the digestive tract of greenlip abalone *Haliotis laevigata* suggest that the environment is microaerobic or anaerobic. Furthermore, the pH of the crop and the stomach has been recorded as pH 5.3 and 5.5, respectively (Harris et al., 1998).

Intensive aquaculture of abalone was developed during the 1950s in Japan to satisfy the need for conservation and human consumption (Ino, 1952). The success of artificial fertilization and (artificial) feed has gone a long way toward establishing the successful culture of abalone. Currently, many countries, including China, the United States, and Australia, have adopted abalone aquaculture (Oakes and Ponte, 1996).

DISTRIBUTION, ABUNDANCE, AND DIVERSITY OF *V. HALIOTICOLI* AND RELATED SPECIES

Currently, five *Vibrio* species have been found in the gut ecosystem of the ruminant-like abalone (Sawabe et al., 1995, 1998, 2003, 2004a,b; Hayashi et al., 2003). The distribution, abundance, and diversity of these vibrios are discussed in this section.

Distribution and Abundance of *V. halioticoli* and Related Species in the Abalone Gut

V. halioticoli was originally described by Sawabe et al. (1998) as an alginolytic, nonmotile *Vibrio* (Fig. 2). *V. halioticoli*-like strains are commonly found in the gut of *Haliotis* spp. in Japan (Sawabe et al., 1995, 1998, 2002, 2004b), South Africa (Sawabe et al., 2003), Australia (Hayashi et al., 2003), and France (Sawabe et al., 2004a). *V. halioticoli* has also been isolated from water in aquaculture facilities raising abalones (Tanaka et al., 2002b, 2003). It is noteworthy that *V. halioticoli* has never been isolated from any other mollusc or echinoderm (Sawabe et al., 2003). Furthermore, protozoans, fungi, and archaea have never been detected in the abalone gut (Tanaka et al., 2004).

V. halioticoli was isolated as an alginolytic, nonmotile, unflagellated facultative anaerobic bacterium from the gut of Japanese abalone *H. discus hannai* (Sawabe et al., 1995). Of interest, there have not been any reports of unflagellated *Vibrio* species before 1998. At the time, it was unclear whether these unflagellated organisms should have been included in *Vibrio* or *Photobacterium*. Later, phylogenetic analysis of the 16S rRNA gene sequence of four *V. halioticoli* cultures clearly demonstrated that the organism should

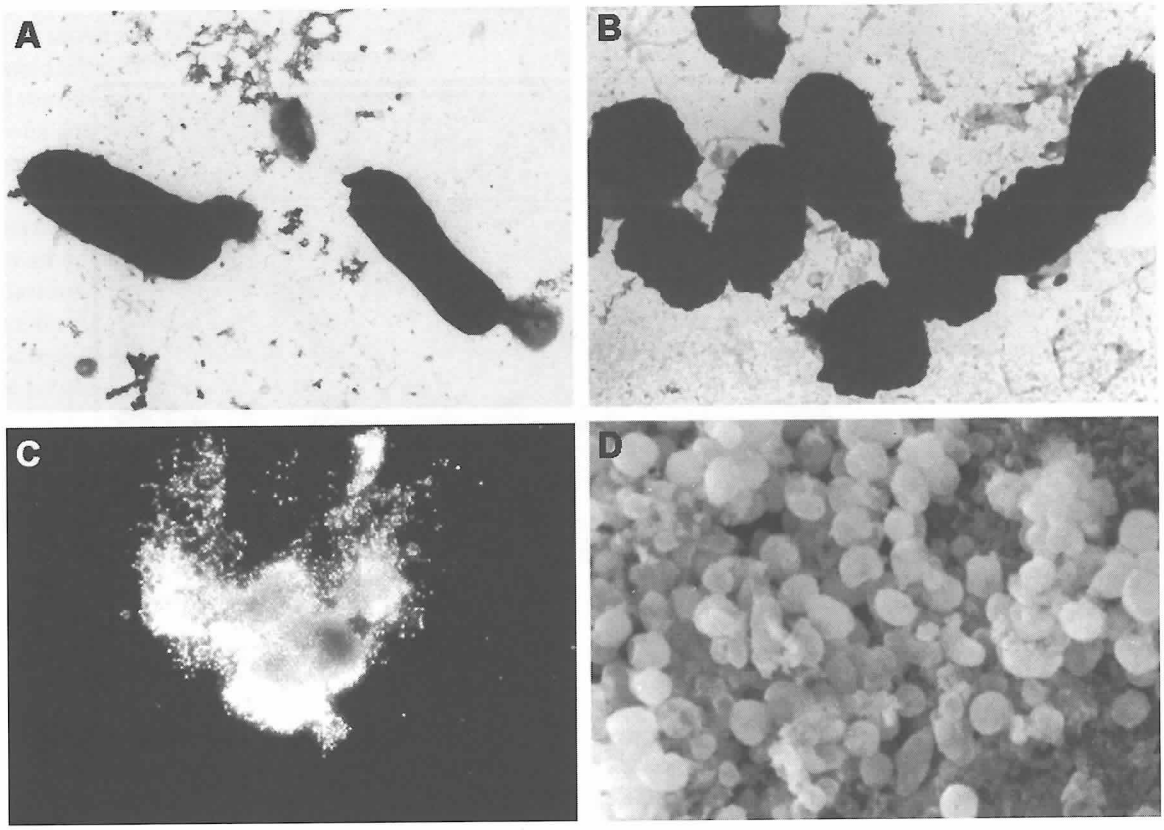

Figure 2. Morphology of *V. halioticoli* IAM14596^T. Negatively stained images of *V. halioticoli* cells cultured in marine broth (A) and broth containing 0.5% (wt/vol) sodium alginate (B). (C) The DAPI (4′,6′-diamidino-2-phenylindole)-stained cells are attached to a seawater-derived alginate gel matrix; the scanning electron microscope image is also shown (D). Parts of panels A, B, and D are reproduced from Sawabe et al. (1998) with permission of the publisher.

be classified into the genus *Vibrio* (Sawabe et al., 1998). Furthermore, polyphasic taxonomy concluded that these isolates belong in a new species, for which the name *Vibrio halioticoli* (the species name is derived from the Latin "from the gut of *Haliotis* abalone") was proposed (Sawabe et al., 1998). The organism utilizes a narrow range of carbohydrates, i.e., glucose, fructose, maltose, mannitol, D-glucosamine, *N*-acetyl-D-glucosamine, laminarin, and alginate. It is noteworthy that the organism grows well on marine agar supplemented with sodium alginate. In terms of population sizes, the number of *V. halioticoli* cells in *H. discus hannai* has been reported to be in the range of 2.6×10^6 to 9.9×10^8 CFU/g of fresh gut, and that they constitute ~70% of the viable bacterial population (Sawabe et al., 1995). It has been noted that *V. halioticoli* produces multiple alginate-degrading enzymes, which are specific for the polyguluronate block in the alginate molecule, and may be involved in the cooperative degradation of seaweed polysaccharide with the host (Sawabe et al., 1995).

In the years immediately after the discovery of *V. halioticoli* (Sawabe et al., 1998), the cells in a variety of abalone species were enumerated with a genomic DNA probe derived from the type strain (Tanaka et al., 2002a; Sawabe et al., 2003). Positive reactions were recorded in *Haliotis discus discus*, *Haliotis diversicolor aquatilis*, and *Haliotis diversicolor diversicolor*, which are all modern Japanese species of abalones. As before, it was noted that all of the *V. halioticoli*-like strains are nonmotile and are capable of alginate degradation. The *V. halioticoli*-like strains have also been isolated from turbo shells, but have never been detected from other seaweed-consuming invertebrates, for example, sea hare, sea urchin, and *Trochidae* and *Littoridae* shells (Sawabe et al., 2003). Subsequently, polyphasic taxonomy, including amplified fragment length polymorphism fingerprinting, and sequencing of the housekeeping gene concluded that isolates regarded as comprising *V. halioticoli*-like taxa could be better elevated into separate species. Thus, *Vibrio ezurae* was established

for the *Haliotis diversicolor* isolates, and *Vibrio neonatus* was named for the *H. discus discus* cultures (Sawabe et al., 2002, 2004b). The proportion of *V. halioticoli*-related species to total microflora in the gut is 42.4, 64.3, 40.6, and 19.2% for *H. discus discus*, *H. diversicolor aquatilis*, *H. diversicolor diversicolor*, and *Turbo cornatus*, respectively (Sawabe et al., 2003).

Nonmotile alginolytic vibrios have been isolated from wild populations of Australian greenlip and blacklip abalones, *H. laevigata* and *Haliotis rubra*, collected at Clifton Springs, Victoria (Hayashi et al., 2003). The bacterial strains were classified as *Vibrio* spp. and made up 96.4% of the 9.4×10^6 CFU/g of the fresh gut material. However, nonmotile alginolytic vibrios consisted of only 10% of these populations. Polyphasic taxonomy of these nonmotile vibrios led to the description of *Vibrio superstes*, in which "superstes" is derived from the Latin meaning "a survivor" (Hayashi et al. 2003). The remainder of the 90% of culturable bacteria were tentatively identified as *Vibrio* spp. (Hayashi et al., 2003). It has been speculated that a small population of *V. superstes* might be correlated with the feeding behavior of the Australian abalones, which prefer red algae—these are common along the Australian coast. The gut microflora, which is dominated by "gut group *Vibrio*," might well be adapted to the host feeding behavior. It is noted that *V. superstes* demonstrates an ability to utilize a wide range of carbohydrate sources, more so than other *V. halioticoli*-related species (Hayashi et al., 2003).

V. halioticoli has been found in the gut of the South African abalone *H. midae*, which was collected at Robin Island, Cape Town (Sawabe et al., 2003). The major food source for these South African abalones is brown algae, i.e., *Ecklonia maxima*. Experiments have revealed that the proportion of *V. halioticoli* in *H. midae* amounts to 67% of the total bacterial population of 3.7×10^6 CFU/g of fresh gut.

Vibrio gallicus, the species name of which is derived from the Latin "from France," is the most common vibrio in the gut of the French abalone *Haliotis tuberculata*, specimens of which have been collected from the coast of Brest, Brittany (Sawabe et al., 2004a). The species is regarded as being divergent from *V. halioticoli* on the basis of 16S rRNA gene phylogeny, which revealed a similarity of 97%. Furthermore, isolates clustered with other psychrophilic vibrios (Sawabe et al. 2004a). Experiments revealed that *V. gallicus* constitutes up to 55% of the total bacterial population of 3.0×10^6 CFU/g of fresh gut.

Vibrios from the guts of abalones from California, New Zealand, and Taiwan have not been examined, to date. Japanese *H. discus hannai* has been transplanted to well-controlled sites in several countries outside of Japan. The gut microbial ecosystem of these transplanted abalones could provide good experimental material for studying microbial evolution as affected by man-made environmental conditions.

Diversity of *V. halioticoli* and Related Species

Different phenotypic traits among *V. halioticoli*, *V. ezurae*, *V. neonatus*, *V. gallicus*, and *V. superstes* have been observed in 20 out of 78 features examined (Sawabe et al., 2004b); most differences are in utilization patterns of carbohydrates and organic acids. It has been noted that *V. ezurae*, *V. neonatus*, and *V. gallicus* do not utilize as wide a range of carbon compounds as *V. halioticoli* does (Sawabe et al., 1998). In contrast, *V. superstes* is more active metabolically and is capable of utilizing many carbon compounds (14 have been documented) (Hayashi et al., 2003; Sawabe et al., 2004b). Intraspecific phenotypic variations have been regarded as insignificant in *V. halioticoli*, *V. ezurae*, *V. neonatus*, and *V. gallicus* (Sawabe et al., 2004b).

V. halioticoli, *V. neonatus*, *V. ezurae*, *V. gallicus*, and *V. superstes* are included in a single robust clade that does not feature any other validly described species, as determined from small subunit rRNA gene sequences (Sawabe et al., 2004b). These 16S rRNA gene sequences are most similar for *V. halioticoli*, *V. ezurae*, and *V. neonatus*. In contrast, the 16S rRNA gene sequence of *V. gallicus* is more distant (<98%) from the other four *V. halioticoli*-related species (Hayashi et al., 2003). A robust clade has also been observed by *gap* (glyceraldehyde-3-phosphate dehydrogenase) phylogeny (Sawabe et al., 2004b).

V. halioticoli, *V. gallicus*, and *V. superstes* are most definitely separate species, insofar as the DNA:DNA similarities are <70% (Hayashi et al., 2003; Sawabe et al., 2004a). Conversely, both pairwise and reciprocal DNA:DNA similarity values among *V. halioticoli*, *V. ezurae*, and *V. neonatus* are at the boundary of a species definition (Sawabe et al., 2002, 2004b). However, the genomic diversities of the species are different, as determined by amplified fragment length polymorphism and repetitive extragenic palindrome-PCR fingerprinting (Sawabe et al., 2002) and the sequencing of housekeeping genes (Sawabe et al., 2004b).

Motility of *V. halioticoli* and Sister Species

The genus name *Vibrio* is derived from the Latin for "vibrate" (Farmer and Hickman-Brenner, 1999). All 63 currently recognized species of *Vibrio* possess sheathed flagella (Baumann and Schubert, 1984; Thompson et al., 2004), with the exception of *V. halio-*

ticoli-related species (Sawabe et al., 1995, 1998, 2003, 2004b; Hayashi et al., 2003) and *V. rumoiensis* (Yumoto et al., 1999). It is interesting that most of the nonflagellated vibrios are found in the gut of abalones. In addition to morphological observations on the lack of flagella among the *V. halioticoli*-related species (Sawabe et al., 1998, 2004a,b; Hayashi et al., 2003), PCR amplifications of genes responsible for *Escherichia coli* flagellin (*fliC*) and *Vibrio parahaemolyticus* flagellins (*flaA* and *flaC*) have not been detected in *V. halioticoli*, *V. neonatus*, or *V. ezurae* (unpublished data).

A possible explanation for the absence of flagella in *V. halioticoli*-related species is being sought by comparing the organism with the lifestyles of *V. fischeri* (Visick and McFall-Ngai, 2000) and *Bordetella* spp. (Parkhill et al., 2003; Nierman and Fraser, 2004). *V. fischeri* is a well-known symbiont colonizing the light organ of the Hawaiian bobtail squid *Euprymna scolopes*. It has been observed that *V. fischeri* loses its flagella after colonization of the light organ crypt (Visick and McFall-Ngai, 2000). Another possibility resides with whole-genome sequence analysis of *Bordetella pertussis* and *Bordetella parapertussis*, which are respiratory tract colonizers. The analyses have revealed that there are large-scale inactivations by frame-shift mutation or transposon insertion in the flagellar operons (Parkhill et al., 2003; Nierman and Fraser, 2004). Downregulation of flagellar expression by long-term symbiotic association or parasitic colonization might well lead to loss of flagellation or gene disruption of flagellar operons.

Detection Methods

Species-specific detection methods are available for *V. halioticoli*, *V. neonatus*, and *V. ezurae* (Sugimura et al., 2000b; Tanaka et al., 2001, 2002a; Sawabe et al., 2004b). In situ PCR specific to an alginate lyase gene of *V. halioticoli* was capable of discriminating between *V. halioticoli* and related vibrios (Sugimura et al., 2000b). The method was considered to be possibly effective in detection of single cells of *V. halioticoli*. Other detection methods include 16S rDNA PCR-restriction fragment length polymorphism and colony hybridization with the *V. halioticoli* genome as probe (Tanaka et al., 2001, 2002a). Both methods are reliable and rapid and should be useful for studying the ecology of *V. halioticoli*. However, it should be emphasized that cultures of *V. halioticoli* could not be discriminated from *V. ezurae* and *V. neonatus* (Sawabe et al., 2003). In fact, sequencing of the *gap* gene is the only effective method for differentiating *V. halioticoli* from *V. neonatus* and *V. ezurae* (Sawabe et al., 2004b).

ECOPHYSIOLOGICAL ROLES OF *V. HALIOTICOLI*

Abalones contain dense populations of *V. halioticoli*. The questions to be answered concern the nature of any contributions that *V. halioticoli* may make to the well-being of the host abalone. This aspect is considered in the following sections.

Cooperative Degradation of Carbohydrates

Algal polysaccharide, cellulose, alginate, and laminarin may well be important energy sources for abalones (Leighton and Boolootian, 1963; Takami et al. 1998). In particular, alginate degradation has been intensively studied as a key biochemical process in the digestive processes of abalone. Alginate is a linear heteropolymeric polysaccharide consisting of a uronic acid backbone (Gacesa, 1988) and is a major component of the cell wall matrix of brown algae (Kloareg and Quatrano, 1988). There are three heterogeneous block structures: polymannuronate, polyguluronate, and mannuronate- and guluronate-mixed blocks. The first characterization of abalone alginate degradation enzymes occurred during the 1960s and involved Japanese and Californian abalones (Tsujino and Saito, 1961; Tujino, 1962; Nakada and Sweeny, 1967). At least two alginases with different substrate preferences have been characterized (Nakada and Sweeny, 1967). As there are technical difficulties with the complete separation of the digestive tract, especially the crop and stomach, cross-contamination of enzymes from gut microbes and host abalone could occur. So the overriding concern is the origin of any enzyme studied. Quite simply, is the enzyme microbial or abalone?

It is apparent that *V. halioticoli* and related species produce alginate lyase (Sawabe et al., 1995, 1998, 2004a,b; Hayashi et al., 2003). In addition, *V. halioticoli* has been recognized to produce polyguluronate-specific alginases (Sawabe et al., 1995). The alginate-loving bacterium also shows unique behavior during alginate degradation in vitro (Fig. 2B-D). Thus, the bacterial cells attach and grow onto a calcium-induced alginate gel matrix and make dense clusters (Fig. 2C,D). The insoluble gel matrix is gradually shrunk by vigorous degradation by *V. halioticoli*. On gels, the organism forms rounded shapes and is chained (Fig. 2B). These cells are larger than those recovered from marine broth without carbohydrate (Fig. 2A). Attempts to purify the alginate-degrading enzyme(s) have not been successful owing to an unsatisfactory separation of the enzyme(s) on size filtration chromatography. However, three kinds of genes responsible for polyguluronate-specific alginases have been

cloned (Sugimura et al., 2000a). In short, *V. halioticoli* is a polyguluronate-specific-alginase producer.

It is clear that abalone secretes alginase (Shimizu et al., 2003), and a polymannuronate-specific alginase (HdAly) has been purified from the hepatopancreas of *H. discus hannai* by Professor T. Ojima (Hokkaido University). Moreover, the gene responsible for the purified enzyme has been cloned from an abalone hepatopancreas eukaryotic mRNA gene library. HdAly has an active pH range of 6.5 to 9 and a pH optimum of 8.0. The major end product is tri-uronide (Shimizu et al., 2003). It is noteworthy that abalones produce their own polymannuronate-specific alginase, which is active at neutral pH (Nakada and Sweeny, 1967; Heyraud et al., 1996; Shimizu et al., 2003). Therefore, it is speculated that there may well be cooperative (symbiotic) degradation between *V. halioticoli* and the abalone in the sharing of energy-rich alginate.

Entry of Vibrios into the Gut Ecosystem

The entry of key symbionts into the environment of the aquatic host gut or symbiotic organs is an uncertain procedure (Lee and Ruby, 1994; Ruby and Lee, 1998; Gros et al., 1996; Millikan et al., 1999). The spatial distribution of symbionts outside of the gut environment and the timing to colonize the gut need to be understood in terms of the development of the host.

Postlarval development of polysaccharide degradation activity in the gut of *H. discus discus* was measured by Takami et al. (1998) using the diatom *Cocconeis scutellum* in well-controlled feeding experiments. Enzyme activities of *H. discus discus* for cellulose, alginate, and laminarin larvae were detected after 17 days of settlement in the case of juveniles, which were fed with diatoms. The enzyme activities increased gradually until 37 days after settlement at 20°C and rapidly increased after 37 days. Chrysolaminarin, which is a β1, 3-glucan homologous to laminarin, is one of the major polysaccharides in *C. scutellum*. Although laminarinase activity might be induced by the diatom laminarin, other polysaccharide-degrading enzymes, i.e., cellulase and alginase, could be constitutively expressed without substrate induction. This means that abalones are ready to eat macroalgae within 45 days in standard rearing conditions (Takami et al., 1998).

Takami et al. (1997) reported on the induction of settlement and the effect of growth on the trail mucus. Commonly, wild abalone larvae swim on trail mucus; these workers designed experiments involving use of (i) trail mucus only, (ii) diatoms only, and (iii) trail mucus and diatoms, in an attempt to better understand the behavior of abalone settlement. The best system for settlement and growth involved use of trail mucus plus diatoms. This led to 97.3% settlement and 70% survival during the 4 weeks of the experiment. Finally, it was apparent that the abalone larvae normally grew up to 1.4 mm in length.

Changes in the gut microflora have been reported during different stages of abalone development (Tanaka et al., 2003). It has been revealed that the gut microflora of 80-day-old juveniles of *H. discus hannai* is affected by microflora of the surrounding seawater, which is composed largely of strictly aerobes, notably *Pseudomonas* and *Alteromonas* spp. The first appearance of *V. halioticoli* in the gut environment is at 80 days. Then by 110 days, the gut becomes populated predominantly by *Vibrio* spp., with *V. halioticoli* accounting for >50% of the total culturable bacterial numbers. *V. halioticoli* cells have also been recovered from water (3 CFU/liter), diatom beds used to culture juveniles (8×10^2 CFU/g), and feces (up to 10^5 CFU/g) (Tanaka et al., 2001, 2002a,b).

So far, scientists have only snapshots of the microecology of *V. halioticoli* in terms of abalone development (Fig. 3). It is interesting to speculate upon the likely timing of *V. halioticoli* entry into the host gut ecosystem. It may well be that the best time is when juveniles start feeding on diatoms, which are covered with the trail mucus of the mother abalones (Fig. 3). This timing is supported by the fact that the survival of juvenile abalones is extended when feeding on diatoms covered with trail mucus (Takami et al., 1997). Furthermore, dense populations of *V. halioticoli* have been observed on diatom beds (Tanaka et al., 2002b). Host abalones start ingesting seaweed, preferring brown algae, and this is accompanied by development of the host digestive system. It is reasoned that sustainable nutrient supplies for *V. halioticoli* and/or other gut microbes lead to stable microbial ecosystem in the gut of abalones (Fig. 3). Increased populations of *V. halioticoli* could lead to distribution into the aquatic environment, via feces, and thus to the seafloor. The nonmotile vibrios could then be available for future entry into and colonization of the abalone gut (Fig. 3).

Fermentation: an Extending Concept for Vibrio-Abalone Symbiosis

Volatile short-chained fatty acids (VSCFAs) are available to the host animal as fermentation products converted from energy-rich carbohydrates. VSCFAs are recognized as important energy sources not only in herbivorous ruminant animals (Hungate, 1966; Stewart and Bryant, 1988; Russell and Rychlik, 2001) and termites (Breznak, 1982) but also in omnivorous human beings (Hooper et al., 2002). Vibrios ferment

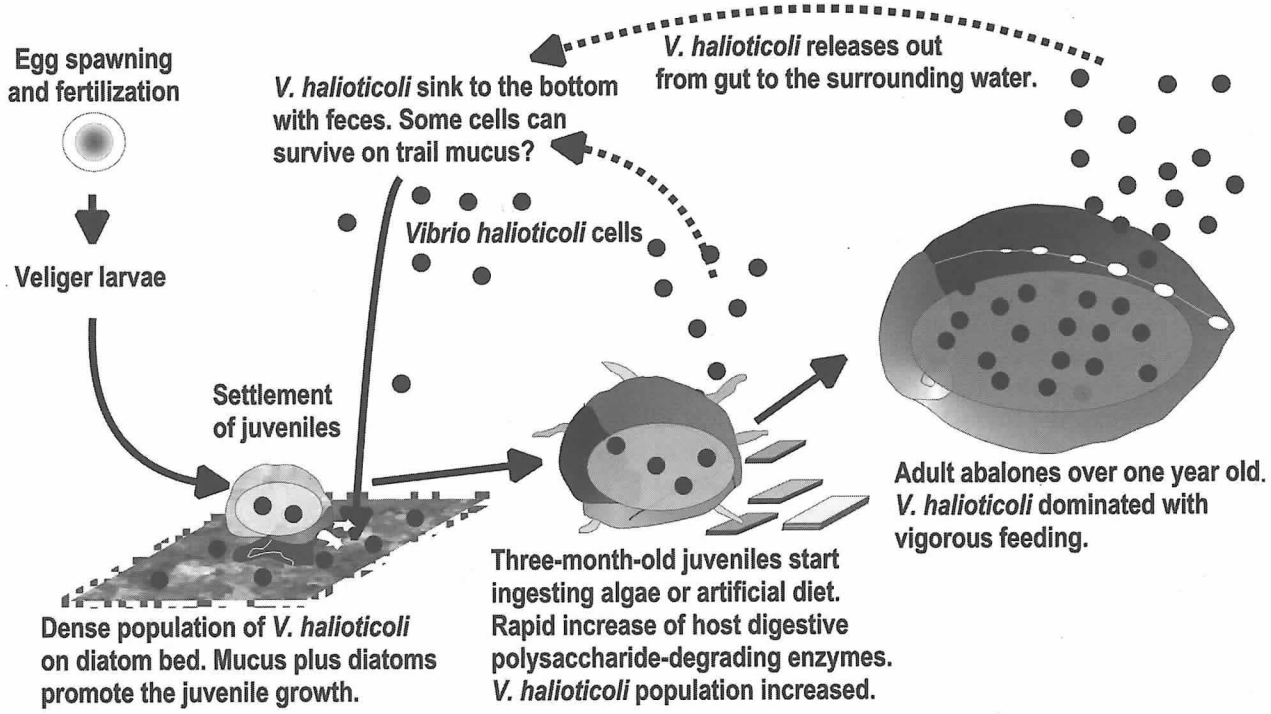

Figure 3. The likely ecophysiology of *V. halioticoli* in terms of the abalone life cycle.

a wide variety of carbohydrates (Baumann and Schubert, 1984). In particular, *V. halioticoli*, *V. neonatus*, and *V. ezurae* are capable of fermentation not only of simple hexoses but also of seaweed carbohydrates through the acetic acid/formic acid pathway (Sawabe et al., 2003). Acetic acid is probably involved as an available energy source or metabolic precursor for abalones (Sawabe et al., 2003). In the case of gut microbial ecosystems, formic acid should be eliminated by methanogens to reduce the possibility of toxicity. However, there is no evidence for the presence of methanogens in the abalone gut ecosystem (Tanaka et al., 2004). Moreover, in situ fermentation experiments of alginate by mixtures of abalone gut microbes with or without the presence of the vibriostatic agent O/129 revealed a reduction in acetic acid and formic acid production in cultures when O/129 was present (unpublished data). It is likely that vibrios may be responsible for alginate fermentation. Amounts of acetic acid and formic acid were also detected from gut homogenates of *H. discus hannai*, which had been fed with artificial diets and *Laminaria* (unpublished data).

The oxygen level inside the gut of *Haliotis* spp. is <0.38 mg dissolved oxygen per liter, as determined by microelectrode analysis (Harris et al., 1998). The gut environment, which could be anaerobic or microaerobic, has a pH of 5.3 to 5.6 in the crop and pH 5.5 to 6.5 in the stomach (Erasmus et al., 1997; Harris et al., 1998). The pH of the intestine is >pH 6.0. Thus, it is possible that *V. halioticoli* and related species are capable of fermenting carbohydrates inside the crop and/or stomach for production of VSCFAs, which may then be absorbed in the posterior part of the stomach and intestine, leading to small pH increases (Ino, 1952).

Contribution of Gut Microbes to the Nitrogen Cycle of Abalone

The carbon and nitrogen balance is an important aspect of animal physiology. In ruminants, it is believed that a major amount of nitrogen is provided by whole cell fractions of rumen microbes (Hungate, 1966; Stewart and Bryant, 1988). Proteolytic enzyme activities have been reported in five species of *Haliotis* (García-Carreño et al., 2003). The Californian green abalone, *Haliotis fulgens*, when fed with the brown alga *Macrocystis purifera*, demonstrated the highest growth rate but the lowest protease activity. Conversely, the lowest growth and highest protease activity occurred as a result of feeding with the red alga *Gelidium robstum* (García-Carreño et al., 2003). Moreover, the total protein content is higher in *G. robstum* than in *M. purifera*. Thus, it seems likely that the protease activities of abalones depend directly on the pro-

tein content of the food. However, nitrogen metabolism is only just beginning to be considered.

COEVOLUTION OF VIBRIO–ABALONE SYMBIOSIS

Host-parasite systems are intrinsically interesting to evolutionary biologists because they potentially signal a long and intimate association between two or more groups of organisms that are often distantly related and quite dissimilar biologically (Page and Holmes, 1998). This long history of association often leads to reciprocal adaptation in the host and parasites (classical coevolution or coadaptation) as well as contemporaneous cladogenetic events in two lineages (cospeciation) (Page and Holmes, 1998). Reconstructing the history of host–parasite systems by comparing their gene trees could test hypotheses of evolutionary events in which host and parasite have cospeciated, the parasites have switched to the host, and a population of parasites has become extinct (Hafner and Nadler, 1988; Page and Holmes, 1998).

The host–parasite theory has also been applied by many biologists to reconstruct marine host–microbe symbioses. Reconstructing the history of host–microbe symbioses has been tried in (i) squid and the light organ symbiotic *Vibrio* (Nishiguchi et al., 1998), (ii) deep-sea clam and the gill symbiotic sulfur oxidizers (Peek et al., 1998), and (iii) algal and gill ectosymbionts (Ashen and Goff, 2000). Parallel evolution (coevolution) between host and symbiont has been observed in *V. fischeri* and squid (Nishiguchi et al., 1998) and the sulfur oxidizer and clam (Peek et al., 1998).

Abalones have a long life history, as supported by fossil records (Geiger and Groves, 1999). It is interesting to estimate that the observed diversity among *V. halioticoli* and related species might be attributed to long symbiotic associations. I have attempted to reconstruct the phylogenetic history of the *Vibrio*–abalone association using available internal transcribed spacer (ITS) sequences for host abalones (Coleman and Vacquier, 2002) and the *gap* gene sequences for the symbiotic vibrios (Sawabe et al., 2004b) (Fig. 4). Both maximum likelihood trees were incongruent, but potentially ancient *V. halioticoli* populations could be divided into at least four populations, with one population cospeciated to French and Australian lineage. The other three populations could be inherited through Japanese and South African lineages with a number of host-sorting and extinction events (Fig. 4). These events might be supported by the fact that mixed populations of *V. halioticoli* and *V. neonatus* are observed in the Japanese abalone *H. discus discus* (Sawabe et al., 2002).

It is not known whether the reconciled tree is real or not because the phylogenies of both vibrio and

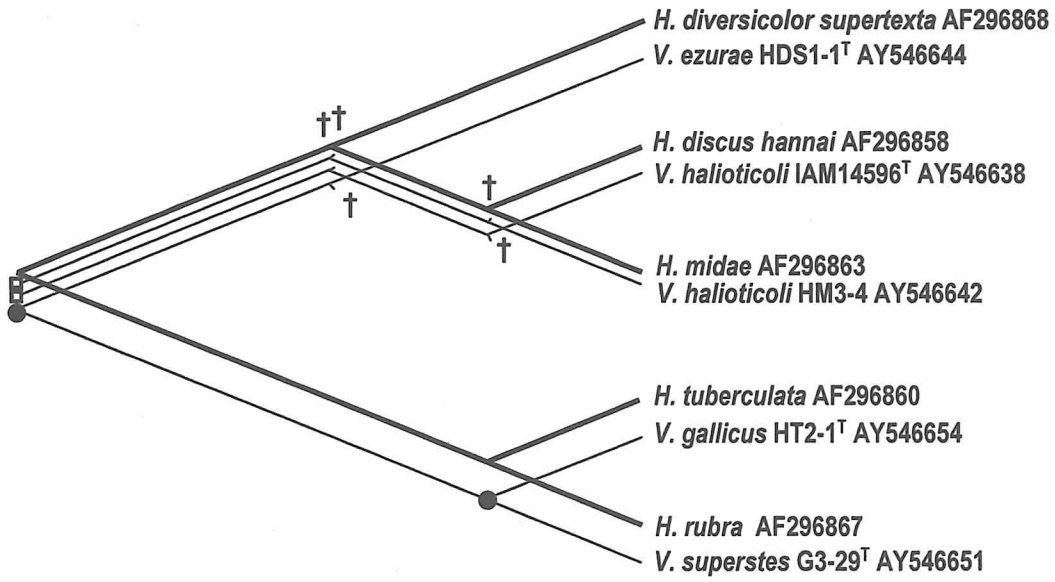

Figure 4. Reconciled tree of *Vibrio*–abalone symbiosis. Each phylogenetic tree was constructed by the maximum likelihood method based on *gap* and ITS genes for symbiotic *Vibrio* and host, respectively. Maximum likelihood trees were compared with the TreeMap program (Page, 1995). The reconciled tree estimated cospeciation (closed circle), duplication (open square), and a sorting event (short branch plus sword mark).

host are not yet fixed. Coleman and Vacquier (2002) simply grouped (i) the North Pacific species, (ii) the European species, and (iii) the Australian species; ungrouped *H. midae* and *H. diversicolor* organisms both diverged below that of the European and Australian species, as determined by ITS phylogeny. The age of *Vibrio–Escherichia* (or *Enterobacteriaceae*) radiation cannot yet be estimated. Global comparative genome analysis would provide good information on resolving the evolutional events of vibrio–animal symbiosis.

THE FUTURE

Out of the chaos of the abalone gut microbial ecosystem, symbiotic harmony has been found between *V. halioticoli* and its host. The central part of this harmony is the cooperative metabolism of algal carbohydrates, specifically alginate. Abalones are clearly capable of breaking down part of the alginate molecule (polymannuronates) to series of di- and trisaccharides as sources of energy. The symbiotic gut vibrios utilize another part of the alginate molecule, polyguluronate, which the abalone is incapable of using, and convert it to host-available waste, i.e., acetic acid. It has been determined that the vibrios are unwilling to use the waste product (Sawabe et al., 1998). Thus, the vibrios appear to be effective partners of the abalones. This vibrio–abalone symbiotic harmony is a good example of vibrio–aquatic animal interactions.

However, many aspects of the vibrio–abalone symbiosis have been determined from snapshots obtained from field material. The absence of sophisticated experimental models has been an obstacle for studying these interactions. Certainly, there is an excellent well-established model for *V. fischeri*–squid symbiosis (McFall-Ngai and Ruby, 1991; Nyholm et al., 2000), and this should be useful for the future. The goal of further work will be to determine whether these vibrios contribute to host morphogenesis, and whether the abalone transfers the vibrio to the next generation. This knowledge may be directly applicable to aquaculture and the conservation of wild stocks.

Acknowledgments. I thank Dr. Yoshio Ezura (Professor Emeritus, Hokkaido University) and Dr. E. P. Ivanova (Swinburne University of Technology) for critically reading the manuscript.

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