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SAN DIEGO

ANTIMICROBIAL METABOLITES PRODUCED BY EPIBIOTIC BACTERIA: THEIR ROLE IN MICROBIAL COMPETITION AND HOST DEFENSE

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Oceanography

by

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The dissertation of Maria Sofia Gil-Turnes is approved,
and it is acceptable in quality and form
for publication on microfilm:

Chairperson

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This work is dedicated to all those organisms who collaborate.

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ABSTRACT OF THE DISSERTATION

Antimicrobial Metabolites produced by Epibiotic Bacteria: their Role in Microbial Competition and Host Defense

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Although many bacteria have the ability to produce antimicrobial compounds in culture, it is not clear whether bacterial metabolites have an adaptive function in microbial competition. The work described in this thesis represents a new approach to microbial chemical ecology.

Three examples are described where antimicrobial compounds produced by epibiotic bacteria protect the host from encroachment by other microorganisms. Embryos of the caridean shrimp Palaemon macrodactylus are protected against infection by the pathogenic fungus Lagenidium callinectes by at least one strain of epibiotic bacteria. This strain produces 2,3-indolinedione, a strong antifungal agent. The American lobster, Homarus americanus, uses a similar strategy of defense against the fungus. Healthy embryos are

covered by a dense layer formed by a bacterial monoculture. These bacteria produce the compound p-hydroxyphenyl ethanol, which also inhibits fungal growth. Three strains of pigmented bacteria which occur in exclusive association with Microcoleus lyngbyaceus, a filamentous species of cyanobacteria, produce a potent antimicrobial compound. The compound, which had not been described previously, has considerable inhibitory activity against a number of fungi and bacteria.

The results of this research indicate that antimicrobial secondary metabolites indeed mediate microbial competition and strongly influence the establishment of associations between antimicrobial-producing bacteria and a variety of hosts.

CHAPTER I

INTRODUCTION

As more is learned about the bacteria associated with all types of organisms, it is apparent that the composition of these microbial communities is seldom haphazard. The external surfaces of virtually all organisms and the internal tissues and organs of many have their particular microbiota (Sonea and Panisset, 1980). Specificity of such associations is probably intensified in aquatic environments where dilution of nutrients makes it advantageous to conglomerate around the sources. On the other hand, because microorganisms are easily transported by water, contact with other, potentially competing microorganisms is continuous. Nevertheless, specific populations are able to maintain their stronghold on their host. Metabolic specialization can explain competitor exclusion in some cases, but the majority of bacteria known seem to share a wide area in the general spectrum of metabolic requirements.

The study of microbiology was born out of the need to isolate, characterize and identify microorganisms pathogenic to humans and animals of interest to humans. With the discovery of antibiotics, enormous efforts were placed into culturing techniques to increase antibiotic production and modification of culture media to achieve production of new antibiotics. A great amount of

information became available on laboratory-reared microorganisms, and the advent of molecular biology brought the detail of this information to incredible refinement. But, we have remained ignorant of the processes that mediate the relationships among bacterial populations, and the pressures that drive the adaptations undergone through time by the oldest organisms on our planet. In this century ecology achieved its recognition as a science and in the past 20 years it has become increasingly obvious that chemical ecology is probably an aspect of every organism's adaptive ability. It is puzzling that we have not looked more vigorously at the role of defensive metabolites, or allelochemicals, in bacterial ecology.

The size and structure of bacteria limit the competitive interactions to metabolic and chemical processes. Even advantageous metabolic characteristics can be reduced to a set of chemical properties. Thus, the study of microbial ecology is necessarily a study of chemical interactions. The fact that bacteria prefer to live in association with other organisms, including other microbial species, enhances our ability to understand the elements of their ecology (Price *et al.*, 1986). We cannot easily observe one individual cell or even a colony directly in nature without displacing or destroying them, but we can measure the effects of the bacteria in many ways and, what is most valuable, the effects of their absence on their host organisms.

There are many examples of beneficial and specific associations that affect our daily life. A property of many bacteria is that they can attach and form colonies or discrete microbial layers in specific locations on the internal and external surface tissues of animals. The best known example of associations involving non-specialized bacteria and a host that seems not to have invested any adaptive effort to favor the associations, is our own human body. The effect of disrupting these associations is quite devastating, as is clearly illustrated by the results of excessive amounts of antibiotics on overall health. Distinct populations of bacteria inhabit the human surface, internally and externally and it is apparent that, to a large extent, the equilibrium which these populations have somehow achieved among themselves and the rest of the microbial population determines the body's capacity to resist microbial pathogens. Some strains of the genus Streptococcus, for example, adhere to clearly delimited 'habitats' within the mouth (Ter Steeg et al., 1987; Robrish, 1986; Gibbons and Van Houte 1971), the gastrointestinal tract and the skin (Mackowiak, 1983; Marples, 1969). The normal flora plays a critical role in the protection of the host from invasive microorganisms (Price et al., 1986) and its general health rapidly deteriorates with the indiscriminate use of antibiotics. Many of these bacteria produce antimicrobial compounds, as is the case of skin bacteria which use sebum as a substrate

(Marples, 1969), vaginal bacteria and some enteric bacteria. Recent research on human enterobacteria shows that colicins, plasmid-coded proteins of high molecular weight, probably regulate species composition and relative abundance of each strain in the human intestine (de Lorenzo et al., 1984; Farkas-Himsley, 1980). It had previously been suggested (Asensio et al., 1976) that the intestinal microbial flora of newborns undergoes a succession that is regulated by microcins, antibiotics of small molecular weight (Aguilar et al., 1983, Duro et al., 1979), and by the selective production of non-specfic agents such as fatty acids and hydrogen peroxide. Whether these are 'antibiotics' or not is an unresolved issue. As Janzen (1977) notes in his paper on the adaptive causes of fruit, seed and meat decomposition, ... "while simple organic acids and alcohols produced by yeasts are not generally called antibiotics, from an ecological viewpoint they surely are".

There are approximately 5000 different types (grouped in some 2000 species) of bacteria known to produce antimicrobial compounds in culture. Because the discovery of penicillin represented such a significant breakthrough in the treatment of bacterial diseases, great efforts have led to the systematic isolation of bacteria and fungi that would produce highly active compounds specifically antagonistic to microorganisms pathogenic to humans and to animals and plants of importance to humans. Since most active compounds are toxic to humans, fewer than 100 antibiotics have a

clinical use and these are produced mainly by filamentous fungi and actinomycetes (mostly Streptomyces). Among the non-filamentous bacteria, only the genus Bacillus is considered important as a producer of antibiotics (de Lorenzo and Aguilar, 1984). Because this work has been so narrowly focused, it has been of little use in elucidating the chemical ecology of microorganisms.

Do bacteria, in nature, produce secondary metabolites which have the specific adaptive 'purpose' of inhibiting competitors? Can we show, or at least suggest that, as in other organisms, there is a chemical element in microbial ecology? Soil microorganisms, for example, produce a variety of antibiotics and microbial inhibitors in vitro, but their true interactions in their complex native ecosystem are extremely difficult to observe and quantify. Barbier (1978) expresses the difficulty of these questions: "when a metabolite has not evolved in order to maintain the ecological niche of an organism (the adaptive advantage), its interaction on the growth of another organism is incidental... when one looks for new antibiotics, a search for interactions and space competition among species may be a real path to discovery". Haslam (1986) further expresses the basic concept that has impelled all ecological research: ..."the concepts of purpose and function are essential to an understanding of living systems since it is invariably assumed that the characteristics of an organism -from the morphological to the molecular- have been selected because

of their functional advantage".

The antagonistic effects that microorganisms have on other microorganisms have probably been put to practical use for as long as agriculture has existed. The use of mulches to protect crops against diseases exploits the fact that microorganisms growing on these mulches produce antifungal compounds (Dixon, 1986). Many strains of soil-inhabiting Pseudomonas optimize the growth of beans, beets, potatoes and other vegetables. The bacteria produce siderophore antibiotics which compete with the iron-binding capacity of deleterious bacteria and fungi (Schroth and Hancock, 1982). Interactions among siderophore-producing bacteria are quite complex (Vanderbergh et al., 1983; Kloepper, et al., 1980). In general, these bacteria are associated with plant roots. Under natural aerobic conditions most of the iron in soil exists in the insoluble ferric form, which is not available to plants without the associated bacteria (Nambiar and Sivaramakrishnan, 1987). The symbiotic bacteria of leguminous plants have also been found to inhibit other soil microorganisms, particularly fungi pathogenic to their host (Gagne and Antoun, 1985).

Soil microbiology has been intensely studied for many years and it is from this field that the first pieces of evidence and questions regarding microbial chemical ecology arose. It was early recognized (Whittaker and Feeny, 1971) that microbial secondary metabolism had a role in competition at least as important as differential

abilities in nutrient uptake, survival during starvation periods and chemotaxis towards favorable substrates (McEldowney and Fletcher, 1987; de Lorenzo and Aguilar, 1984; Veldkamp et al, 1984; Woodruff, 1980; Chet and Mitchell, 1976; Bell and Mitchell, 1972; Mitchell et al., 1972) and away from unfavorable ones (Young and Mitchell, 1973). Antimicrobial compounds are produced by a variety of bacteria and fungi found in the soil and in the rhizosphere of most plants (Lindow, 1987; Whittaker and Feeney, 1971). In addition, many soils contain accumulated substances which exert a general inhibitory action on bacteria and fungi. Seawater, also, has inhibitory effects against a variety of terrestrial and aquatic microorganisms (ZoBell, 1946). The lack of knowledge concerning the significance of antimicrobial compounds in nature and the lack of information about the distribution and ecology of known or potential producers have made the selection and sampling of natural environments essentially a random process (Williams and Vickers, 1986).

The growing awareness regarding microbial antagonism in nature is even changing the treatment of sludge generated at wastewater disposal plants (Millner et al., 1987), as it is recognized that unsterilized sludge is not colonized by pathogenic bacteria, such as Salmonella. This information has resulted in the inclusion of sludge in fertilizers. Research by Paul et al. (1980) suggests an interesting association between a nematode that is parasitic on a

variety of insects and enteric luminous bacteria that produce antimicrobial compounds. The nematode completes its reproductive cycle within the insect and, at the onset of this period, the luminous bacteria are excreted into the insect's hemoceol. The insect is killed but does not decompose until the nematode has completed its cycle. The bacteria responsible for putrification are presumably kept in check by the antimicrobial compounds produced by the symbiotic bacteria. A pioneer researcher of the fungus-culturing attine ants, N. A. Weber (1955, 1972) speculated that the fungi might produce substances to inhibit the growth of microbial competitors in the fungus gardens (Wiemer, 1985; Angeli-Papa, 1984). In another study (Hervey and Nair, 1979), a fungus of the genus Lepiota, cultured by ants of the genus Cyphomyrmex was shown to produce in liquid culture the antibacterial compound lepiochlorin, a small lactol.

The marine environment in general, in comparison to many terrestrial environments, is dilute or sparse in its nutrient sources. It is thus more likely that different organisms will form associations to utilize any available energy and nutrient sources more efficiently and thoroughly. Furthermore, the chemical composition and the physical constraints of the ocean are radically different from those on land. We might, therefore, expect to find correspondingly different metabolites being produced by marine organisms (Okami, 1986; Fenical, 1982; Barbier, 1978;

Nigrelli, 1962). Research on bacterial associations in the ocean has intensified during the last few years, with the emphasis on those associations that are endosymbiotic and highly host-dependant. Most of this research has been concerned with detailed descriptions of the physical characteristics of the associations and some very interesting work has been done on the symbionts at the molecular level.

Bacteria with Unique Metabolic Capacity/Highly Dependant Hosts

On one extreme of the spectrum of bacterial assocciations are symbiotic bacteria with the ability to derive energy from unusual substrates. Of course, as isolating techniques become more sophisticated, it may become apparent that these organisms are common, but at this point, they are considered rare.

The discovery of deep-sea hydrothermal vents (RISE Project Group, 1980) showed that rich and diverse communities of organisms thrive at immense distances from the euphotic zone. These environments are rich in reduced sulfur compounds, and the waters from the vents and the surrounding rocks are laden with bacteria capable of fixing CO_2 chemoautotrophically by oxidizing reduced sulfur compounds (Felbeck and Somero, 1982). These bacteria are presumably eaten by the abundant filter feeders which in turn may be consumed by carnivores. A large proportion of

the community (Reid and Bernard, 1980) is composed of large tube worms of the species Riftia pachyptila Jones, a pogonophoran (Vestimentifera) which is devoid of mouth, digestive tract and anus, and with a morphology extremely unfavorable for an absorptive mode of nutrition. These worms have developed a tight association with bacteria capable of oxidizing hydrogen sulfide to fix CO2 (Southward et al., 1981) and there is evidence that the host produces a factor that regulates symbiont cell division (Vetter, pers. comm.). The bacteria are found in small intracellular vacuoles in the trophosome, an extensively vascularized organ (Cavanaugh et al., 1981). Large crystals of elemental sulfur are found within the trophosomal tissue which indicates another drastic adaptation on the part of the host. Hydrogen sulfide is the source of reductant energy but is also highly poisonous to eukaryotic cells as it inhibits mitochondrial respiration. The current hypotheses are that the host either produces a binding enzyme to transport the hydrogen sulfide to the bacteria or that it converts the hydrogen sulfide and sulfate to thiosulfate, which is not poisonous to eukaryotic cells (Vetter, seminar October 2, 1987). The discovery of this extreme example of the role of reduced inorganic elements in animal nutrition led to research in other environments rich in organic matter, which potentially harbor hydrogen sulfide and are thus potential habitats for similar symbioses. The reducing marine and salt marsh muds have yielded a variety of invertebrates associated with

sulphur-oxidizing bacterial symbionts (Cavanaugh, 1983; Jorgensen, 1982; Howart and Teal, 1979). In general these organisms have obvious adaptations to facilitate the symbioses: pogonophorans and the oligochaete P. leukodermatus lack a mouth and digestive tract. Bivalves of the genera Solemya (Reid and Bernard, 1980) and Calyptogena (Childress and Mickel, 1982) have very small guts and feeding appendages and thick gills where they harbor the bacteria. These invertebrates have adapted morphologically to internal chemoautotrophic fixation of CO_2 to the extent that survival without the symbiotic bacteria would be impossible. The distribution of these animals is determined exclusively by environmental conditions which favor specialized bacteria.

In the terrestrial environment, several symbioses include enteric bacteria with specialized metabolisms such as cellulolytic, methanogenic and vitamin-producing capacities (Mason and Richardson, 1981; Fonty et al., 1987; Haanstad and Norris, 1985; Lee et al., 1987; Martin, 1987). Analogous associatioins are found in aquatic environments. The mysid shrimp Mysis stenolepis obtains nourishment from food containing large amounts of cellulose but loses its ability to do so in the presence of antibiotics (Wainwright and Mann, 1982). Wood-boring bivalves of the family Teredinidae, known as shipworms, can live on a diet of wood alone, without filter feeding (Gallager et al., 1981). Shipworms are involved in the first association known in

which the cellulolytic bacteria are lodged in a specialized organ (the gland of Dehayes) and not in any part of the digestive tract (Waterbury et al., 1983). Furthermore, the same bacteria are capable of fixing nitrogen, which would explain how these organisms live solely on cellulose. Other shipworms (Carpenter and Culliney, 1975) possess nitrogen-fixing bacteria found in large numbers in their gut. The isopod Gnathia calva, a fish parasite, has a peculiar structure in its digestive tract. This structure is filled with bacteria (Juilfs and Wagele, 1987) which are probably related to the hematophagous way of life of the isopod. Symbioses with nitrogen-fixing bacteria analogous to those of legumes and Rhizobium, also occur in marine plants. Bruguiera gymnorrhiza (L.) Lamk., which is a mangrove tree species from Japan harbors nitrogen-fixing bacteria of the familiy Enterobacteriaceae in wart-like structures in its bark (Uchino et al., 1984).

In recent years, it has become necessary to revise models for nitrogen fixation in the ocean. It was formerly thought that only heterocystous blue-green bacteria (cyanobacteria) were capable of nitrogen fixation. It is now believed that many of the non-heterocystous cyanobacteria also fix nitrogen and one of the ways in which they exclude oxygen from the fixing tissues is by association with aerobic bacteria (see Chapter VI).

Bacteria with no known specialized metabolism adapted to life within animal and plant tissues and organs.

Some organisms have evolved special structures to enclose heterotrophic bacteria. Such adaptations may be the result of a long period of coevolution, but the nature of the interactions between host and bacteria are so far conjectural.

The diatom Rhizosolenia can be very abundant in oligotrophic waters, with very low concentrations of nitrogenous compounds usable by algae. There is evidence that this mat-forming diatom is associated with large numbers of intracellular bacteria which are probably responsible for the high rates of acetylene reduction measured in the mats (Martinez et al., 1983).

Holland and Nealson (1978) examined eight echinoderm species, representative of the five classes in that phylum and found that all except the two holothurians studied contained subcuticular bacterial populations consisting of one or two morphotypes. Feral (1980) subsequently found similar bacteria in holothurian species. The bryozoan Palmicellaria skenei (Ellis and Solander) has hypertrophied vestibular glands. This hypertrophy is related to the presence of curved rod-shaped bacteria that inhabit the vestibular gland in great numbers (Lutaud, 1965). Other bryozoans possess special bodies suspended from the main funicular strands which are laden with bacteria. The microorganisms, which differ morphologically in different

species of bryozoans are effectively isolated in these pouch-like structures (Lutaud, 1969). It is not known how these bacteria are transmitted from one generation to the next and what their role is in the association but it has been proposed that they may have a role in larval metamorphosis (Woolacott, 1981). Bryozoan larvae of the genus Bugula carry large numbers of bacteria within the pallial sinus. This structure exists only during the larval stage. Evagination of the pallial sinus occurs during metamorphosis and concomitant with involution of the larval locomotory organ which might carry some bacteria to the interior of the metamorphosing individual. The bacteria may have a role in metamorphosis completion or substrate conditioning. The bryozoan species that possess bacteria are the most successful in colonizing new environments and are considered important fouling organisms. Another bryozoan, Watersipora arcuata, has recently been reported to be associated with an unusual prokaryotic partner, Acholeplasma, a true mollicute (Boyle et al, 1987; Zimmer and Woolacott, 1983). In this association the bryozoan larva carries the bacteria in a circular groove and the adult host forms encapsulated packets within its tissues surrounding groups of bacteria, in a fashion similar to that of the Bugula association . There are a few visual reports of unidentified 'mycoplasma-like' prokaryotes in the tissues of oysters (Harschbarger et al., 1977) and bryozoans (Zimmer and Woollacott, 1983) but identification of the prokaryotes

was not established. Mollicutes, the smallest known organisms capable of self-reproduction, have been observed and identified in terrestrial vertebrates, insects and plants. They are found in highly anaerobic microhabitats (rumen, acidic coal wastes) and have primarily been linked with animal diseases (Bove and Duplan, 1974; Hayflick, 1972).

There are several reports of sponges associated with a variety of eubacteria and cyanobacteria (Santavy, 1985; Bergquist and Bedford, 1978; Imhoff and Trupfer, 1976; Sara, 1971), but, relatively little is known about the functional details of these relationships. In some sponges, bacteria occupy up to 50% of the host's volume (Barbier 1978, Vacelet and Donadey, 1977; Wilkinson, 1978) and some researchers have proposed that the natural products isolated from many sponges are produced by the bacterial symbionts (Faulkner, 1978). One sponge, however, stands because of its adaptation to transmission of the associated bacteria to its offspring (Levi and Levi, 1976). The oviparous sponge Chondrosia reniformis Nardo insures the transmission of symbiotic bacteria by a remarkable mechanism involving amoebocytes which act as carriers of bacterial cells during embryogenesis. The amoebocytes carrying the symbiotic bacteria are incorporated into the blastocoele of the swimming larva and after metamorphosis the bacteria are released into the mesohyl of the young sponge.

Luminous bacteria are another example of highly

adapted symbionts. Some groups of fishes and squid have specialized organs whose only function is to harbor luminous bacteria (Young, 1977; Haneda and Tsuji, 1971). The presence of these bacteria is intimately related to the behavior of the hosts. There is evidence that the host actually regulates symbiont density and the amount of luminiscence by regulating the concentration of free iron made available to the bacteria (Haygood and Nealson, 1984). Some tunicates also carry luminous symbionts (Mackie and Bone, 1978) in specialized organs. This is the only known case in which the bacteria (or bacteroids) are intracellular and probably have undergone considerable biochemical specialization. Control of luminiscence in this symbiosis is apparently determined directly by the host, giving the bacteria an evolutionary position intermediate between organelles (Margulis, 1981) and true symbionts.

Associations where neither host nor symbiont has undergone any obvious adaptation

Many marine organisms have a normal bacterial flora living on their surface (Ducklow, 1983; Wilkinson et al., 1981; Wimpenny, 1981; Colwell and Liston, 1962; Liston, 1957). The host organisms are not known to have specialized structures or behaviours that can be related to the presence of the bacterial epibionts.

Microscopic examination of algae almost always produces evidence of bacteria (Round, 1983). The

filamentous bacterium Leucothrix mucor has a worldwide distribution on algae but many other types colonize algal surfaces as well. Apart from the specific association with nitrogen-fixing bacteria mentioned earlier, there are many observations of interdependence between cyanobacteria and eubacteria. All investigators agree that algae and bacteria form a tight system which must be understood in order to estimate total productivity and biocycles in the ocean and in lakes (Mikhaylenko and Kulikova, 1972). Most studies have been done on fresh water systems and the results suggest that the cyanobacteria act as substrate for the eubacteria and produce metabolites that have a growth-stimulating effect on the eubacteria. The bacterium responsible for Legionnaires disease, Legionella pneumophila, for example, has a widespread distribution in nature (Tison, et al., 1980), but it could be grown only very sparsely and under restricted conditions in laboratory culture (Freeley et al., 1978) until it was found that it always occurs in association with a species of cyanobacteria from the genus Fischerella. In studies of phytoplankton cells in natural seawaters, it was found that bacteria attached to phytoplankton different from those in the surrounding sea water (Kogure et al., 1982; Cole, 1982; Shiba and Taga, 1980). Studies on the epiphytic bacterial flora of seaweeds, showed very complex and diverse microbial communities associated with several algae, and a large number of strains were found to produce compounds inhibitory

to other strains in the community (Lemos et al., 1985). Blooms of the unicellular alga Phaeocystis pouchetii tend to strongly depress the abundance of cooccurring species (Davidson and Marchant, 1987). This depression appears to be related to an association with manganese-binding bacteria which presumably cause a localized depletion of manganese and possibly some other micronutrients. In some associations the bacteria are quite obviously providing factors that are essential for the development of the algae (Thomas and Allsopp, 1983). Colonies of the freshwater alga Volvox aureus are not viable unless provided with Pseudomonas fluorescens, a bacterium isolated from mucilage surrounding the colonial alga (Kochert and Olson, 1970). The specificity of this association was shown by the inability to infect the Volvox with any other bacterial species. The same occurs with the planktonic alga Asterionella (Kain and Fogg, 1958). The macrophyte Ulva lactuca does not grow if treated with antibiotics unless cultures of a bacterium isolated from its surface are subsequently added to the medium (Waite and Mitchell, 1976). Interactions between algae and bacteria may play a major role in succession and dominance of individual algal species in a given habitat. Jolley and Jones (1977) have shown that some bacterial strains stimulate the growth of algae with which they coexist in nature. However, the same bacteria either inhibit or do not affect other species (Delucca and McCracken, 1977). Furthermore, some species of algae respond

differently to single- and multiple-member bacterial cultures. For example, in laboratory experiments with Pseudomonas and Flavobacterium, each strain alone stimulated the growth of Chlamydomonas, whereas in combination they were inhibitory to the algae. The distribution of the microbial populations of some macroalgae is so well regulated that specific bacterial types are consistently found on different parts of the alga (Lobban and Baxter, 1983; Cundell et al., 1977).

Cyanobacteria and eubacteria have been observed in many marine sponges and it has been suggested that they play an important role in sponge nutrition (Sara, 1971; Vacelet and Donadey, 1971). In some sponges, bacteria occupy up to 50% of the mesohyl volume (Wilkinson 1982). In a study of 11 species of demospongia belonging to seven different orders it was found that in general, massive sponges with high tissue density contain numerous bacteria belonging to a few (4 to 7) distinct types of bacteria with unusual complex cell walls, and sponges with low tissue density contain fewer bacteria of one morphological type which are usually thread or rod-shaped and with conventional cell walls (Vacelet and Donadey, 1977). The sponge species studied are markedly different systematically, morphologically and ecologically which suggests that the occurrance of intimately associated bacteria is a general phenomenon in sponges, although the particular physiological relationships between the symbionts probably are quite different. For

example, the marine sponge Halichondria panicea produces a lectin which is essential for the growth of its bacterial symbiont Pseudomonas insolita but has no effect on the physiology of other Pseudomonas species isolated from six other marine sponges (Muller et al., 1981).

Species of Vibrio are often found in the gut and on the surface of zooplankton organisms, particularly copepods (Huq et al., 1984, 1986). The observation that zooplankton blooms are related to the seasonal cycle of V. parahaemolyticus (Huq et al., 1983; Kaneko and Colwell, 1978), the causative agent of cholera, led to experiments by Huq et al. (1984) that showed that the bacterium has great affinity for the copepod and that it promptly colonizes the oral area and the surface of the egg sacs.

Some species of cyanobacteria have been found on the external surface of ascidians (Lewin, 1975; Kott et al., 1984). An association between the ascidian Trididemnum tegulum (Cox et al., 1985; Kott et al., 1984) and an unusual cyanobacterium was recently described. Compound ascidians of the family Didemnidae often harbor prokaryotic photosynthetic symbionts from the genus Prochloron in their tissues.

Some studies suggest that among the coelenterates the composition of surface bacterial floras varies according to species (Doores and Cook, 1976). In some cases, specific bacteria seem to be required for the proper development of the organism. For example, in the scyphozoan Cassiopea

andromeda, a Vibrio species apparently induces the changes necessary for settlement and metamorphosis in the planula larva (Neumann, 1979). The mucus surrounding polyps of octocorals and scleractinian corals are known to contain bacteria of diverse types (Rublee et al., 1980; Ducklow and Mitchell, 1979). These bacteria utilize compounds from the coralline mucus and may play a role in processing the mucus for utilization by reef detritus feeders. The bacterial species Vibrio alginolyticus which grows rapidly on the coral Heteroxenia fuscesens is specifically attracted to its mucus and possesses a mechanism to maintain itself on the coral surface (Ducklow and Mitchell, 1979).

Associations between molluscs and bacteria are known to occur outside the cellulose digesting species discussed previously. The tusk shell (Scaphopoda) Dentalium dentale is associated with bacteria that are transmitted throughout the embryonic divisions (Geilenkirchen et al., 1971). Although there is some evidence that molluscan larval settlement and metamorphosis are dependant on bacterial cues (Hadfield, 1978; Weiner and Colwell, 1982), with the exception of the luminous bacteria associated with some deep-sea squids, there is no knowledge of specific bacteria living in association with the molluscs other than in the digestive tract.

In some cases, the associations involve more than two types of organisms as with the polychete Janua (dexiospira) brasiliensis Grube which usually live where the

green alga Ulva lobata occurs. It was reported (Kirchman et al., 1982) that settlement and metamorphosis of the pelagic Janua larva is induced by bacteria isolated from the surface of Ulva. Other polychete larvae that settle preferentially on different algae may be dependant for metamorphosis on cues given by bacteria associated with the algae. The external microflora of a marine wood-boring isopod (Boyle and Mitchell, 1981) is apparently important to the host's nutritional capability.

Antimicrobial Compounds from Marine Bacteria

The number of observations involving groups of microorganisms associated with other organisms in the ocean continues to grow. As for studies of secondary metabolites with antimicrobial activity produced by marine bacteria, the record is not very abundant. Even though ZoBell (1946) and Burkholder (1973) recognized that many surface bacteria had inhibitory activities against other microorganisms of the same general area, research on microbial chemical ecology has not attracted many natural products chemists. The general approach in this area has been, once again, to look for unusual and/or highly potent compounds with activity against principally human pathogens (Bonar et al., 1986; Stanley and Stanley, 1986; Stellwag and Brenchley, 1986; Okami, 1982; von Berlepsch, 1980; Woodruff, 1980; Gauthier and Breittmayer, 1979; Grant and Mackie, 1977; Okazaki and Okami, 1972), and other activities with medical application

(Umezawa et al., 1983; Jacobs et al., 1981; Kaul, 1981; Keleti et al., 1979; Okutani, 1977; Imada et al., 1973).

Appendix A (p. 161) shows the structures of antimicrobial compounds produced by some strains of marine bacteria. The first antimicrobial compound from marine bacteria to be reported was 2-(3',5'-dibromo-2'-hydroxyphenyl)-3,4,5-tribromopyrrole (I) produced by Pseudomonas bromoutilis, a strain isolated from Thalassia blades collected in Puerto Rico (Burkholder et al., 1966). This compound showed activity against several marine bacteria and it is highly active against Gram positive (G+) bacteria pathogenic to humans (Staphylococcus aureus, Diplococcus penumoniae and Streptococcus pyogenes). Because of its high toxicity to mice, however, the compound is not amenable to therapeutic use. Andersen et al. (1974) reported the production of the same compound by a Chromobacter sp. isolated from a water sample from the North Pacific. They found that, among the secondary metabolites isolated, the tribromopyrrole (I), tetrabromopyrrole (II) and p-hydroxybenzaldehyde (III) were all autoinhibitory.

Wratten et al. (1977) isolated a yellow Pseudomonas sp. from a La Jolla tidepool and found that the strain produces the following antimicrobial compounds: p-hydroxybenzaldehyde (III), 2-n-pentyl-quinolinol (IV) and 2-n-heptyl-4-quinolinol (V) which was the major active secondary metabolite in the extract.

Okazaki et al. (1974) and Kitahara et al.(1975),

isolated a benzanthracene (VI) derivative produced by an actinomycete, Chainia sp., from marine muds. This compound inhibits G+ bacteria and its action has been studied quite thoroughly.

Kohl et al. (1974) isolated the magnesidins (VII), a group of compounds derived from the antimicrobial pigment prodigiosin (VIII), which is produced by terrestrial bacterial strains and by at least one marine bacterial strain, Alteromonas rubra (Laatsch and Thomson, 1983). Gandhi et al. (1976) isolated a pigmented bacterial strain from washings of the alga Caulerpa peltata collected near Bombay, India, which also produces the magnesidins.

In 1976 Okami et al. studied another actinomycete, Streptomyces griseus (a very common soil species) isolated from shallow sea mud near the mouth of Sagami Bay. This strain showed antimicrobial activity when grown in a yeast extract medium with high concentrations of NaCl (5%). The activity was optimized by the addition of dried Laminaria (Kobu Cha) to the medium. The isolated active compound, aplasmomycin (IX) inhibits G+ bacteria (including mycobacteria) and it owes its name to its activity against the malaria-causing agent Plasmodium berghei (Chen et al., 1981; Sato et al., 1978; Nakamura et al., 1977). Like other ionophoric compounds, it affects both prokaryotic and eukaryotic cells due to its ability to transport cations across the lipid portion of biological membranes.

A new group of aminoglycoside antibiotics was

reported by Okami (1979) and Hotta et al. (1980, 1983, 1985). These compounds, the istamycins (X), are produced by the newly described actinomycete species Streptomyces tenjimariensis, also isolated from samples of marine mud at Sagami Bay. Istamycins A and B show strong inhibition of G+ and G- bacteria, including many strains that are resistant to known aminoglycoside antibiotics. Current research is on modification of this compound in search of heightened activity and lowered toxicity. Toxicity is lowered by 3-O-demethylation of istamycin B and the resulting compound is effective against bacteria resistant to other antibiotics.

Gauthier and Flatau (1976) reported the isolation of an uncharacterised antibiotic substance from a marine violet-pigmented Alteromona. The compound is described as a polyanionic polysaccharide, weakly bound to cells and with the tendency to diffuse into the medium on agar plates. In 1977 Ballester et al. reported the isolation of a high molecular weight, uncharacterized, antibiotic produced by a marine bacterium. Recently, Simidu et al. (1987), isolated tetrodotoxin (Narita et al., 1981; Mosher et al., 1964), the neurotoxin accumulated in fish livers and other organisms, from cultures of several strains of Vibrio species frequently found in seawater and in association with marine organisms. The role of the toxin in the bacteria is not known.

Focus of Research

We see, then, that there is quite a bit known about associations of bacteria with other organisms in the ocean and some known about secondary metabolites produced by marine bacteria. The significance of most of these symbioses for the partners is not known. Moreover, the degree of specificity, the nutritional and other exchanges, and the way in which these associations keep their integrity among other competing organisms remain a mystery. How do these populations of bacteria manage to colonize the surface of different organisms and maintain this 'habitat' in an environment where there is continuous contact with other, potentially competing, microorganisms? There are very few studies on competition strategies among microorganisms, and, indeed, the question is rarely asked. Unless the bacteria have very specialized metabolisms and the association constitutes a tight symbiosis, the mechanisms of competitor exclusion are not at all obvious. Thus the experimental aspects of such a question seem unsurmountable. By focusing on what might be called 'loose' associations, however, and by examining the effects of the elimination of the bacterial symbionts on the host, the natural mechanism may become accentuated and measurable.

Working Hypothesis

The questions that the work presented in this thesis seeks to answer are thus:

Are antimicrobial secondary metabolites important in marine microbial ecology?

Are these compounds a factor in the establishment and maintenance of bacterial associations with other organisms?

Do the hosts benefit from the availability of these metabolites?

The foregoing questions were tested against the following hypotheses:

MANY BACTERIA PRODUCE ANTIMICROBIAL COMPOUNDS AS A MEANS OF COMPETITION WITH OTHER MICROORGANISMS.

BACTERIA LIVE IN ASSOCIATION WITH ALL KINDS OF ORGANISMS, PARTICULARLY IN THE OCEAN.

MANY OF THESE ASSOCIATIONS INVOLVE BACTERIA THAT PRODUCE ANTIMICROBIAL COMPOUNDS.

THE HOST ORGANISMS BENEFIT FROM THESE ASSOCIATIONS IN BEING PROTECTED AGAINST POTENTIALLY PATHOGENIC OR ENCROACHING MICROORGANISMS.

IN THE ABSENCE OF THE ASSOCIATED BACTERIA, THEHOSTS BECOME VULNERABLE TO INVASION BY MICROORGANISMS.

Outline of Research Strategies

The working hypothesis assumes that the benefit flowing from the bacteria to the host is the 'free' availability of antimicrobial compounds specific to locally abundant potentially pathogenic or encroaching microbes. One must, therefore, establish first that there is in fact a

stable association between symbionts and host and secondly, that the stability of the association is dependant on these antimicrobial metabolites. Following the work of the pioneer researchers of insect-microbe symbioses, Buchner, Cleveland, Hungate, Trager and Martin (Martin, 1987), the research strategy followed was based to an extent on Koch's postulates for identification of a disease vector (Stanier et al., 1986). .

1. Separate the microbe from its host and determine whether the symbiont-free host is adversely affected.

2. Reestablish the association of the two partners and determine whether the problems of the aposymbiotic host are alleviated.

3. Establish the mechanism by which the association of the two partners is normally sustained and perpetuated.

4. Determine the underlying cause of the problems experienced by the aposymbiotic host.

5. Identify and culture the microbial symbiont, and determine whether it has characteristics that would make it able to mitigate the difficulties experienced by the aposymbiotic host.

6. Determine the quantitative significance of the microorganism's contribution to its host's needs.

7. Conduct a comparative study to determine whether comparable associations occur with other hosts. This survey should include both closely related species and species that are ecologically similar but not closely related.

To give focus to this new area of chemical ecology, and to reduce the number of unknown factors affecting the associations, the examples to be studied were chosen because they possess some unexpected quality that suggested the type of relationship sought. Thus, the embryos of the shrimp Palaemon macrodactylus were studied (Ch. III) because treatment with penicillin greatly increased their vulnerability to microbial pathogens; embryos of the lobster Homarus americanus were chosen (Ch. IV) because the larvae and juveniles easily become infected by the fungal pathogen Lagenidium callinectes, whereas its embryos are practically invulnerable to it; the colonial filamentous cyanophyte Microcoleus lyngbyaceus was selected from the initial survey (Ch. VI) because all of the 65 specimens studied contained a group of bacteria not found in any of the other samples, and because the filaments were unusually free of epibiotic fungi and other microorganisms.

Each study case had different intricacies and opened different questions, but, in each case, the underlying mechanisms of the associations and the chemical, biological and microscopic evidence obtained, support the working hypotheses of this dissertation.

CHAPTER II

PRELIMINARY WORK

Screening

The first step in this research was to confirm that bacteria of diverse types can be isolated consistently from a variety of marine organisms and that there is a high incidence among these bacteria of antimicrobial activity against other bacteria and fungi from the same environment. Table II.1 is a list of the organisms sampled.

The general procedure was as follows:

1. Organisms were collected in individually numbered plastic bags.

2. At the site of collection, the number was recorded as well as information on depth of site, location of organism with respect to neighbors and substrate, and any unusual growth form or coloration.

3. Organisms were brought to the laboratory within less than one hour and rinsed three times in sterile filtered seawater. Small pieces of tissue were ground in an Elvejmeer grinder with sterile sea water. In some cases, both internal and external tissues were sampled.

4. In most cases three dilutions each 1/10 of the previous one, were plated (using sterile cotton swabs) on agar plates. Initially four different media were used (see section on growth media).

5. All the strains that seemed morphologically different on the plates were isolated.

6. All bacteria were tested against a set of bacteria and fungi from the surrounding water and sediments and against Beneckea harveyi and Vibrio anguillarum provided by Dr. K. Nealson.

7. When host organisms could not be easily identified, a voucher was saved for eventual identification.

Growth Media

There are several reasons to use a variety of media to screen bacterial types thoroughly (Simidu and Tsukamoto, 1980; Hall et al., 1984; Hirsch and Christensen, 1983). The strain of Pseudomonas insolita mentioned before (Muller, 1981) could not be cultured successfuly until a lectin isolated from the host sponge was added to the culture medium. Aplasmomycin, the antibiotic isolated from cultures of Streptomyces griseus, is produced only when the bacterium is grown in a low-nutrient medium to which an extract of Laminaria blades is added (Okami et al., 1976). Anaerobic phototrophic bacteria were found to exist in great quantities in sponges only after media with specially-tailored ingredients were tested (Imhoff and Truper, 1976). Similarly, an unusual association between bryozoans and mollicutes was recently found only after using a growth medium that contained a homogenate of the host and penicillin, which does not affect mollicutes as they do not

have a cell wall (Boyle, 1987).

For the present work, it was not important to detect specialized bacteria such as anaerobes, chemotrophs or bacteria strictly dependant on some factor from the host. The main object was to screen surface bacteria which must have developed some drastic form of defense against competitors. From an adaptive point of view those organisms are perhaps still in a fairly early stage of coevolution with their host, and it can be speculated that at some future time they may develop more intimately dependant exchanges with their hosts. Nevertheless, at an early stage of the research it seemed important to compare the effectiveness of different media with respect to the number of strains isolated and the activity of isolated strains. The four different media (Difco Manual, 1953) used were chosen following Dr. K. Nealson's advice to cover a relatively wide spectrum of nutrient compositions.

A) 2216 Difco Agar - this medium is based on the original ZoBell's marine medium and it contains bactotryptone, yeast extract, glycerol and agar. It is prepared with distilled water as it contains all the necessary salts.

B) TCBS Agar - contains yeast extract, pancreatic digest of casein, peptic digest of animal tissue, sodium citrate, sodium thiosulfate, oxgall, sodium cholate, sucrose, sodium chloride, ferric citrate, thymol blue, brominated thymol blue and agar. It is prepared with 75%

filtered seawater, 25% distilled water.

C) Thioglycollate medium - this medium permits isolation of anaerobes and microphyls as well as aerobic microorganisms. It contains tripticase peptone, dextrose, sodium chloride, dipotassium phosphate, phytone peptone, sodium thioglycollate, methylene blue, agar, and it is prepared with 75% filtered sea water, 25% distilled water.

D) Mycophil agar - generally, this neutral medium is prepared with addition of a number of antibiotics to isolate fungi. We thought it might favor actinomycete isolates. It contains papaic digests of soybean meal, dextrose and agar. It is prepared with 75% filtered sea water, 25% distilled water.

All the strains that were isolated in media B, C and D, were also isolated in medium A, although the opposite was not true (Table II-1). Consequently, medium A was used for all subsequent isolations. Medium B (TCBS agar) has a blue-green tint and, in order to compare the few strains that grew in this medium, it was necessary to transfer them to another medium (we chose 2216 MA). It was noticed in two instances that bacteria strains transferred directly from medium B were active against some of the test organisms but, upon subsequent transfers in 2216 MA, they lost this activity.

Media prepared with fish, conch, crab and algal broths were also used to test the possibility that some of the associated bacteria might have specific needs provided

only by the host or, at least, by an exclusively marine organism. Our screening, which at this stage of the research may have been rather superficial for the sake of covering large numbers of specimens, indicates that diversity and detection of active strains in those specialized media is comparable to results on 2216 MA.

Testing Techniques

The choice of test organisms was different for each collection location. Allelochemicals represent a mechanism designed for competition with a restricted group of potential competitors. Antimicrobial compounds in particular, as opposed to physical defenses (spines, exoskeletons, claws), are targeted defenses constituting a specialized competitive mechanism. To address the ecological function of these compounds, it is important to test the activity of the bacteria against microorganisms that are potential competitors in nature. Thus, bacteria collected in Puerto Rico were tested against a set of water and sediment bacteria and fungi from the same area. Occasionally, interesting strains were tested against Beneckea harveyi and Vibrio anguillarum, two strains commonly used to assess antimicrobial activity. Several methods were tried:

1. A lawn of test organism either from liquid culture or from agar colony, is streaked with unknown bacterial colonies.

2. Agar is prepared with test organism incorporated into it. Unknown colonies are streaked on the surface of the solidified agar.

3. Individual liquid cultures of test organism are mixed with liquid culture of unknown and after some days the liquid is streaked on agar to see which colony has survived.

4. Streaks of both colonies, test and unknown, placed side by side on agar.

5. Several colonies to be tested are streaked across streaks of the test organism.

6. Identical-size plugs containing colonies of test and unknown strains placed side by side on agar plates.

Method 6 proved to be the simplest and most reliable and reproducible testing technique.

Results

A total of 244 individual organisms (approximately 140 species) were collected and screened for active bacteria (Tables II-2, IV-1 and VI-2). Four (20%) of the 20 organisms collected in different locales near La Jolla and Point Loma contained bacteria with antimicrobial activity. Approximately one third of the 100 organisms screened from reefs in the Bahama Islands, and half of 57 organisms (not including the 62 specimens of Microcoleus lyngbyaceus collected in the area of La Parguera, Puerto Rico) contained active bacterial populations. The different species screened

TABLE II-1

List of specimens screened for the presence of bacteria. The symbol (+) indicates that one or more strains of the bacteria isolated from the specimen inhibited the growth of one or more test organism; the symbol (-) indicates that none of the bacteria isolated from that specimen inhibited any of the test organisms.

Code No.	Species or Description	
	Chub Cay, Bahamas - 20 m	
S1	yellow sponge	
	internal tissues	+
	external surface	-
S2	orange sponge	
	internal tissues	-
	external surface	-
S3	rubbery orange sponge	
	internal tissues	-
	external surface	-
S4	mushroom-like brown sponge	
	internal tissues	-
	external surface	+
	Hogsty Reef - 3 m	
P1	small sabellid	
	tube	+
	worm	+
S5	truffle sponge	+
BG1	cyanophyte	+
M1	*Turbinaria* sp.	+
S6	sponge	-
S7	sponge	+
	Hogsty Reef - 7 m	
CA1	*Laurencia* sp.	-
CA2	*Dictyota* sp.	+
T1	ascidian	+
CA3	*Halimeda* sp.	-
CA4	*Penicillus capitatus*	-
CA5	*Rhipocephalus* sp.	-
CA6	*Stypopodium* sp.	-
O1	*Eunicia* sp.	+
M2	spotted seahare	-
O2	*Muricea* sp.	-
Z1	*Palythoa* sp.	-
T2	*Didemnid* sp.	+
M3	*Tridachia crispata*	-
P2	red worm (found in sponge)	+
P3	brittle worm	+

Table II-1 (contd.)

	Auklin Island dropoff - 25 m	
RA1	red alga (epiphyte of gorgonian)	-
I1	orange crinoid (Nemaster rubiginosa)	
	arm	-
	body	-
P4	green worm(in Pseudopterogorgia cyst)	-
M4	Strombus gigas stylus	-
M5	parasitic clam (on gorgonian)	
	gill	-
	gut	+
CA7	Valonia	
	inside liquid (no bacteria)	
	wall	+
	Crooked Island Mangrove Channels	
U1	crab	
	carapace scrape	-
	gill	-
	testes	-
	cecae	-
	hepatopancreas	-
	squashed carapace	-
T3	ascidian	+
CA8	Dictyosphaeria	+
	Rum Cay - 25 m	
T4	Clavellina sp.	
	body	-
	gelatinous sheath	+
T5	ascidian, purple blue	-
RA2	red alga	-
M6	mollusc eggmass	-
L1	Mycetophilia aliciae	+
L2	Meandrina meandrites	-
L3	Acropora cervicornis	-
L4	Porites sp.	-
L5	Eusmilia fastigiata	-
M7	Cyphoma gibbosum on 03	+
03	Pseudopterogorgia sp.	-
CA9	Avrainvillea nigricans	
	stem	+
	blade	-
Y1	hard red bryozoan	-
P5	polychaete, dark pink	-
04	Eunicia sp.	
	polyp	+
	skeleton	-
P6	Hermodice carunculata	
	integument	+
	guts and head	-

Table II-1 (contd.)

	Little San Salvador, NW - 8 m	
F1	grouper	
	small intestine	-
	cecae	-
	stomach	-
	spleen	-
	liver	-
	gonads	-
	kidney	-
S8	orange sponge	
	internal tissues	-
	external surface	-
05	Briaerum asbestinum	
	internal tissues	-
	external surface	+
S9	yellow-brown sponge	
	internal tissues	-
	external surface	+
T6	green-gray ascidian	+
S10	purple flute sponge	-
S11	sponge, iridescent purple out, yellow in	
	internal tissues	-
	external surface	-
CA10	Penicillus sp.	-
	Little San salvador, N - 13 m	
Y2	bryozoan	-
S12	Mycale laevis	
	internal tissues	-
	external surface	-
RA3	encrusting coralline alga	-
06	Erythropodium sp.	+
T7	brown large ascidian	
	internal tissues	-
	external surface	+
T8	small brown rubbery ascidian	-
T9	grey ascidian	-
T10	small orange ascidian	+
S13	yellow demosponge	-
S14	greyish demosponge	-
07	Erythropodium sp.	
	internal tissues	-
	external surface	+
08	Pterogorgia citrina	-
RA4	encrusting coralline alga	-
H1	hydroid	-
P7	Filograna implexa	-
Y3	red bryozoan	-
S15	red thin sponge	-
CA11	Udotea cyathiformis	-

Table II-1 (contd.)

	Little San Salvador, mangrove inlet	
H2	hydroid	-
CA12	curly green alga	-
CA13	fuzzy green alga	-
CA14	Dictyopsphaeria cavernosa	
	internal liquid	-
	wall surface	+
CA15	green alga	-
T11	green ascidian	-
T12	brown ascidian	+
S16	sponge, Thalassia epibiont	+
M8	snail eggs (collars)	-
RA5	Galaxaurus sp.	-
T13	black ascidian	-
Z2	Zoanthus sociatus	-
SC1	Cassiopeia	-
X1	sediment, entrance of channel	-
X2	sediment, middle of channel	-
X3	sediment, at Acetabularia grove	-
T14	grey ascidian	-
M9	Murex sp.	-
M10	Pinna carnea	
	gill	-
	hepatopancreas	-
	gut	-
M11	Cassis tuberosa	-
TH1	holothurian (small with oral tentacles)	
	gut	-
	respiratory tree	-
	integument	-
	Chub Cay - 16 m	
S17	Agelas sp., orange	-
S18	Agelas sp., darkbrown	-
T15	brown ascidian	-
	La Jolla/Point Loma, California	
YA1	Macrocystis sp.	
	healthy broad blade	-
	healthy thin blade	-
	float	-
	unhealthy leaf	-
	rhizoid	-
RA6	red alga	+
CA16	Dictyota sp.	-
YA2	Laminaria sp.	+
YA3	Laminaria sp.	
	pneumatocyst	-
	rhizoid	-
	blade (only one morph, requires tissue)	-
L6	coral	-

Table II-1 (contd.)

M12	Hypselodoris porteriae	-
M13	white nudibranch	-
M14	Doriopsilla albopunctata	+
M15	Aplysia sp., red	-
M16	Cadlina luteomarginata	
	foot	-
	gut	+
M17	Mytilus edulis	
	gonad	-
	gill	-
	foot	-
	gut	-
X4	unhealthy leaf	-
BG2	cyanophyte	-
RA7	red alga	-
AN1	tidepool anemona	-
YA4	brown alga	-
S19	sponge	-
	Bodega Bay, California	
U2	kelp crab eggs	-
U3	Hemigrapsus oregonensis	-
	Mangroves shore,Parguera, Puerto Rico	
CA17	Brachitrichia quojii	-
M18	mangrove clam	+
S20	Tedania ignis	-
X5	mangrove sediment	-
CA18	filamentous alga	-
X6	dry mangrove soil	-
	Caracoles Island	
X7	rotten mangrove branch	+
CA19	Valonia sp.	+
X8	sediment	+
	Enrique reef lagoon	
M19	Tridachiella sp.	+
SC2	Cassiopeia sp.	-
AN2	Phymanthus crucifer	+
T16	ascidian	-
CA20	filamentous alga	+
Z3	Zoanthus sociatus	-
AN3	Phymanthus crucifer	+
AN4	Stoichactis helianthus	-
AN5	anemona	-
AN6	Phymanthus crucifer	
	crown	+
	foot	-
AN7	Phymanthus crucifer	

Table II-1 (contd.)

	crown	-
	foot	-
AN8	Phymanthus crucifer	
	crown	-
	foot	-
	Enrique Reef, 13 - 20 m	
09	Briareum asbestinum	-
010	Telesto testudinum	+
AN9	green anemona	+
AN10	pink anemona	-
S21	pink sponge	+
T17	black ascidian	-
L7	Mycetophilia aliciae	+
L8	Eusmilia fastigiata	-
CA21	Cladophoropsis sp.	+
AN11	Phymanthus crucifer	-
AN12	anemona	-
AN13	Phymanthus crucifer	+
Z4	Zoanthus sociatus	-
AN14	Stoichactis helianthus	-
CA22	filamentous green alga	-
AN15	Phymanthus crucifer	-
CA23	green filamentous alga	+
S21	pale blue sponge	-
	Collado island, shallow	
Y4	pink bryozoan	+
T18	white ascidian	+
M20	Aplysia sp.	-
Y5	orange bryozoan	-
A16	Phymanthus crucifer	+
H3	Obelia sp.	-
Z5	brown zoanthid	-
Z6	light-brown zoanthid	+
Z7	green zoanthid	-
AN17	Aiptasia tagetes	+
	Medusa Dock, 4 m	
T19	orange ascidian	+
S23	Tedania ignis	-
AN18	Condylactis sp.	-
Y6	black bryozoan	+
T20	black large tunicate	-
CA24	Caulerpa racemosa	-
P8	small sabellid worm	+
S24	pale blue sponge	+

included 3 cyanophytes, 7 red algae, 4 brown algae, 24 green algae, 24 sponges, 3 hydrozoans, 2 scyphozoans, 20 octocorals 8 scleractinian corals, 10 anemones, 7 zoanthids, 8 polychaetes, 20 molluscs, 5 crustaceans, 1 holothurian, 1 crinoid, 6 bryozoans, 20 tunicates and 1 fish. Only in two cases were no bacteria found: in the internal liquid of the spherical alga Valonia and on the green alga Rhipocephalus. In most cases, only one or two of the strains isolated from a particular host showed activity. Several of the organisms tested yielded bacterial morphs that could not be distinguished visually from morphs found in other organisms. In some host species, however, the bacterial flora composition seemed quite constant. A peculiar lemon-colored bacterial strain was repeatedly isolated from the mangrove anemone, Phymantus crucifer. This strain was active against most test organisms but, unfortunately, it lost its activity through repeated transfers. The most impressive example of specificity is described later (Ch. VI) where bacteria associated with the filamentous cyanophyte Microcoleus lyngbyaceus were always found on the 62 host specimens screened and were never found anywhere else.

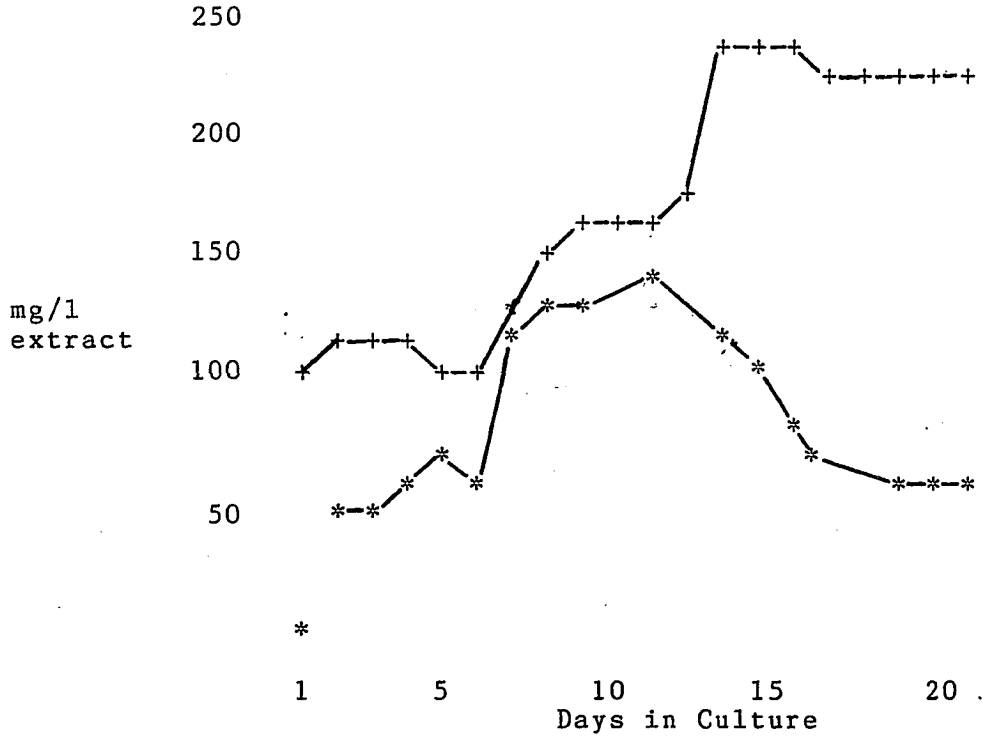
Optimal Culture Time

At the beginning of the research, each culture of interest was grown in several (usually 30) 100-ml flasks and extracts of both the cells and the medium were compared to assess the optimal point of 'harvest' and whether the

activity came from intracellular or extracellular compounds. It was found that, in general, peak cell productivity was achieved at about 12 days and medium productivity plateaus at 15 to 21 days. Activity was often found both in cell extracts and medium extracts. For some of the strains, however, activity was found in medium extracts but not in cell extracts. The yield of cell extracts was always much lower than the yield of medium extracts.

Fig. 2.1

Diagram of extract yields (mg/l of culture) for medium (+) and cells (*) based on 100 ml cultures of strain #25.



Plasmids

The production of some antibiotic compounds is governed by bacterial plasmids (Hopwood, 1979; Hopwood, 1978; Novick, 1969). For example, aureothricin, kasugamycin, kanamycin and chloramphenicol are all plasmid-governed compounds (Beringer and Hirsch, 1984). In general, the interest in isolating these plasmids is based on the possibility of transferring them to strains with higher metabolic capacity in order to increase production of the antibiotics. However, if any of the strains studied for this work have such a plasmid, we thought that it would be a powerful tool to confirm the involvement of the antimicrobial compound in the association. Reinfection of sterile hosts with normal and cured cultures would allow the elimination of only one parameter in the study, namely the production of the antimicrobial compound. Three different methodologies were tested to look for plasmids in five strains of active bacteria. The simplest and most trustworthy method was learned at Dr. Helinski's laboratory and is described below. Strain J166 (see Ch. III) was the only isolate that seemed to contain a plasmid. This approach was abandoned, however, because the bacteria did not respond well to the curing treatments used to eliminate the plasmid (acriflavine, high and low temperatures). The most satisfactory procedure used to separate plasmid DNA was:

1. Transfer a large colony of cells with sterile

toothpick into 150 ul of 2% triton (a detergent with pH 12.4) in an Eppendorf tube. Twirl cells against side to remove from toothpick and to expose to solution for cell lysis.

2. Add an equal volume of phenol saturated chloroform (150 ul) to each tube, cap and vortex well to mix. Membranes and protein remain in the lower chloroform phase and the extracted DNA is on the upper layer.

3. Spin tubes five minutes in a microfuge; the bottom phase will be phenol-CHC13, topped by a clear interface containing protein and cell membranes.

4. Remove up to approximately 100 ul of the upper layer being careful not to disturb lower layer. Add dye buffer and load and run on an 8% agarose gel (150 V for 3 hours).

CHAPTER III

EMBRYOS OF *PALAEMON MACRODACTYLUS* AND ASSOCIATED BACTERIA OF THE GENUS *ALTEROMONAS*

The caridean shrimp *Palaemon macrodactylus* was introduced from the Orient to the West Coast of the United States within the past 30 years. Because of its tolerance to a relatively wide range of salinities, this organism is thriving in estuaries along the coast, where its preferred habitats are algal mats attached to piers or hard substrates. Although in several Asian countries the shrimp is appreciated as food, its rather small size (6 cm length x 1 cm width) has made it unattractive to the American consumer. The shrimp is used mostly for bait by local sport fishermen. It has, however, characteristics that make it a very good experimental organism: its sturdiness in captivity, its wide tolerance for different salinities and temperatures, its short gestation time (16 days) and its long reproductive period (April to October).

William S. Fisher, from Dr. W. H. Clark's laboratory at the Bodega Marine Laboratory (U. C. Davis) studied *P. macrodcatylus* extensively and in 1982 he completed his PhD dissertation on the interaction of the brooding embryos and aquatic microorganisms. After an extensive contamination of the BML's installations in 1974 by the fungus *Lagenidium callinectes* (Fisher *et al.*, 1975), a pathogen of several crustaceans (Armstrong *et al.*, 1976;

Bland et al., 1976; Cook, 1971; Feigenbaum, 1975; Couch, 1942) Fisher became particularly interested in the defense strategies of the embryos against microbial encroachment (Fisher and Clark, 1983). Larvae and juveniles are more often affected by microbial pathogens but embryos appear to be more resistant to diseases. The shrimp are external brooders (Fig. 3.1) and the 16-day brooding period provides ample opportunity for microorganisms to colonize the embryonal surface. Maternal preening and embryonal envelope thickness were the classical explanations for the remarkable sturdiness of some of these brooded embryos. Preening, however, only affects the outermost layer of embryos on the cluster and the periembryonal sheath is not particularly thick or tough. Furthermore, while investigating the response of isolated embryos to treatment with Penicillin, Fisher (1983, III) was surprised to find that treated embryos became diseased, while those untreated remained healthy. He found that the pathogenic agent most commonly causing death of embryos treated with penicillin was the fungus L. callinectes (Fisher, 1983, II). Among several strains of bacteria isolated from homogenates of healthy embryos, he found three strains that were sensitive to penicillin and inhibited the growth of the fungus. This information was extremely interesting to us as it was what we might expect to observe in a case fitting our hypothetical model. Dr. Fisher kindly provided us with the isolated strains. Two of the strains, labelled I-2 and

J166, survived and were successfully cultured in liquid media.

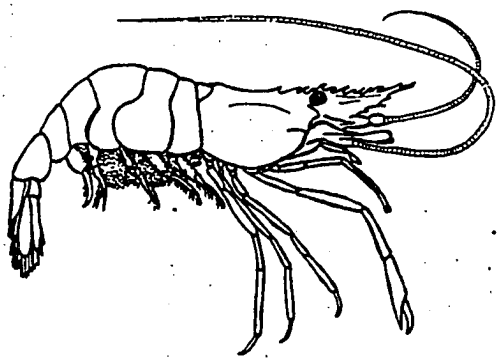


Figure 3.1. Palaemon macrodactylus female carrying embryos.

The experimental details of the research are explained in this chapter's section on Methods. After some assessment of different culture conditions, the two bacterial strains, I-2 and J166, were cultured in slightly different liquid media in large batches. The media were extracted with organic solvents and the biologically active compounds were isolated by following their activity against the pathogenic fungus of interest. Final purification of the active compounds was done by HPLC and characterization was achieved by a combination of spectral analyses: proton and carbon nuclear magnetic resonance, and infrared, ultraviolet and high resolution mass spectra. Strain I-2 produces one major active compound, 2,3-indolinedione (Fig.

3.2) and strain J166 produces the major active compound, anthranilic acid (Fig. 3.3). It must be noted, however, that recent research by Dr. Dianne Tapiolas showed that, when cultured in a fermenter, the major compound produced by J166 is pyrrole 2-carboxylic acid.

To assess the extent of the embryos' dependance on the associated bacteria and the involvement of the antimicrobial compounds in maintaining their health, embryos detached from the maternal pleopods were sterilized and reinfected with pure cultures of both strains and exposed to the pathogen. Of the different active compounds isolated only 2,3-indolinedione was chosen for this experiment because it is produced in larger quantities and it is many times more active than the other compounds. Furthermore, we suspect that I-2 may be the dominant strain, since it is slightly inhibitory to J166 in vitro. This experiment was crucial to the central hypothesis of this research, allowing the confirmation of two central issues in these associations: namely the process protecting the embryos against pathogenic microorganisms and the detailed mechanism (the production of antimicrobial compounds) by which the protection is achieved. All steps of this experiments were followed by scanning electron microscopy (Fig. 3.8) and both the experimental information and SEM evidence show that:

1. Both strains, I-2 and J166, protect the embryos against invasion by the fungal pathogen and possibly

pathogenic bacteria.

2. Absence of these and other penicillin-sensitive bacteria make the embryos extremely vulnerable to microbial infection.

3. 2,3-indolinedione, the major active compound produced by strain I-2, protects the embryos against infection by the pathogenic fungus and possibly some pathogenic bacteria.

4. The antifungal compound 2,3-indolinedione is not toxic or otherwise harmful to the embryos at concentrations 200 times higher than those recommended for most antifungal agents.

5. There may be more strains of penicillin-sensitive bacteria involved in beneficial associations with the embryos.

6. In nature embryos are covered by a large number of bacteria identical in appearance to I-2 and J166. This visual data is complemented by several bacterial isolations done previously by Dr. W. Fisher and by spot isolations in which strains identical to I-2 and J166 were always present. This association clearly illustrates how metabolites produced by bacterial symbionts determine the ecological status of both the host organism and the bacteria involved. The evidence gathered through this research has allowed the fulfillment of almost all the conditions defined on Chapter I.

1. The bacteria were separated from the host and it

was determined that the aposymbiotic host is adversely affected.

2. The association with two of the symbionts was successfully reestablished and the problems of the aposymbiotic host were significantly alleviated.

3. The mechanism that mediates the association is the production of antimicrobial compounds.

4. The aposymbiotic host is vulnerable to infection by pathogenic microorganims.

5. Two of the microbial symbionts were identified and cultured and it was determined that they do produce the compounds that mitigate the aposymbiotic host's problems.

6. The quantitative significance of the microorganisms' contribution to their host's needs was not determined, but it seems to be a major fraction of the host's total needs.

7. A comparative study to determine whether comparable associations occur with other hosts was done and is described in Chapter IV.

Bacterial Strain I-2

General Characteristics of I-2: This strain, identified as belonging to the genus Alteromonas (Baumann and Baumann, 1981) is characterized by acquiring a sooty color after three days of growth, both on agar and in liquid medium. The individual cells are approximately 2.2 um long, rod-shaped and have a single polar flagellum.

Culture Conditions: I-2 grows well on MA2216 and this is the medium that was adopted for transfers and tests. In order to test the strain's ability to survive on shrimp exudates, a medium was prepared by boiling mashed shrimp and filtering this 'broth'. Both the liquid medium and the solid medium (1.5 % agar added to the broth and reautoclaved) promoted a healthy growth of the strain. In yet another experiment, a whole autoclaved shrimp was half-immersed in agar in a Petri dish, and after gelification, the whole plate was smeared with healthy I-2 cells. There was a 1 mm halo of very abundant bacterial growth around the shrimp but nowhere else on the plate. The colony survived under these conditions for more than a month, but the area of growth did not change appreciably. For batch culture, several liquid media were tested (see Ch. II) . A medium including starch and glycerol, produces the highest yield of total extract (approximately 700 mg per 16 liters), but the highest yield of the compound that was later found to be active is achieved by using only starch as a source of carbohydrates (total extract approximately 450 mg per 16 liters). The composition of the final culture medium is:

5 g bactopeptone]
3 g yeast extract] to 1 liter of filtered
10 g starch] seawater

Optimal growth time was estimated to be two weeks.

Activity Tests: In liquid culture, I-2 always inhibited and outcompeted L. callinectes, whether it was introduced at the same time, or after the fungus had been

growing in the medium for some time. On agar plates, if equally large plugs of I-2 and the fungus are placed adjacently on an agar plate, there is no fungal growth and I-2 will eventually grow over the original plug containing fungal hyphae. If a new plug of I-2 is placed adjacent to a plug of L. callinectes which had been growing on the plate for three days, hyphal spread is inhibited on the side of I-2, but it continues, albeit more slowly than the control's, on the side opposite the I-2 plug.

Extraction and Isolation: I-2 cultures were grown for two weeks in 16-liter carboys with aeration. Final pH of culture medium fluctuated between 7.6 and 8.0. The medium was extracted, using forceful stirring, with three liters of ethyl acetate per carboy. Extraction was repeated a second time. Yield was usually between 500 and 700 mg per carboy. The first separation was done on a silica flash column with a solvent regime of ethyl acetate/isooctane. Ten fractions were collected, ending with pure ethyl acetate. Activity of the fractions was followed by placing 1.0 mg of the initial fractions on paper discs (0.5 cm in diameter) and testing against L.callinectes plugs on agar plates, together with the appropriate solvent control, in this case, methanol. Activity was found in fractions 6, 7 and 8 (60, 70, and 80 % ethyl acetate/isooctane) of the flash column. These fractions were further purified by normal phase (Porasil) HPLC with a solvent system of 60% ethyl acetate and 40% isooctane. After HPLC, only 0.5 mg of the

fractions to be tested were used on each disc.

Characteristics of 2,3-indolinedione: reddish-orange prisms soluble in methanol and DMSO; m.p. 201-203 °C; HRMS: requires 147.01 for $C_8H_5NO_2$ requires, found 147.03; ^1H NMR (360 MHz, $CDCl_3$): δ8.2 (s, 1H); 7.68 (dd, 1H, J = 7.7 Hz); 7.51 (d, 1H, J = 7.8 Hz); 7.18 (dd, 1H, J = 7.7 Hz); 7.01 (d, 1H, J = 7.8). The ^1H NMR spectrum, melting point and IR spectrum were identical to those of the commercial compound. This compound (Fig. 3.2), commercially known as isatin, has been known for over 100 years (Erdmann, 1841) as an oxidation product of the plant pigment indigo, which is related to violacein, a molluscan pigment. Isatin is used in the manufacture of dyes and certain acridine and quinoline derivatives. There are no reports of this compound occurring as a natural product and no reports of its biological activity. There is ample information regarding antiviral, antibacterial and antifungal activities of derivatives of 2,3-indolinedione (Maysinger *et al.*, 1980; Maysinger, 1978; Varma and Nobles, 1975; Alexandrescu, 1969). The most likely biosynthetic precursor of this compound is the aminoacid tryptophan.

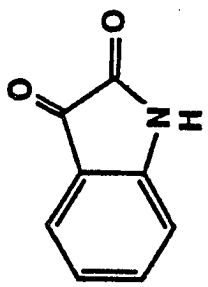
Activity against *L. callinectes* : In liquid medium assays, 0.1 mg of 2,3-indolinedione in 10 ml of medium totally inhibits the growth of *L.callinectes*. After 1 week, however, growth of the fungus is resumed. Assays on agar plates show that, when the compound is incorporated in the agar, concentrations as low as 0.001 mg/ml are inhibitory.

Figure 3.2

^1H NMR Spectrum of 2,3-indolinedione (isatin)

$C_8H_5O_2$

orange prisms



360 MHz in $CDCl_3$

If the compound is placed on discs, 0.1 mg/disc inhibits the growth of 3-day old fungal colonies (5 mm growth outside the plug edge). If the disc is placed on growing hyphae, 0.5 mg/disc of the compound are necessary to clear or kill the fungus. In vivo activity is discussed in Chapter V.

Activity Spectra of I-2 and 2,3-indolinedione: As suggested by the information shown on Table III-1, this strain probably produces other active compounds besides 2,3-indolinedione, or is capable in other ways of outcompeting other bacteria. Twenty five strains of bacteria collected in the Bodega Bay area were tested against I-2 by streaking each strain on a fresh lawn of I-2, and against 1,2-indolinedione by placing discs with 0.3 mg of the compound adjacent to healthy growing colonies. The experiment was duplicated and the results were identical.

TABLE III-1

Strains from Bodega Bay tested against I-2 and 2,3-indolinedione

Test Strain No.																								
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	*	*	*	*	*	*	*	-
-	-	-	-	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-

- no inhibition
* inhibition by I-2
+ inhibition by 2,3-indolinedione

Bacterial Strain J166

Characteristics: This species of Alteromonas is

characterized by a dry white appearance and the formation of scalloped edges in the colonies. The odor is also quite characteristic of this strain. Individual cells are rod-like and have a polar flagellum.

Culture Conditions: Several media were tested for this organism and the one eventually chosen was standard seawater medium without glycerol:

5 gm bactopeptone
3 gm yeast extract
17 gm agar
250 ml deionized water
750 ml filtered seawater

Since in preliminary separations with small volumes of culture it was apparent that there were several active compounds, it could not be assessed which medium was optimal. Furthermore, the total yield of extract was similar in all media (approximately 700 mg per 16 liters). Carboys containing 16 liters of culture medium were seeded with 100 ml of pure one-day old cultures grown and were kept under aeration for ten days to two weeks. The culture medium's final pH was 8.4 to 8.5.

Activity against L. callinectes and bacteria: The type of biological activity of J166 against L. callinectes is similar to that of I-2. In liquid culture, J166 inhibits the growth of L. callinectes, and on agar plates, plugs of J166 placed adjacent to equally sized plugs containing fungal hyphae inhibit the growth of the fungus completely. Activity against the same set of bacteria matched against I-

2, shows a different pattern (Table III-2).

TABLE III-2

Activity of J166 against Bacteria collected at Bodega Bay

Test Strain No.																								
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
-	*	-	-	*	*	*	-	-	*	*	*	*	*	-	*	-	*	-	*	*	-	-	*	-

- no inhibition by J166
* inhibition by J166

Extraction, isolation and activity of active compound: The culture medium was extracted with three gallons of ethyl acetate per carboy (16 liters) with stirring. The procedure was repeated twice. The average yield was 600 mg of extract per carboy. The extract proved very difficult to work with because the active compounds decomposed rapidly. Tracking of activity was unreliable because of its inconsistency. The most conspicuous spot, though not the major compound under the growth conditions described, stained first brown and then purple with H_2SO_4/vanillin stain on silica TLC. This compound apparently decomposed and remained attached to the silica of the columns as evidenced by the fact that it appeared in all fractions from 50% to 100% ethylacetate. Its activity seemed dependant on time of storage before testing, and NMR spectra of the very small amounts that could be isolated always showed a mixture of compounds. Elution in Sephadex columns and preparative TLC techniques were tried but it was

never possible to obtain sufficient amounts of pure compound to get any information of use. Information on this compound was finally available by increasing production in fermentor culture conditioins and the compound was eventually identified by Dr. D. Tapiolas, as pyrrole 2-carboxylic acid (Fig. 3.4).

The major active compound isolated from carboy cultures was o-aminobenzoic acid (Fig. 3.3) or anthranilic acid. This compound forms pale yellow crystals upon purification by HPLC (40% ethyl acetate/isooctane) from a fraction eluted from a silica flash column with 60% ethyl acetate/isooctane. Spectral data on this compound are as follows: HRMS: requires 137.13 for $C_7H_7O_2N$, found 137.13 . MP 146 °C. ^1H NMR (C_3D_6O) : δ7.8 (dd, 1H, J = 6.5, 1.5 Hz); 7.2 (ddd, 1H, J = 6.9, 5.8, 1.5, 1.5, 1.1 Hz); 6.8 (d, 1H, J = 7.9 Hz); 6.5 (ddd, 1H, J = 7.3, 7.3, .7,.7 Hz). ^{13}C NMR ($CDCl_3$): δ169.3 (s),151.9 (s), 134.0 (d), 131.5 (d), 116.5 (d), 115.6 (s), 115.0 (d). IR(CHC13): 3500, 1670 cm^{-1} Comparison with commercially available anthranilic acid (Sigma Chem. Co.) showed identical spectral characteristics. This compound constituted approximately 1% of the total organic extract obtained from J166's culture medium.

Testing the Host's Dependance on the associated Bacteria and the Protective Properties of 2,3-indolinedione

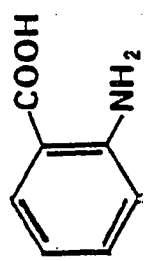
In order to assess the effectiveness of each strain of bacteria and of the major active compound in protecting

Figure 3.3

^{1}H NMR Spectrum of o-aminobenzoic acid (anthranilic acid)

$C_7H_7O_2N$

pale yellow crystals



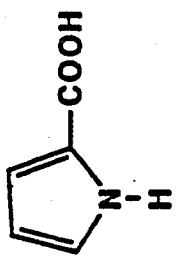
360 MHz in $CDCl_3$

Figure 3.4

^1H NMR Spectrum of 2-pyrrole carboxylic acid

$C_5H_5NO_2$

white crystals



360 MHz in MeOH-d_4

Dianne Tapiolas

the embryos against the pathogenic fungus, the following experiment was carried out at the Bodega Bay Marine Laboratories (Figs. 3.5 and 3.6). isolated, 2,3-indolinedione, we decided to test the effect of the absence of these elements on the bacteria. The experiment was carried out at the Bodega Bay Marine Laboratories.

Experimental Conditions: Twenty females of approximately the same size and bearing embryos at different stages of development were kept in separate compartments of a continuous-flow water table for three days. One cluster of embryos was detached from each one. Each cluster was divided in five smaller clusters, containing some 20 embryos each. Each cluster was kept separate, suspended by a thread in a Nalgene centrifuge tube (50 ml) with individual aeration. The water used was autoclaved 50/50 seawater/pondwater. The treatment groups were as follows:

Group C is the control. The clusters were taken out of the water for a few minutes every day to be examined under the dissecting microscope, but were otherwise left untouched and the water was not changed.

Group P was treated with a penicillin solution (100 mg/l for 6 hours) and then transferred to clean water on day 1 and again on day 2 of the experiment.

Group I was treated identically to Group P until day 2. On day 3, each cluster was dipped for 2 hours in a liquid culture (2 loopfulls of bacterial cells were

Figure 3.5

Effect of Bacterial Strains and 2,3-indolinedione against Fungal Infection: Treatment Schedule for Each Experimental Group

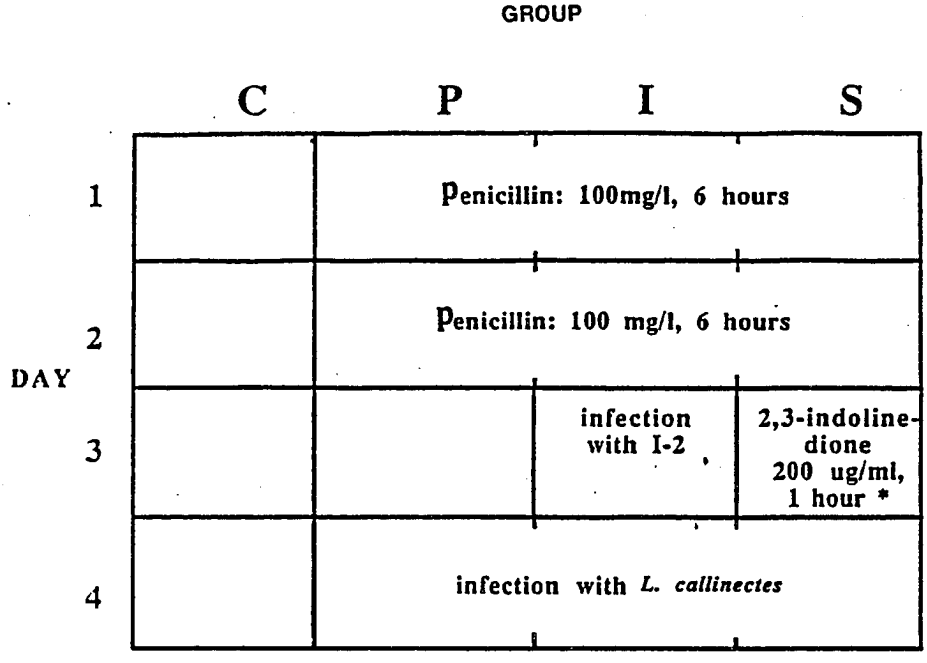
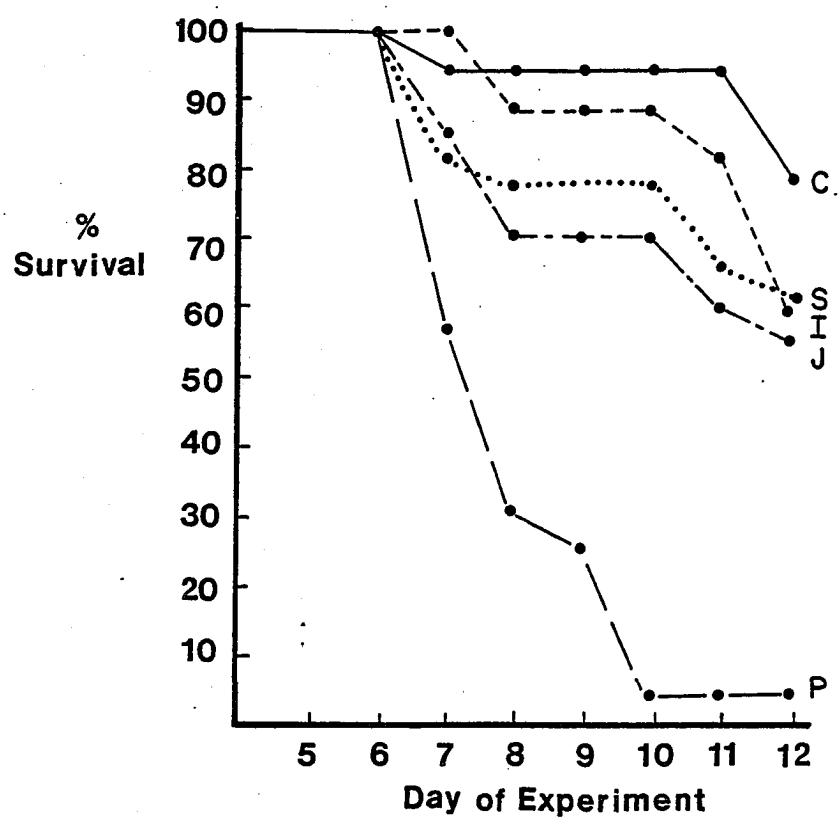


Figure 3.6

Percent Survival of Embryos after Infection with *Lagenidium callinectes* (on day 4 of experiment).



cultured for two days in 50 ml of 2216 marine broth; 50 ml of sterile water were added just prior to immersion of the embryos) of pure I-2 and then returned to clean water.

Group J was treated identically to Groups P and I until day 2. On day 3, each cluster was dipped for 2 hours in a liquid culture (2 loopfulls of bacterial cells were cultured for two days in 50 ml 2216 marine broth; 50 ml of sterile water were added just prior to immersion of the embryos) of pure J166 and then returned to clean water.

Group S was treated identically to Groups P, I and J until day 2. On day 3, clusters 1 to 10 were dipped for 1 hour in a solution of 200 ug of 2,3-indolinedione per ml of water and then returned to clean water. Clusters 11 to 20 were not treated until day 5. All embryos were treated again on day 7.

On day 4, all clusters were examined, and they all appeared to be healthy. Later that day, groups P, I, J and S were inoculated with 0.5 ml aliquots of liquid culture of L. callinectes. (the original stock was prepared by placing four plugs of 0.5 mm in diameter containing hyphae from the edge of the fungal growth in 50 ml of 2216 marine broth and cultured for three days), previously shaken vigorously to attempt to break up the hyphae in even pieces.

On day 5, all embryos looked healthy. Group S was all treated with 2,3-indolinedione, clusters 1 to 10 for the second time in the experiment and clusters 11 to 20 for the first time.

On day 6 no changes were observable in the embryos.

On day 7, the situation is as follows:

Group C	18 clusters alive,	1 dead, 1 dried
P	9 clusters alive,	10 dead, 1 dried
I	15 clusters alive,	3 dead, 1 dried, 1 hatched
J	14 clusters alive,	6 dead
S	17 clusters alive,	2 dead, 1 dried

Some of the clusters died of dessication when irregularities in the air flow caused splattering of the water out of the containers.

From day 8 through 11, clusters in Group P continue to die and some of the embryos in Groups I, J, S show enlarged, pale cells. Even though they do not disintegrate, they do not look healthy.

The final tally on Day 12 is as follows:

Group C: 15 alive, 4 dead (embryonal coat intact)
P: 1 alive, 18 dead (disintegrated)
I: 10 alive, 8 dead (not disintegrated)
J: 9 alive, 11 dead (not disintegrated)
S: 10 alive, 9 dead (not disintegrated)

Some additional observations worth noting are:

- Only those embryos that were advanced (eyespot stage) at the beginning of the experiment actually hatched. Less developed embryos were not observed to hatch during the period of the experiment, even though those cohorts that remained with the mother did hatch.

- Not all the embryos that died looked like they had succumbed to the fungus. Approximately one third of the dead embryos seem to have been attacked by bacteria first.

Assessing the Effect of Penicillin: Figure 3.7 shows the results of a subsequent experiment done to assess the effect of penicillin on the embryos. The experimental setting in this case was as follows:

- Ten embryos from each of four different females were placed in a large jar with sterile 50/50 seawater/pondwater with individual aeration. Each of the nine jars contained approximately 40 embryos from four different females and at four different stages of development. Treatment doses and lengths were identical to those described in the previous experiment. Each jar was subjected to a different treatment as follows:

1. Jar C - no treatment
2. PC - treatment with penicillin only
3. P - penicillin plus addition of fungus
4. PI - penicillin treatment, infection with pure I2 culture and addition of fungus
5. I - infection with pure I2 culture, addition of fungus
6. PJ - penicillin treatment, infection with pure J166 culture and addition of fungus
7. J - infection with pure J166 culture, addition of fungus
8. PS - penicillin treatment, treatment with 2,3-indolinedione, addition of fungus
9. S - treatment with 2,3-indolinedione, addition of fungus

The experiment was allowed to proceed for six days. Each embryo was picked up with a Pasteur pipette and observed daily under the microscope. The results suggest that penicillin itself does not affect the embryos but handling and addition of culture medium to the water are probably detrimental.

Fig. 3.7

Effect of Penicillin on Embryos of *P. macrodactylus*

	C	PC	P	PI	I	PJ	J	PS	S
Day 1 No. Live Embryos	42	32	55	43	41	40	45	41	39
Day 2 (Penicillin Treat.)	--	P	P	P	--	P	--	P	--
Day 3 (Add Pure Cultures)	--	--	--	I2	I2	J166	J166	--	--
Day 4 (Add *L. callinectes*)	--	--	Lc	Lc	Lc	Lc	Lc	Lc	Lc
Day 7 No. Live Embryos	42	21	6	32	29	33	31	18	24
Percent Survival	100	65	10	77	70	80	64	44	61

SEM Confirmation of Experimental Data: Progress of the two previously described experiments was followed by scanning electron microscopy as well. One or two embryos from each experimental group were fixed at different stages of the experiment, observed and photographed. A representative photographic sequence of events during the experiment is shown in Figure 3.8. The fixation procedure of the specimens was as follows:

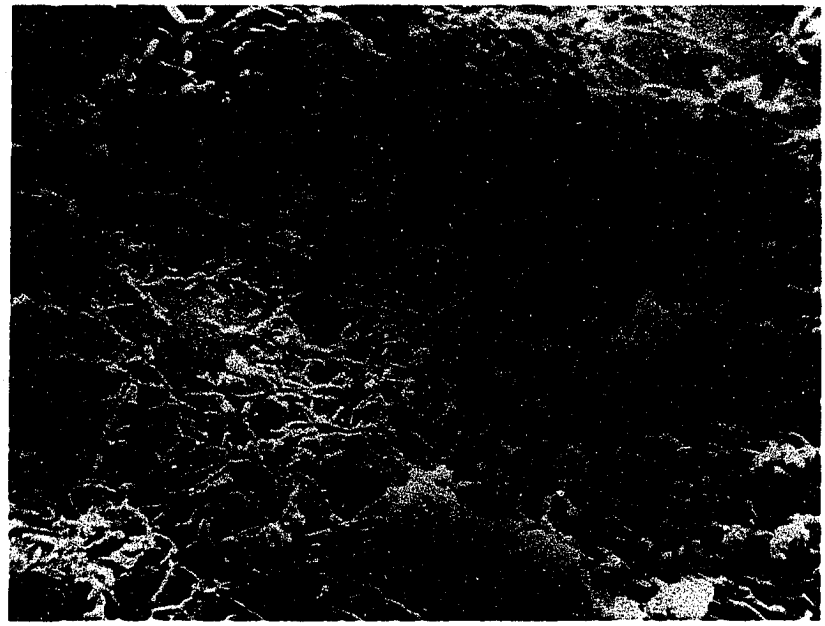
- 2.5% glutaraldehyde in sterile filtered sea water for 24 hours
- three rinses in sterile filtered sea water
- half an hour in 1% Os04 in filtered sterile sea water

Figure 3.8

Scanning electron micrographs following the experiment described on page 62.

A) Surface of healthy embryo recently collected from the Russian River estuary; X2000.

B) Surface of embryonic coat after treatment with Penicillin.



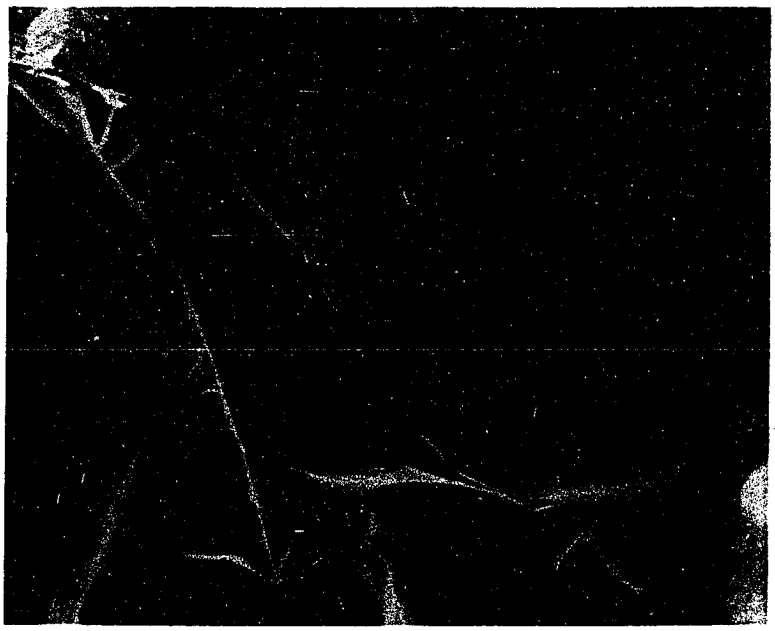
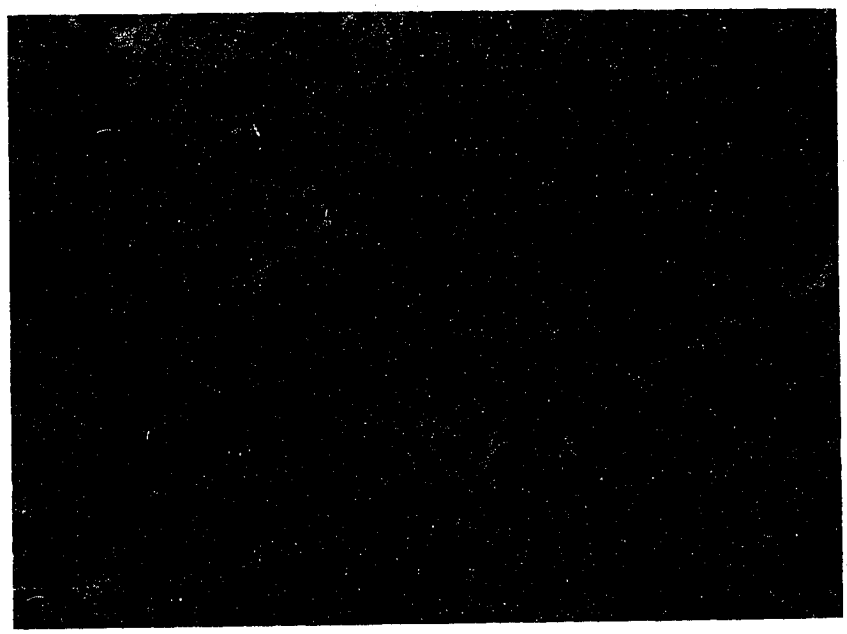


Fig. 3.8 (contd.)

C) Surface of embryonic coat of embryo from Group I (treated with Penicillin and infected with pure cultures of I-2), day 11 of experiment, X1000.

D) Surface of embryonic coat of embryo from Group J (treated with Penicillin and infected with pure cultures of J166), X1000.



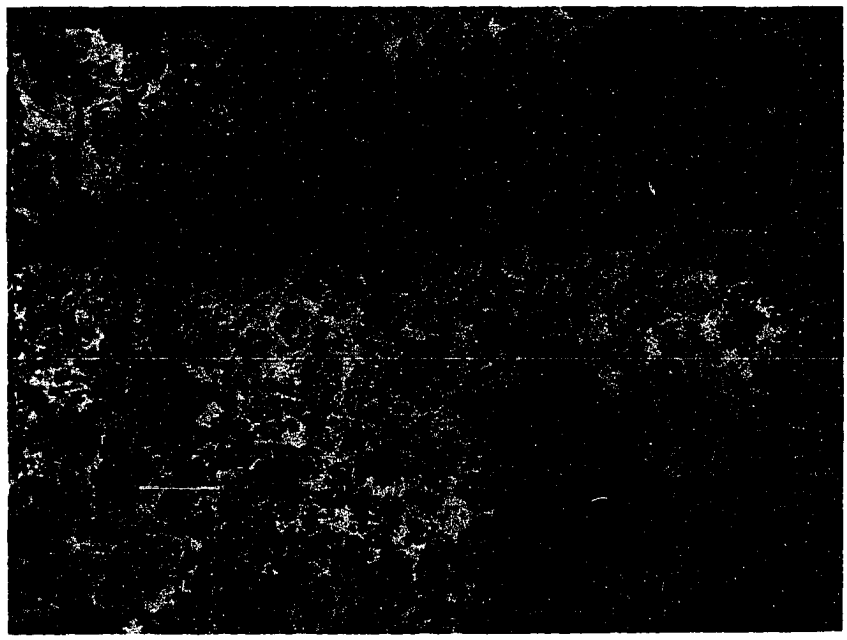
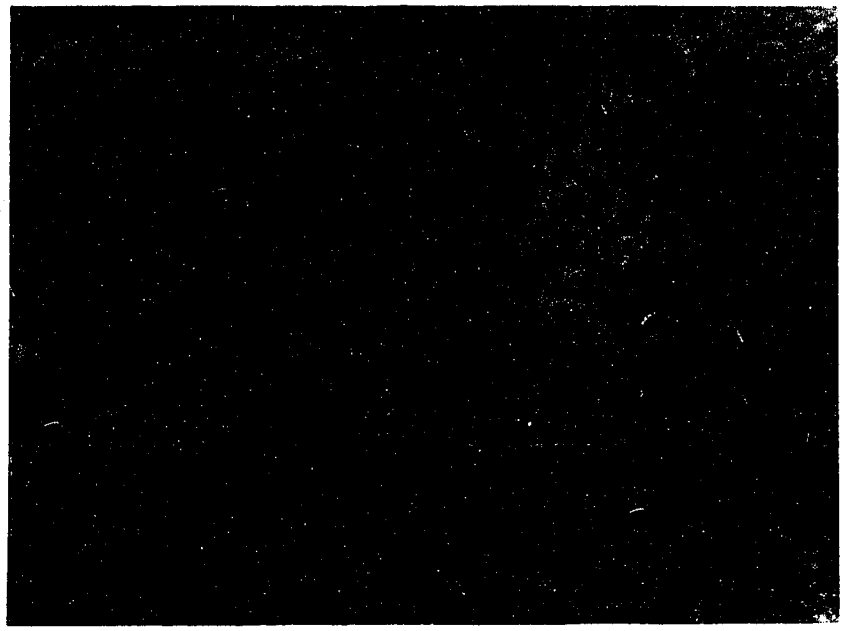
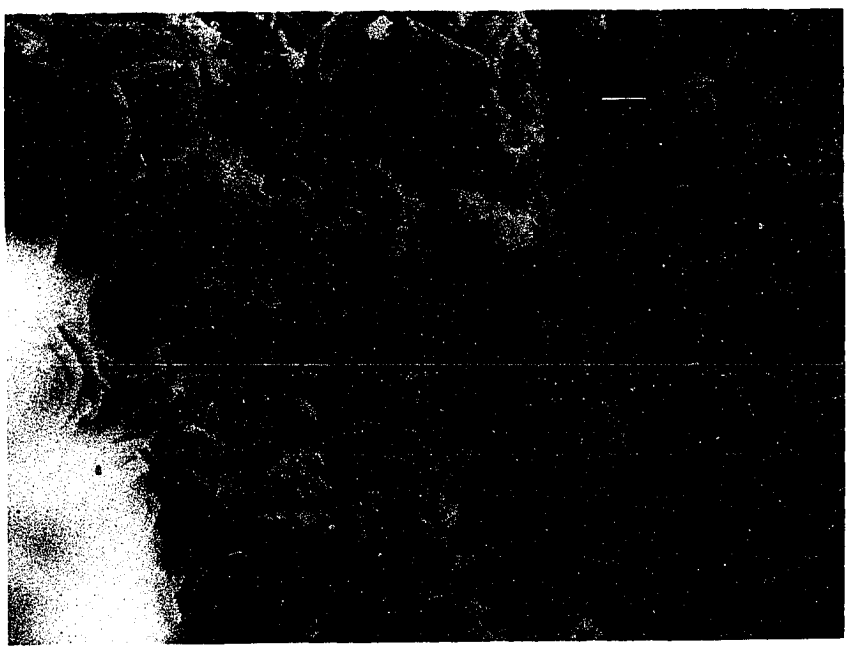


Fig. 3.8 (contd.)

E) Surface of embryo from group P (treated with Penicillin). Hyphae of L. callinectes have penetrated the coat and invaded the embryonal tissues, X1000.

H) Surface of specimen P16 on day 11 of experiment, X5000. It appears that these embryos were not successfully sterilized. P16 was the only survivor from group P.





- three rinses in sterile filtered sea water

- dehydration with ethanol series, 20 minutes each: 50%, 75%, 95%, 100% (three times)

The embryos were then critical-point dried in CO2 in individual specially modified microcentrifuge tubes. Gold coating was done for 3 minutes at 5 mAmp (300A coating). Each embryo was then observed in a Hitachi 539 SEM. Notes were made of all observations, and several photographs were taken.

CHAPTER IV

HOMARUS AMERICANUS (AMERICAN LOBSTER) EMBRYOS AND THEIR ASSOCIATED BACTERIA

Embryos of Homarus americanus are brooded externally (Fig. 4.1) for nine to twelve months (Hughes, 1973; Cobb and Wang, 1985). Up to 60,000 embryos can be extruded by one female . The first layer of embryos is attached to the female's swimmerettes, and subsequent batches are cemented to each other and finally to the abdomen. Preening by pleopods may account for ridding the outer layer of embryos of encroaching microorganisms, but the deeper layers have no mechanical defense.

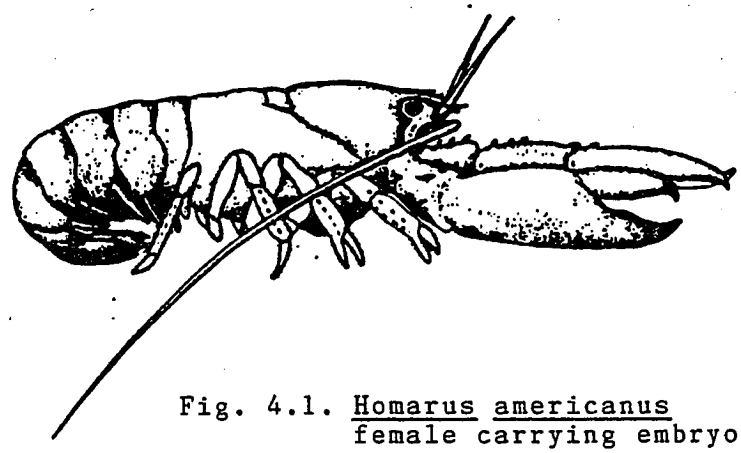


Fig. 4.1. Homarus americanus female carrying embryos.

Considering the lack of obvious protection against microbial colonization and the long duration of exposure to sea water and bottom sediments, it is quite remarkable that most embryos hatch. Furthermore, it is known that the pathogenic fungus Lagenidium callinectes can destroy the

tissues of lobster larvae in a very short time (Nilson et al., 1976). Infections of juveniles and adults have also been reported to a lesser extent (Provenzano, 1985; Cobb and Phillips, 1980; Fisher et al., 1978). Data on lobster diseases in the wild are not extensive but in recent years it has become more important to understand, prevent and cure diseases that are likely to disturb aquaculture operations, particularly at the first four larval stages (see Ch. V).

The remarkable resistance of the embryos to a pathogenic agent that affects later stages of the same organism living in the same environment suggests that the embryos possess a defense mechanisms that, in nature, the free-swimming forms do not require. In the confining conditions of aquaculture operations, however, larvae and juveniles do not survive if exposed to the pathogen. In an experiment similar to that described for P. macrodactylus, embryos from females caught at the East Coast and held in captivity at the Bodega Marine Laboratories were exposed to large quantities of L. callinectes. The embryos proved to be completely uninfectable, even after repeated additions of the fungus. In fact, the cotton thread by which the embryos were suspended was colonized by hyphae, but no sign of fungal encroachment or impaired health was apparent on the lobster embryos. After five weeks, the experiment was abandoned without achieving infection. Microscopic examination of these embryos showed that all were free of fungal and protozoan epibionts. On closer observation with

the scanning electron microscope, it became obvious that they were extensively covered by a mosaic-like layer of bacterial rods (Fig.4.2.A) forming a tight crust around the embryos.

Scanning electron micrographs of specimens at different stages of development showed that older successful (i.e., attached to the mother) embryos have the densest and most extensive coverage by these bacteria (Fig. 4.2.B). Some embryos occasionally are sparsely colonized by three other morphotypes, all quite characteristic and recognizable (Fig. 4.2.D). Harper and Talbot (1984) examined by SEM the epibiotic bacteria flora of embryos from 66 Homarus lobsters collected at different locations, including laboratory-reared specimens. They sought to correlate bacterial coverage of the embryos with loss of embryos from the pleopods (Perkins, 1971). Throughout the study, they found only four different morphotypes of bacteria associated with the embryos. To their surprise, they noticed that health and successful retention of the embryos was correlated to a high coverage by one of the strains, namely the 'colonial rod' strain resembling the one we found.

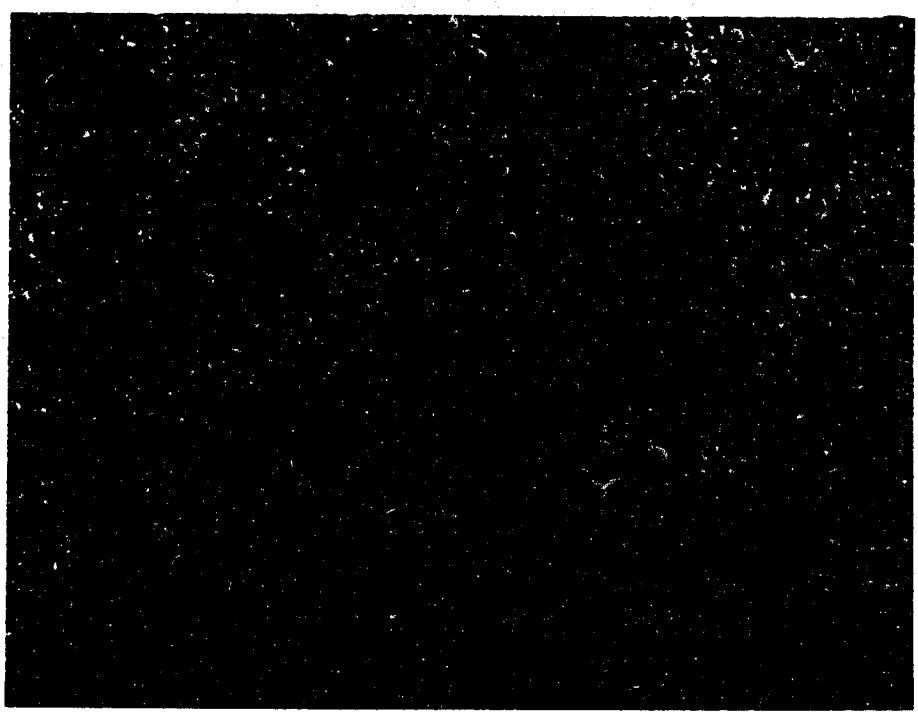
Platings of homogenates of several specimens (embryos from different females), consistently resulted in the isolation of four, sometimes five, different morphotypes. Only one of these, a salmon-colored, extremely slow-growing strain, showed in vitro activity

Figure 4.2

Embryos of Homarus americanus.

A) Surface of young embryo; X5000.

B) Coverage on healthy embryo near hatching time, X5000.



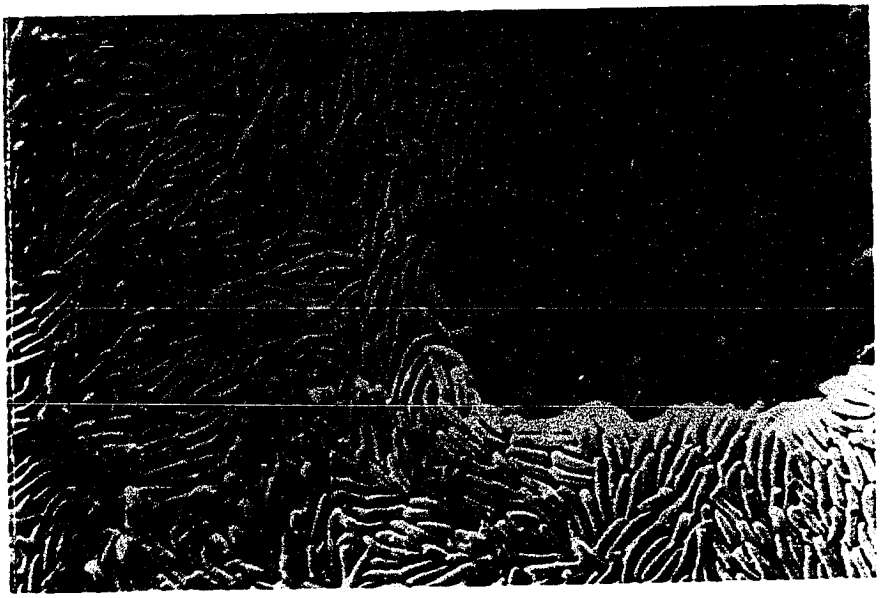
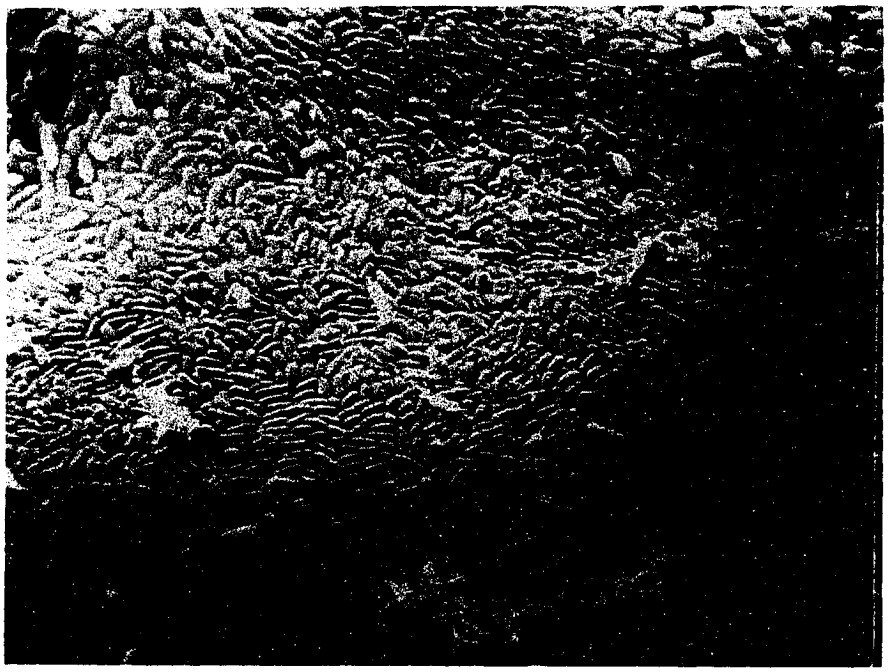
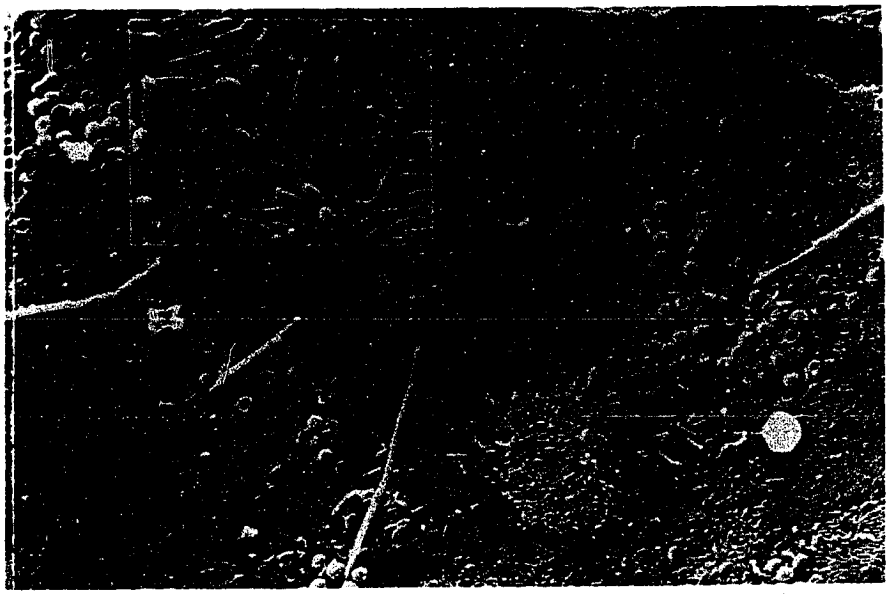


Fig. 4.2 (contd.)

C) Pure culture of strain L-E grown on millipore filters, X2000.

D) Embryo showing some coverage by three other strains of bacteria found occasionally, X1000.





against L. callinectes. These bacteria, in liquid culture, produce a compound, p-hydroxyphenyl ethanol (Fig. 4.3) which is active against the pathogenic fungus. Only relatively small quantities of this compound are produced, but its activity is comparable to that of 2,3-indolinedione. If these laboratory results reflect what occurs in nature, the unusual densely-packed coverage by these bacteria may explain why they constitute such an impenetrable barrier, even though each cell's production of antifungal compound is relatively low. This compound, interestingly, has been reported to be produced by a number of fungi and it is involved in antifungal defense.

The strength of this association lies in the remarkable amount of coverage by a bacterial symbiont that produces small amounts of a powerful antifungal compound. The fact that there is a clear correlation between length of retention of the embryos and general health with their coverage by this particular strain indicates that the host depends on this association for its health under conditions that involve lengthy exposure to other microorganisms.

Experimental Conditions

Infection with L. callinectes: Ten groups of about five embryos each were detached from berried females (caught in the USA East Coast with embryos already extruded, and kept in aquaria at the Bodega Marine Laboratory subsequently) and were then placed in three consecutive

rinses of sterilized sea water. Each cluster was suspended from a thread and placed in 125 ml Erlenmeyer flasks containing 75 ml of sterilized sea water, with aeration through an airstone. The liquid culture of L. callinectes used to infect the embryos was prepared by introducing a 0.5 cm in diameter core of fungal hyphae obtained from the edge of a 3-day colony, into 10 ml of 2216 Difco Marine Broth medium and allowing the fungus to grow for one week. The culture was shaken until the hyphae appeared to have broken into approximately equal-size pieces, and 1 ml of this thick culture was added to each experimental flask. By the fourth day, there was no apparent colonization of the embryonal surface. Subsequently, and for a period of two weeks, 1 ml of fungal culture was added daily to each flask, so that the seawater became viscous with fungus. The cotton thread was in fact becoming colonized by hyphae, but no sign of fungal encroachment or declining health was apparent on the lobster embryos.

Isolation of Active Bacterial Strain: Approximately five embryos from each specimen were homogenized in a sterile Elvejmer tissue grinder with 10 ml of sterile sea water and this homogenate was plated onto Difco 2216 Marine Agar plates. In most cases, platings of 1/10 and 1/100 dilutions were also plated out. Consistently, only four (once five) different bacterial morphotypes could be observed on the plates. Individual colonies were transferred to plates and purified. Agar cores of 0.5 cm in diameter

containing pure cultures were placed on agar plates approximately 1 cm away from agar cores containing L. callinectes. Only one of the colonies was inhibitory to the fungus. The visible characteristics of the active bacterium were: slow-growing, crust-like shape, salmon color. Because colony pattern is sometimes a characteristic of different strains (Whittaker and Drucker, 1970), we observed the pattern formed by the pure strain grown on Millipore membranes. Although the results were not totally satisfactory (see section on SEM methods), it is apparent that the bacteria tend to form the characteristic mosaic-like arrangement. The scanning electron micrographs of these bacteria confirm that the shape and size of the isolated bacteria is identical to those of the bacteria observed to cover the lobster embryos (Fig. 4.2.C). The rods measured about 1.3 x 0.4 um and do not have any obvious appendages.

Batch Culture: This strain was very difficult to culture in large quantities and the crude extract yields were extremely low. The strain did not grow at all in the standard sea water medium, but it did grow slowly in 2/3 2216 marine broth and in standard sea water medium where no source of carbohydrate. The best yield was obtained with a medium prepared with 5 g of yeast extract and 3 g of bactopeptone per liter of sea water. Table IV-1 shows the different media tested with varying carbohydrate composition.

TABLE IV-1

Liquid media tested for batch culture of strain L-E

Medium No.	BP	YE	GY	SU	FR	GL	days	pH	mg crude in 16 lt
1	x	x	x				15	4.6	100
2	x	x		x			15	4.3	125
3	x	x			x		15	4.4	90
4	x	x				x	15	4.4	0
5	x	x					15	8.1	250
6	x	x					21	8.8	420

BP-bactopeptone; YE-yeast extract; GY-glycerol; SU-sucrose; FR-fructose; GL-glycerol

A loopfull of pure bacteria was added to 500 ml of liquid medium and cultured at room temperature (approx. 25 oC) in a shaker for three days. After confirmation of bacterial purity and identity on agar plate overnight, 150 ml of the culture were added to each of three 16-liter carboys, which were maintained at room temperature with aereation for three weeks.

Isolation of Active Compound: Every 16 liters of medium were extracted with about 4 liters of ethyl acetate for 24 hours with periodic shaking. The extraction was repeated twice. The solvent was evaporated in a rotary evaporator and the crude extract was brought to complete dryness under nitrogen. Activity of the crude extract was confirmed by saturating a 0.5 cm in diameter paper disk with 1 mg of extract dissolved in methanol. The dry discs where then placed at the edge of 3-day old colonies of L. callinectes and observed for zones of inhibition for about

three days following. A disc saturated with methanol only was always placed on the same plates as a control. The initial fractions of the crude extract were separated in a small silica column with ten sequential solutions of ethyl acetate/isooctane from 10% to 100%. The active compound was found on the fraction eluted by the 80% solution. Further purification was done by HPLC using 80% ethyl acetate/isooctane as eluant. The final purification was done on a Sephadex LH20 column using the following solvent system: hexane/methylene chloride/methanol, 2:5:1.

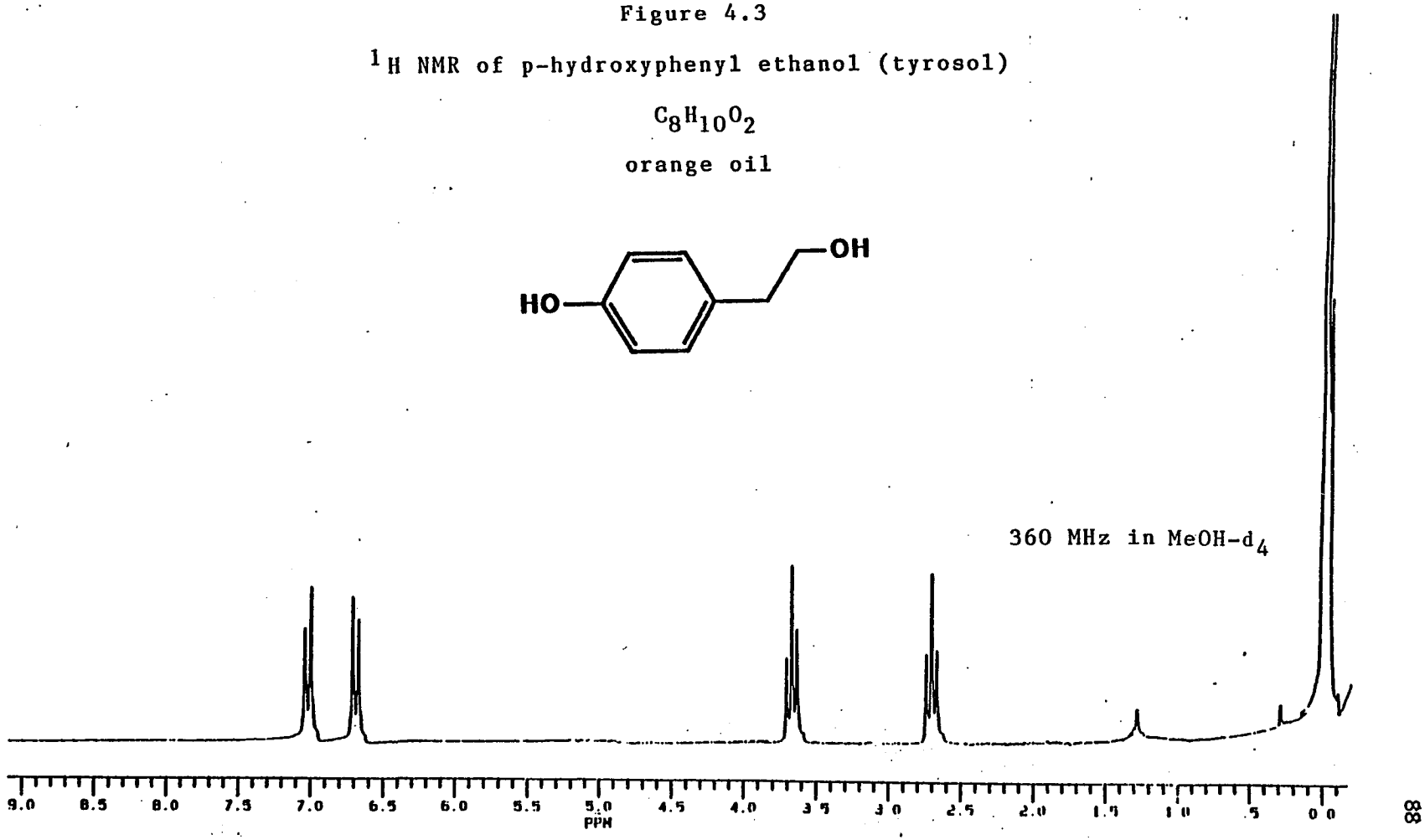
Characteristics of Active Compound: orange oil; IR: 3400, 3150 cm^{-1}; HRMS: requires 138.04 for $C_8H_{10}O_2$, found 138.06. ^1H NMR, 200 MHz (C_3D_6O): δ 7.0(d, 2H, J = 8.6 Hz), 6.6(d, 2H, J = 8.6 Hz), 3.7 (t, 2H), 2.7 (t, 2H); ^{13}C NMR, 200 MHz (C_3D_6O): δ 155.0, 131.0 (2x), 130.0, 115.5, 115.4, 63.8, 39.1. This compound, p-hydroxyphenyl ethanol (Fig. 4.1), had not previously been isolated as a natural product from bacteria. It had, however, been isolated from cultures of the mold *Ceratocystis fimbriata*, a parasite of carrots and other vegetables (Stoessl, 1969). The presence of the fungus on the carrots precludes infection by other pathogenic fungi. The compound was also isolated from cultures of the fungus *Candida albicans* (Narayanan and Rao, 1976). A related compound, 2-phenylethanol, was reported (Lingappa *et al.*, 1969) to be one of two major compounds responsible for the phenomenon of autoinhibition in *C.*

Figure 4.3

^1H NMR of p-hydroxyphenyl ethanol (tyrosol)

$C_8H_{10}O_2$

orange oil



albicans. Experiments by Narayanan and Rao (1976) who cultured C. albicans with L-tyrosine as the only source of nitrogen, indicate that p-hydroxyphenyl ethanol is synthesized from L-tyrosine in the fungus. Another interesting activity of this compound is at the core of a rather complex symbiotic association (Claydon et al., 1985). The bark beetle Scolytus sp. bores into elm trees carrying the pathogenic fungus Ceratocystic ulmi, the causative agent of Dutch elm disease. On the bark of healthy trees, however, the fungus Phomopsis oblonga can be found. This fungus rapidly invades the wound left by the boring beetle and effectively outcompetes the pathogenic fungus. Once P. oblonga is inside the tree's phloem, the beetle does not find the tree suitable for breeding and abandons it.

Activity of Compound

The active compound inhibited growing cultures of L. callinectes in liquid medium at a concentration of 0.01 mg per ml of medium. On agar plates, discs containing 0.1 mg of the compound inhibited the growth of the fungus (8 mm inhibition zones) effectively.

SEM techniques: The first embryos observed (Fig. 4.2.A) were kept in a solution of 2.5% glutaraldehyde in 3% NaCl/distilled water for 24 hours, then rinsed in 3% NaCl/distilled water solution for half an hour, and transferred to a solution of 1% osmium tetroxide in 3% NaCl/distilled water for an hour and a half. Specimens were then stored in the NaCl solution overnight and subsequently

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dehydrated in an acetone/distilled water sequence, as follows: 35%, 15 min; 50%, 15 min; 75%, 30 min; 95%, 1 hr; 100% 1 hr; 100% overnight. The micrographs were obtained with a Hitachi 539 (Bodega Marine Laboratory). Embryos observed more recently (Figs. 4.2.B and D) were fixed in a similar same manner (Felgenhauer, 1987), and then critical-point dried with Freon, coated with 300A of gold and observed in a Cambridge 360 scanning microscope. Isolated pure colonies (Fig. 4.2.C) were fixed differently (Shapiro, 1985; Afrikian et al, 1973). A drop of a 3-day liquid culture was deposited on a piece of millipore filter (0.25 u pore size) on an agar plate. As soon as growth was visible, the specimens were prepared as follows: fixation in 3% formaldehyde, 3% glutaraldehyde in 0.2M sodium cacodylate trihydrate buffer (solution kept at pH 7.2 to 7.4) for one hour; three washes of 5 min in 0.2M cacodylate solution; transfer to 2% osmium tetroxide in 0.2M cacodylate solution for one hour; six rinses of 5 minutes in 0.2M cacodylate solution; dehydration in a sequence of ethanol/distilled water as follows: 30%, 50%, 70%, 80%, 90%, 100% each for 5 to 10 minutes. The specimens were then critical-point dried under CO_2, coated with gold (300 A) and observed with a Hitachi S405A scanning electron microscope.

CHAPTER V

MICROBIAL DISEASES IN AQUACULTURE: THE USE OF BACTERIAL COMPOUNDS AS ANTIMICROBIAL AGENTS

Modern therapeutic strategies against microbial diseases are shifting from massive treatment with ultrapotent chemicals having radical effects and unknown long-term consequences to less powerful but more naturally-balanced, ecologically-sound treatments. In some areas, such as commercial aquaculture, it has become a necessity to completely abandon the old methods of microbial disease control and look instead for biologically sound alternatives, because the common antibacterial and antifungal treatments are no longer considered safe to humans and to the environment in general.

Crustacean and fish aquaculture is a fast-growing industry in the United States, Japan and in most of the developing world (Shigekawa and Logan, 1986; McCraren, 1985; California Seagrant, 1982; Rosenthal and Oren, 1981; Glude, 1977). As the demand increases and the natural stocks decline, more effort is invested in developing aquaculture systems with reasonable production economics. Intensive aquaculture facilities require high holding densities, relatively warm water which is partially recirculated and some degree of handling, all of which enhances the likelihood of injury and disease susceptibility and contagion. Fungal and bacterial infections cause severe

losses, particularly in crustacean larval stages and juveniles and in fish eggs and fingerlings (Lewis et al., 1982; Neish and Hughes, 1980; Fisher et al., 1975). Crayfish farming is the largest aquaculture industry in the United States, with 50,000 ha in production (Scott and Thune, 1986). Efforts to implement aquaculture of highly priced marine crustaceans, such as lobster, shrimp and crabs have increased in the last years and much research is being directed towards the management of diseases particular to the culture of these crustaceans (Castille and Lawrence, 1986; Taki et al., 1985; Lewis et al., 1982)..

Studies show that 41% of the red swamp crayfish Procambarus clarkii (Girard) from commercial ponds contain high levels of deleterious bacteria in their circulatory system (Scott and Thune, 1986). The same has been found for the American lobster, Homarus americanus (Cornick and Stewart, 1966) and the blue crab, Callinectes sapidus (Johnson, 1976, Colwell et al., 1975; Haskell et al., 1975). Successful larval rearing operations of Penaeus monodon, a penaeid shrimp highly appreciated for human consumption, have been devastated by two mycotic agents, a Lagenidium species, and Haliphthoros philippinensis.

There has also been an increase in the number of fish hatcheries, both marine and fresh water and, despite great advances in this area, microbial disease still remains the major concern to the industry (Foo et al., 1985; Geiger and Parker, 1985; Toor, et al., 1983; Austin et al., 1981;

Warren, 1981; Johnson and Finley, 1980; Bullock, 1977). Both fungal and bacterial infections are a serious problem in fish hatcheries, where enormous numbers of eggs and fingerlings are kept in extremely crowded conditions (Meyer and Hoffman, 1976).

Sea-ranching, an aquaculture strategy that is lately gaining recognition among Western countries, requires the ability to produce abundant, healthy juveniles on a steady basis to replenish harvested stocks. The objective is to stock the fishing grounds with hatchery-reared juveniles whose larval stages were cultured under optimal conditions, i.e., absence of predation, appropriate food, controlled temperature, and disease treatment (van Olst et al., 1980). Lobster hatcheries have existed since 1885, and several installations were functioning at the beginning of the century in New England, Eastern Canada and Europe. The difficulties of dealing with a situation that required much investment of research and funds at a time where natural stocks were still plentiful, caused the gradual abandonment of these enterprises. From 22 hatcheries existant in 1920, there are now only two, one in Massachusetts and one in France.

Commercial farming of an aquatic organism requires an adequate consumer demand and profit potential for the species, the ability to reproduce in captivity, simple larval development, high food conversion efficiency, and resistance to disease (Cobb and Phillips, 1980). The

American lobster, H. americanus, meets several of these requirements and has been selected by the U. S. National Oceanographic and Atmospheric Administration as one of four animals having 'high priority' for aquaculture research and development (Glude, 1977). With this official endorsement, funds have become available for research in several areas of lobster, shrimp (Hanson, 1977) and crab biology. Prophylaxis is, of course, the most desirable strategy against disease, and proper space, filters and aereation are the key to this approach (Lightner, 1982; Johnson, 1978). But, treatment of disease is critical in cases of outbreaks that can devastate a whole operation with millions of juveniles and eliminate valuable broodstocks, which in some cases are the product of painstaking genetic selection over many years (H. americanus becomes fertile at 6 years of age and reproduces every two years).

The total worldwide aquaculture of fish and shell fish doubled in the last one-half of the 1970s to about 10% of total fishery landings, and they have doubled again in the following 15 years. FAO predictions based on present demand and production trends are 130 million metric tons for the year 2000, 25 % of which will come from aquaculture operations (Sandifer, 1988; Nash, 1988). Aquaculture organisms, especially if they are sea-farmed, have much lower production costs than terrestrial farm organisms and it is quite apparent that consumption of aquatic organisms will surpass even that of poultry, which is currently the

leading animal product consumed in the U. S.

One element that is quickly shifting consumers' preference towards seafood is the recent discussions concerning the high contents of omega-three fatty acids in organisms that have algae at the base of their food chain. There is evidence that omega-three fatty acids are not metabolized by the human body to cholesterol and other eicosanoids associated with disease and pain (Lands, 1988).

Shrimp aquaculture, in particular, has increased greatly in the last few years. Ecuador, for example, where Incas were farming shrimp 400 years ago, is the major producer and in 1985 it was providing 75% of the world's farmed shrimp (Rosenberry, 1983).

In the U. S., the state of Louisiana has launched a successful crayfish program where in ten years production went from 800 to 50,000 hectars which are presently yielding 55,000 MT of crayfish per year. Large-scale rearing of crustaceans for stocking of natural habitats has also been practiced to replenish or supplement overfished populations (Kurata, 1981). Introduction and establishment of crustacean species in new areas have also been successful, as illustrated by the the large freshwater caridean, Macrobrachium iar, which has adapted to the marine salinities of the Hawaiian Islands (Nolfi, 1980; Singholka et al., 1980; Goodwin et al., 1977). This shrimp has been so successful in aquaculture that it has overshadowed other caridean species, which are cultured in smaller scale and

mostly for research purposes in the United States. Nevertheless, caridean shrimp constitute 15% of the total worldwide catch of shrimps (more than 150,000 MT annually) (Neal and Maris, 1985). In the United States their value is lower than for penaeid shrimp principally due to the small size and the practice of machine peeling, which results in loss of meat (Collins and Kelley, 1969), but they are importnt locally to the fishing communities and internationally as a source of high-priced fishery products. The shrimps are canned, frozen or sold fresh and are a significant proportion of the luxury food market for crustaceans.

Common Microbial Diseases of Aquaculture Crustaceans

Marine shrimp culture has become successful (i.e., profitable) due basically to three breakthroughs in recent years (Glude, 1988): larval culture techniques, the development of high density culture systems, and the control of microbial diseases in these systems. Although there has been steady progress in the first two areas in laboratories worldwide, the third area, the control of microbial and viral diseases has again become a serious issue requiring new strategies (Brock, 1988; Taki et al., 1985; Aquacop, 1977; Vanderzant et al., 1971). The crustacean diseases listed below represent the most significant concern to commercial aquaculture and have best been studied in the American lobster but have been reported to affect other aquaculture organisms as well (Stewart, 1980).

1) 'Shell disease' is caused by chitinolytic bacteria and fungi (Fisher et al., 1976a). Larvae are the most susceptible and show lesions in the exoskeleton which allow entry of other pathogenic microorganisms, causing serious secondary infections.

2) 'Gaffkemia', caused by the Gram-positive bacterium Aerococcus viridians (variety homari), has brought about serious losses mainly in northeast coast lobster ponds (Fisher, et al., 1978; Schapiro et al., 1974). Presumably, the pathogen is introduced through cracks and lesions and, once it reaches the circulatory system, it competes for hemolymph sugars, multiplies quickly and causes septicemia and death. Although the bacterium has no mechanism of invasion, the lobster has a complete lack of defense against it: A. viridians is not cleared from the haemolymph as are other microorganisms injected in healthy lobsters.

3) Fouling by a variety of microbial epibionts which can cause asphyxia by occlusion of egg membranes or larval gills is another common problem. Even though the causative organisms are not identified, this condition is pervasive when nutrient levels rise in the aquarium water. One organism often found to be responsible for this condition is Leucothrix mucor, a filamentous bacterium (Johnson et al., 1971).

4) Fungal diseases can be extremely difficult to treat and often the only course of action to follow is dismantling and total disinfection of the aquaculture

operation (Johnson, 1970; Cook, 1971). The mycotic pathogens most often found to affect crustaceans in aquaculture are:

Lagenidium sp. - This phycomycetous fungus was first reported by Couch (1942) to be parasitic on eggs of the blue crab Callinectes sapidus. It was subsequently found on larvae of the white shrimp Penaeus setiferus (Lightner and Fontaine, 1973), the brown shrimp P. aztecus (Cook, 1971), the Dungeness crab, Cancer magister (Armstrong et al., 1976; Fisher and Nelson, 1977) and in hatchery larvae of several panaeid shrimp (Bland, 1976). L. callinectes can also destroy the tissue of lobster larvae in a very short time (Nilson et al., 1976).

Haliphthoros milfordensis - Also a phycomycetous fungus, this pathogen is a serious threat to both European and American cultured lobster (Fisher et al, 1975), particularly in small juveniles.

Fusarium sp. - This deuteromycetous fungus is a common plant and field crop pathogen which recently has been identified to cause a gangrene-like condition in the appendages and gills of juvenile and adult lobsters and several panaeid shrimp (Solangi, 1976). No chemical agent has been found to be effective against Fusarium as a treatment for culture animals.

Efforts to prevent these and other diseases usually involve treatment of the aquarium water with ultraviolet light (Fisher and Nelson, 1977; Mock and Murphy, 1970) in

conjunction with addition of various chemicals known to inhibit fungi and bacteria. In most cases, however, the minimum inhibitory concentrations of these compounds are toxic to some stage of the organisms being treated (Egidius and Moster, 1987; Lio-Po, 1978; Abrahams and Brown, 1977; Delves-Broughton, 1974; Armstrong et al., 1976; Fisher et al., 1976). Furthermore, some of these compounds are herbicides (Lio-Po and Sanvictores, 1986) or other types of active chemicals with unknown effects on aquatic organisms (Williams et al., 1986; Fisher and Nelson, 1978; USEPA, 1976).

Malachite green, for example, the compound most commonly used for treatment of fungal infections in hatcheries and aquaculture systems (Nelson, 1974), has the advantage of a low minimum inhibitory concentration but it is toxic to most larvae (Fisher et al., 1976b) and was shown to be potentially teratogenic or mutagenic (Lieder, 1961; Nelson, 1974). In 1978, the U. S. Fish and Wildlife Service issued a moratorium on the use of malachite green in Federal hatcheries. Formalin, another widely used antimicrobial compound, is quite toxic and by no means universally effective. Furanace, a common herbicide also used in antimicrobial treatment, has a low MIC in vitro, but its effects on algae and marine plants are questionable (Bailey, 1983, 1984).

Much work has been done in the search for adequate, safe and economical antimicrobial agents to be used with

aquaculture organisms. Chemicals are continuously being tested and new strategies are being investigated (Egidius and Moster, 1987). Some of the latest work involving bacterial infections follow the development of immunity by vaccination (Lamers and van Muiswinkel, 1986; Shotts et al., 1986; Slivka and Steenbergen, 1984; Anderson et al., 1983; Cipriano, 1982; Egidius and Andersen, 1979; Michel 1979; Gould et al., 1978; Paterson et al., 1976; Schapiro and Steenbergen, 1974). Vaccination is a reasonalbe alternative to protect valuable broodstocks but it is not practical for large numbers of animals.

There are other areas in which it is critical to keep healthy populations of organisms. Our approach has been to delve into aquaculture species, because it is considered such an important area with pressing research needs. Other areas of research, such as taxonomy, genetics, population biology, molting physiology, response to environmental factors, and development of bioassays also require large homogeneous populations of contemporaneous organisms, which can only be obteined from laboratory cultures.

The concept of microbial pathogen control by associated microflora has been used to some extent in agriculture (Barrows-Broaddus, 1985; Gagne, 1985) and is actually quite old. Mixing small amounts of 'disease-suppressing' soil in fields where fungal diseases are a problem or rotating crops to include plants that seem to free the fields from pathogens (Spalla and Roncucci, 1987;

Schroth and Hancock, 1982), is nothing but the application of the general concept of microbial chemical competition to protect the vulnerable organism.

The use of 2,3-indolinedione as an antifungal agent in aquaculture

We decided to test the effectiveness and practicality of 2,3-indolinedione as a preventive and therapeutic agent in infections of Lagenidium callinectes on isolated embryos and to assess its effects directly on adult specimens of Palaemon macrodactylus at high concentrations. We chose to test the embryos separately from the females because large-batch embryo culture is one important aim in aquaculture. Separate culture of the embryos permits large densities, avoids cannibalism, eliminates the need for feeding, which in turn makes water maintenance simpler, and requires only a small number of select brooding specimens, since oviposition occurs more frequently when the females lose their broods. Although less vulnerable to fungal infections that larvae and juveniles, adults can also become diseased and the main problem with the classical treatments has been toxicity. We tested concentrations up to a hundred times higher than those found to be effective as an antifungal treatment with the most common compounds used. The experiment resulted in the definition of a minimum inhibitory concentration and treatment strategy for exposures to pathogen concentrations that are probably highly exagerated. Treatment with 2,3-indolinedione was

very effective at concentrations and exposures comparable to usual treatments. Exposure to larger concentrations did not affect the normal development of the embryos. In fact, all embryos hatched into apparently healthy larvae. Similar treatment concentrations were tested on adult females and males and no apparent ill effects were observed.

Defining Treatment: In order to estimate the treatment concentrations to be used as a starting point, information regarding minimum inhibitory concentrations in vivo and recommended treatments for malachite green (Bailey,. 1984; Abrahams and Brown, 1977) was combined and the same correlation was used for 2,3-indolinedione. Two methods were used to estimate MIC in agar, as shown in Figure 5.1. Column A indicates concentrations of 2,3-indolinedione mixed into the agar after it was sterilized, at temperatures of approximately 50 oC. Column B indicates concentrations of the compound added to the agar before sterilization. Results suggest that 2,3-indolinedione is heat-labile. Methanol was used to dissolve the compound at 50 ul per mg. A control plate with only methanol (80 ul, i.e., the highest amount of methanol used) was prepared, as well as a control plate with only medium. A 0.5 cm in diameter core wit hyphae of L. callinectes from the edge of a 2-day old colony was placed on each plate as soon as the agar was solidified.

Figure 5.1

Determination of Minimum Inhibitory Concentration of 2,3-indolinedione on the fungus L. callinectes in vitro. Fungal growth was measured after three days. Compounds were added to medium A after autoclaving and to medium B before autoclaving.

mg Compound per ml Agar	mm Fungal Growth from Plug Edge Medium A	Medium B
0.005	3	9
0.01	0	9
0.10	0	6
1.00	0	9
80.00 (ul) MeOH (control)	10	9
---	9	9

Experimental Procedure: With a MIC of 0.01 mg/ml, the chosen starting treatment was 20 ug/ml of seawater for dips of 2 hours daily. Higher concentrations were also used to test the limits of possible toxicity. Figure 5.2 illustrates the sequence of events in the experiment with isolated embryos. Clusters of about 100 embryos were detached from one single female and suspended from a thread in individually aereated Erlenmeyer flasks containing sterile 50/50 sea water/pond water. One ml of a very thich 5-day old liquid culture of L. callinectes was added to each experimental flask. Figure 5.3 shows embryos treated with 2,3-indolinedione (A) and untreated (B), after exposure to the pathogenic fungus.

Figure 5.2

Treatment with 2,3-indolinedione as deterrant of infections by L. callinectes. Treatment A consists of daily 2-hr dips in a solution of 20 ug/ml of water; treatment B consists of daily 1-hr dips in a 200 ug/ml solution; treatment C consists of daily 1/2-hr dips in a 400 ug/ml solution.

			Experimental Groups/Treatments		
Day #	C	I	II	III	IV
1			---addition of 1 ml fungal culture---		
2			A	B	C
3			A	B	C
4			A	B	C
5		1/3 infected	A	B	C
6			A	B	C
7			A	B	C
8		all dead	A	B	C
9			A*	B	C
10			A	B	C
11			-------no treatment--------		
12			----------		
13			----------		
14	hatch		hatch	hatch	hatch

*one embryo appears to be infected

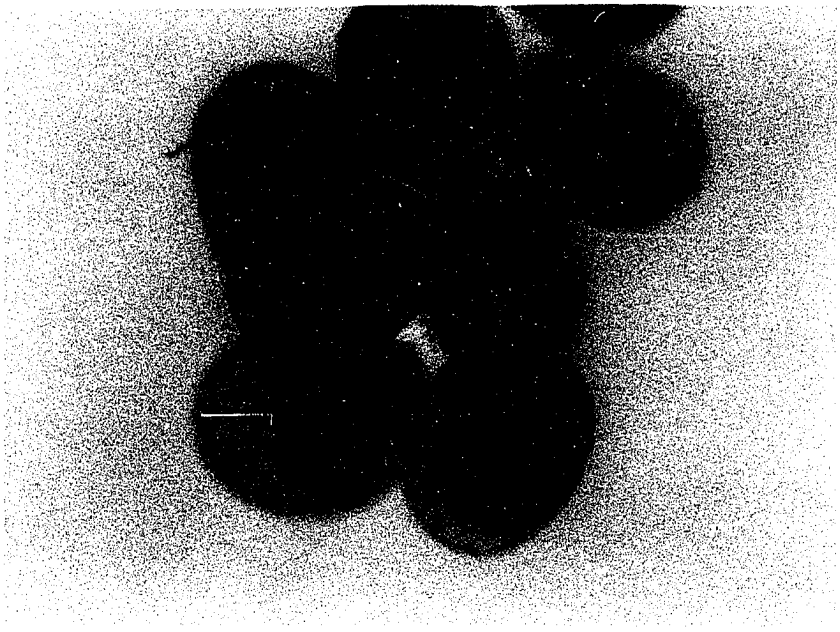
To test the effect of 2,3-indolinedione on the adults, two males and four females were tested using concentrations and treatment durations identical to those used for the experiment illustrated in Figure 5.2. Each animal was transferred to freshly-prepared treatment jars daily. Treatment was continued for 16 days and the specimens were observed for another week. No ill effects were observed. One of the females was carrying embryos and these hatched normally. It is interesting to note that the in vitro MIC of malachite green is much lower that that of

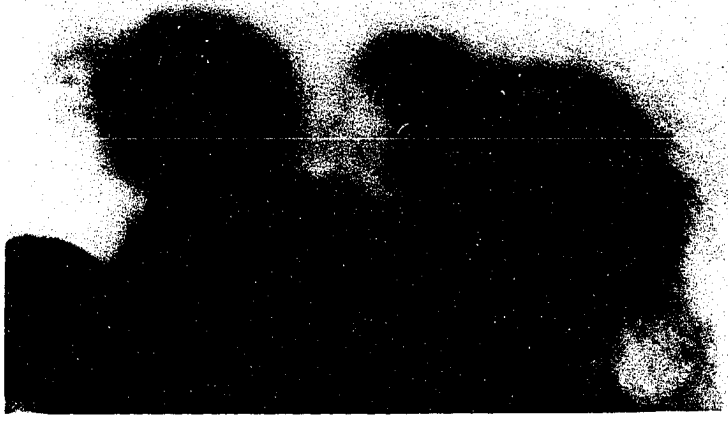
Figure 5.3

Embryos of *Palaemon macrodactylus* at different stages of experiment to assess effectivenes of 2,3-indolinedione as an antifungal agent against *Lagenidium callinectes*.

A) Embryos exposed to the fungus and treated with 2,3-indolinedione; X35.

B) Embryos exposed to the fungus without treatment with 2,3-indolinedione; X35.





2,3-indolinedione whereas the *in vivo* doses are comparable. The fact that malachite green is very soluble in water but 2,3-indolinedione is not, probably accounts for this discrepancy.

CHAPTER VI

THE CYANOPHYTE *MICROCOLEUS LYNGBYACEUS* (*LYNGBYA MAJUSCULA*) AND THREE ASSOCIATED STRAINS OF PIGMENTED BACTERIA

At the earliest stages of this research dozens of organisms were systematically examined for confirmation of our idea that the presence of bacteria with antimicrobial activities on higher organisms is a common occurrance. During one such 'census' in Puerto Rico, Dr. Luis R. Almodovar, Professor of Phycology at the Department of Marine Sciences, University of Puerto Rico, mentioned to me that many years earlier he and Dr. Burkholder had isolated a purple bacterial strain from a piece of *M. lyngbyaceus* mat, and that this strain had shown inhibitory activity towards several microorganisms tested. He further indicated that he had noticed that as the mats age, they show patches of purple and pink coloration and he suspected this may be due to the presence of bacteria. Even though cyanobacteria are prokaryotic organisms, from a physiological and ecological standpoint they function as filamentous algae (Humm, 1980), and thus I decided to pursue this lead and collected and processed several of these mats. Platings of *M. lyngbyaceus* filaments yielded several morphotypes of bacteria, among them three very characteristic for their coloration, purple, red and yellow. These three strains had not been (and were not since) isolated in preparations of any other samples, including sediments from the same areas.

Later it became obvious that they were always associated with M. lyngbyaceus, which suggested that this was indeed some type of symbiotic association.

There is a good amount of information about associations between algae and bacteria with indications that bacteria can actually choose a host (Kogure, 1982), perhaps by recognizing some factor on the host's surface (Imam, 1984; Lupton and Marshall, 1981). Algae may also affect their own bacterial flora by producing selective antibacterial compounds. The spectrum of antimicrobial activity of organic extracts of the same algal species has been shown to vary in as a function of the season and fertility periods (Pesando and Cavam, 1984). In some cases, there is a high degree of species-specificity, as illustrated by the requirement by cultures of an Oscillatoria species for three specific strains of bacteria that that colonize its surface. The three strains are stimulatory in any combination to only this host species and no other (De Lucca and McCracken, 1977). Although this and other examples (discussed in Chapter I) seem to indicate a transfer of useful factors from the bacteria to the host, the most accepted view is that bacteria benefit from the exudates of the alga and produce stimulatory factors that increase the transfer of nutrients. When algae enter a symbiotic association, generally the growth rate decreases while the photosynthetic rate remains unchanged, so that the excess carbohydrate is leaked to the symbiont (Smith et al.,

1969). Because algal surfaces are such nutrient-rich environments where competition for substrate may give rise to interesting strategies, Lemos et al., (1985), looked at the epiflora of five species of green and brown algae and tested the inhibitory activity among the components of the epiphytic community. Interestingly, they found 38 active strains, all pigmented, and all with very different antibacterial activity spectra.

M. lyngbyaceus is classified as belonging to the Oscillatoria, a group of filamentous cyanobacteria that do not form heterocysts (Humm, 1980; Papageorgiou and Packer, 1982). The individual cells associate pill-like (Fig. 6.1), surrounded by a gelatinous sheath of pectic materials and glycoproteins. As the cells divide by binary fision, they form elongated assemblages that resemble the filaments of ekukaryotic algae. Reproduction is by breakage of the filament, or trichome, into smaller daughter segments, known as hormogonia.

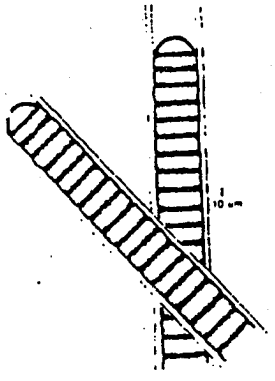


Figure 6.1. Diagram of Microcoleus lyngbyaceus filaments.

Cyanobacteria or cyanophyta or blue-green algae, are prokaryotes and their cell membrane and cellular organization place them without a doubt in the group of the bacteria. But, they also have the unique plant-like characteristic of being able to generate molecular oxygen as a by-product of photosynthesis. Their chlorophyll, rather than bacterial chlorophyll is Chlorophyll-a, the photosynthetic pigment also found in higher plants. The cells of the Oscillatoria group are also larger than bacterial cells and their filamentous arrangement allows them to grow in tufts and to form large mats with complex ecological zones of their own (Paerl and Prufert, 1987). Newly detached trichomes adhere to rocks, mangrove roots, dead coral and piers, and as the filaments elongate, they tangle and form crusty structures. As the mat grows, oxygen becomes trapped within the filamentous web and eventually the complete colony is torn from its stronghold and floats to the surface.

M. lyngbyaceus, devoid of heterocysts, was not thought to play a role in nitrogen fixation. It is, however, an extremely important and abundant component of the tropical ecosystem, particularly in shallow waters. Several researchers have hypothesized, and recently offer some evidence that this species does in fact fix nitrogen (Capone, 1983; Kallas et al., 1983; Pearson et al., 1979).

How this and other non-heterocystous species achieve oxygen exclusion to avoid inactivation of nitrogenase, is not quite understood. Some researchers have suggested that in some cases the nitrogen-fixing cells are located deep in the mat away from high concentrations of oxygen, as is apparent with Trichodesmium, a planktonic Oscillatoria (Bryceson and Fay, 1981). Other strategies may involve buoyancy permitting gliding out of high oxygen zones, or the secretion of thick mucilagenous sheaths where oxygen diffusion is slowed down. Biochemical adaptations may involve the production of high quantities of superoxide dismutase, or carotenoid synthesis which requires large amounts of oxygen. Others have suggested that the several bacterial associates observed to live on the sheaths (Gyurjan et al., 1984; Caldwell and Caldwell, 1978; Paerl, 1976) and concentrated around heterocysts, may play a role in harvesting molecular oxygen (Paerl, 1985; Paerl and Bland, 1982; Jones, 1982). A personal observation in this regard is that M. lyngbyaceus specimens collected from the roots of mangroves growing in 'bird islands' (islands where large numbers of egrets and pelicans come to rest after sundown), grow in very loose, hair-like, tufts. The concentrations of nitrogenous compounds around these islands are extremely high and presumably the cyanophytes would not be fixing nitrogen in these areas.

To the untrained eye, filamentous cyanophytes and filamentous algae are not easily discernible. It was

during the initial period of familiarization with these organisms, that I noticed that, under the microscope, filamentous algae could be seen to be colonized by a variety of diatoms, fungi (Kohlmeyer and Demoulin, 1973) and different types of cyanobacteria, whereas M. lyngbyaceus appeared to be largely free of any epibiotic organisms except, as it became obvious later under the electron microscope, a large number of bacteria. It was apparent that this might be indeed a case of symbiosis involving bacteria and a host which, in some functional and ecological aspects resembles eukaryotic filamentous algae and in others is unique. The first step was to confirm that the association occurs often and exclusively .

Table VI-1 shows the raw data on 62 different collections, indicating, site of collection, rough description of the specimen and presence of bacterial strains. The three pigmented strains were isolated from all the samples except for two that were stored overnight in a refrigerator before being homogenized and plated. The purple and red strains are highly sensitive to temperature. All three strains are rather slow-growing and do not thrive until the third or fourth transfer, which possibly indicates the presence of inducible enzymes. Both the purple and red strains also undergo bleaching, or loss of pigmentation under conditions that we have not been able to specify but which are related in part to the growth medium. Six different isolation media were used for the first

TABLE VI-1

Isolates from *Microcoleus lyngbyaceus*

Sample Origin	Pigmented Strains	Other
Site A: Eastern tip of Enrique reef, mangrove island, shallow lagoon		
1 thin, green veneer on shallow rock	P R Y	W
2 encrusted mat, bubbly, dark green/brown	R Y	W
3 small floating tufts entangled with *Cladophoropsis*	P R Y	W
4 mature mat, brown	P R Y	W
5 same as 4, red patches	P R Y	W
6 thin mat, yellow	P R Y	W
7 hard, old mat, black patch	R Y	W
8 same as 7, pink patch	P R Y	W
9 same as 7, yellow patch	P R Y	W
10 decomposing mat, yellow	P R Y	W
11 mature mat, wine color	P R Y	W
12 reddish, hair-like ecophene from mangrove root	R Y	
13 tuft growing on sand	P R Y	W
14 tuft attached to *Brachitrichia quojii*	P R Y	
15 mat attached to rocky bottom	P Y	
16 reddish, attached to *Thallassia*	P R Y	
17 red filaments, mangrove root	P R Y	
18 green filaments, floating in mangrove lagoon	P	
19 bluish-green, at 12 m	R	W
20 red, 12 m, attached to *Occulina*	P R Y	Z
21 dark, attached at reef crest	P R Y	
22 on mangrove sediment, dark	P R Y	
23 bluish-green, reef lagoon attached to plastic slab	P R	
24 red, attached to dead coral	P R Y	
25 red, on sand	R	
26 red, attached to tire	P R Y	
27 red, attached to mangrove root	P R Y	
28 greenish, on mangrove root	P R Y	L
29 bluish, tangled with filamentous chlorophyte	P R Y	
30 bluish, on *Thallassia*	P R Y	
31 reddish, on mangrove root	P R Y	
32 green tuft	P R Y	E

Table VI-1 (contd.)

33	brown crust, forereef, 6 m	P					
34	bluish crust on Cladophoropsis	P					
35	red, mangrove root	P	R	Y			
36	red, on Acantophora	P	R	Y			
	Site B: Caracoles, shallow mangrove island						
37	reddish tuft	P	R	Y			
38	gree-red filaments						
	Site C: Collado, westernmost reef, mangrove island, pristine deep backreef lagoon with continuous inflow from front reef						
39	green, on mangrove roots	P	R				
40	red filaments	P	R				E
41	green filaments, on sediment		R				
42	red, flimsy crust	P	R	Y			
43	shiny green	P	R	Y			W L
	Site D: bird islands, dense mangroves on first row of reefs, closest to shore						
44	reddish	P	R	Y			
45	greenish-red, on mangrove root	P	R	Y			
46	red	P	R	Y			W
47	deep red	P	R				
48	red	P	R				
49	large red floating mass	P	R	Y			
50	red, at La Gata island	P	R	Y			
51	red, at Naida's pier tire	P	R	Y			L
52	tank, wet tables			Y			L
	Site E: Car Reef, outer chain, 20 m						
53	blanket on sandy bottom	P	R	Y			
54	reddish at reef base	P	R	Y			Z
55	reddish, on Telesto	P					
	Site F: Turrumote lagoon, outermost reef chain, enclosed lagoon with shallow reefs and zooanthid beds						
56	red		R	Y			
58	reddish, on Cladophoropsis	P	R	Y			Z
59	red, on Halimeda	P	R	Y			
60	very small filaments, red	P	R	Y			
61	pink-green (stored in refrig.)						swarming tan
62	green "						swarming tan

collection at site A (Enrique reef lagoon). Since, in almost all cases, the standard sea water medium proved to be the most convenient, only this medium was used for all subsequent collections. The data indicate that: 1) All the specimens processed (except the two ones kept in the refrigerator) yielded the pigmented strains; 2) all three pigmented strains were isolated from 80% of the specimens, the remaining 20 % yielding two or one of the strains only.

Scanning electron micrographs (Fig. 6.2.A and B) showed that the bacteria occur in patches of varying density on the outer surface of the trichome. Transmission electron micrographs (Fig. 6.2.C) confirm that the bacteria are attached to the mucilagenous material surrounding the trichome. H. W. Paerl, from the University of North Carolina, recently studied a local Lyngbya species which shows large concentrations of sheath-associated bacteria where free oxygen was excluded (Paerl and Prufert, 1987). He has not isolated the associated bacteria (pers. comm.) but it is tempting to speculate that the ubiquinone-related compound produced by the strains we isolated bay not only be antimicrobial, but may play a role in oxygen exclusion as well.

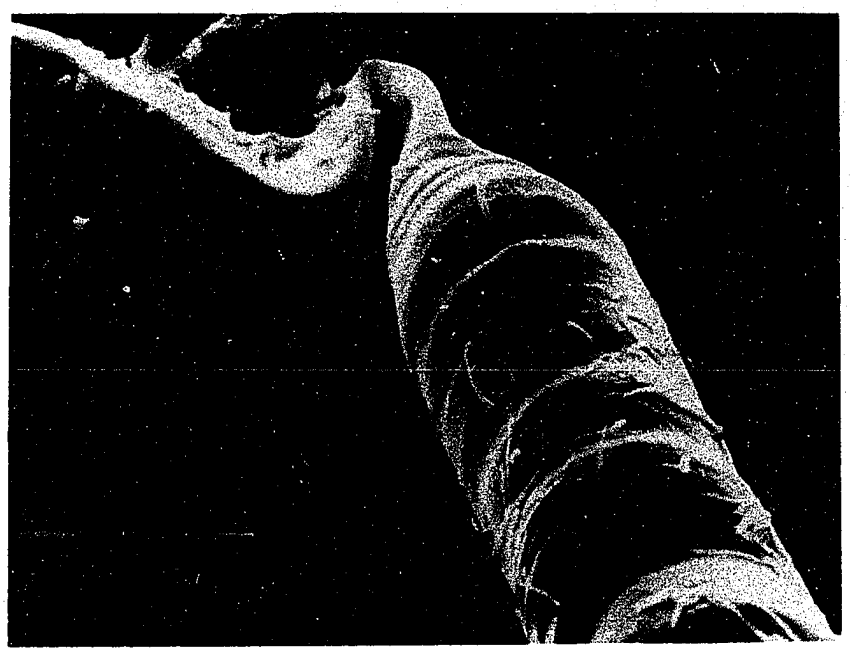
Table VI-2 shows the antimicrobial activities of the three strains, P (Purple), R(red) and Y(yellow), against a set of bacteria and fungi isolated from sediments and water in the same collection sites. Other characteristic strains were repeatedly isolated from the same specimens but in much

Figure 6.2

Electron Micrographs of *Microcoleus lyngbyaceus* filaments and associated Bacteria.

A) Filament sheath covered sparsely by different bacterial strains, X2000.

B) Filament covered heavily by bacteria. Filament on the background with epibiotic diatoms is from a filamentous alga; X 250.



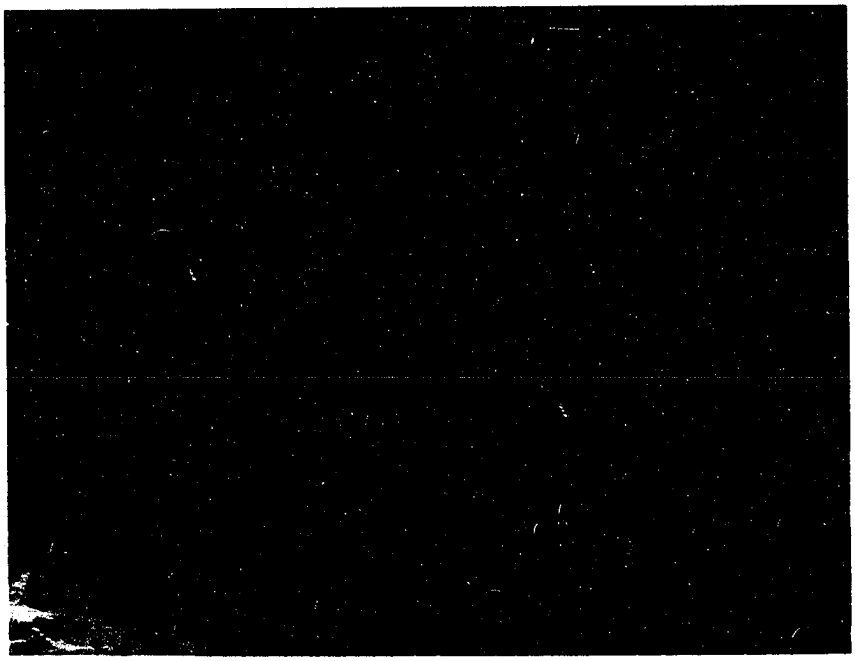


Fig. 6.2 (contd.)

C) Transmission electron micrograph showing bacteria embedded on the mucilaginous sheath; X7000.

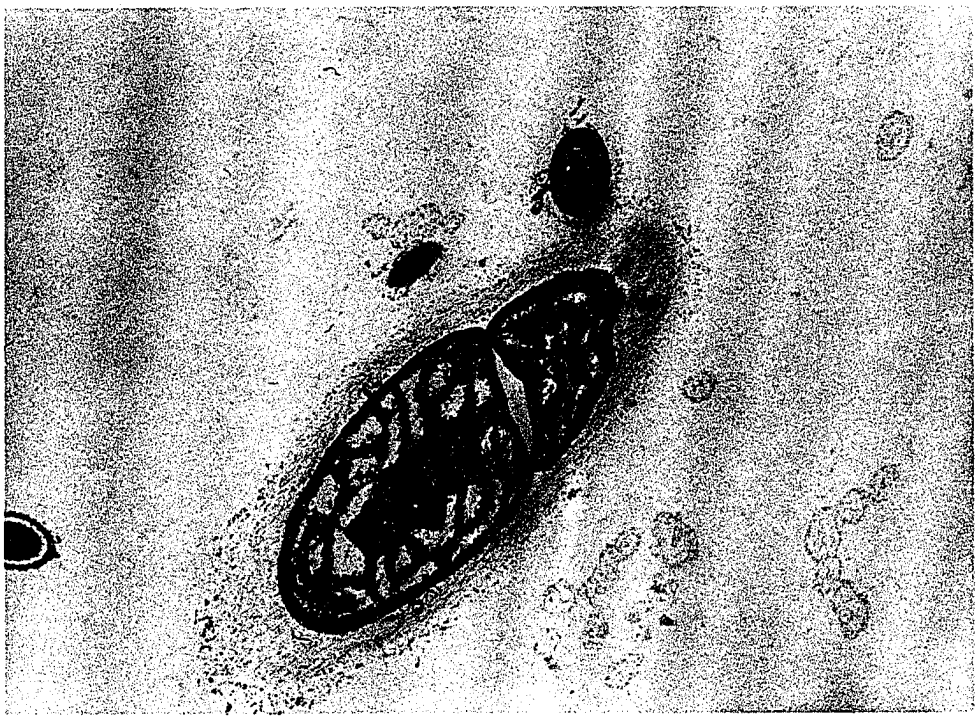


TABLE VI-2

Activity of bacteria isolated from Microcoleus lyngbyaceus against five strains of bacteria and four types of fungi.

Strain Tested	B1	B2	B3	B4	B5	F1	F2	F3	F4
P	+	+	--	--	--	+	+	+	+
R	+	+	+	--	+	+	+	+	+
Y	+	+	+	--	+	+	+	+	+
W	+	+	+	+	+	--	--	--	--
Z	+	--	--	--	--	+	+	+	+
L	--	+	+	--	--	--	+	+	--
Be	+	+	--	+	+	--	--	--	--

+ inhibition of culture by test strain on agar plates
-- no inhibition of culture by test strain on agarplates

lower frequency.

Experimental Conditions

Culture Conditions: Batch culture of strains B and R was difficult and erratic: pigmentation was often lost, yields of crude extract were quite variable and agar cultures often had to be transferred several times before they would yield a vigorous growth in liquid medium. Strain 'Y', behaves in an overall more consistent manner and is a hardier strain under our growth conditions. Strains B and R had to be grown in separate tubes of liquid standard sea water medium for several days before purple and red pigment was visible and only then could the 16-liter carboys be seeded successfully. All three strains were grown for two to three weeks and final pH was consistently between 8.2 and 8.5 The yield of crude extract varied from 500 mg to 1000 mg per carboy. The red strain was generally the most productive strain. The proportion of active compound to crude extract was similar in all three strains, approximately 20 mg per gram of crude extract.

Isolation of Active Compound: The active fraction in all three extracts was identified by testing against the fungal strains. The growth medium was extracted twice with ethyl acetate. The initial separation was done in a silica column with a series of ethyl acetate/isooctane eluants ending with ethyl acetate/methanol. The active factor was found mainly in the 50% ethyl acetate/isooctane fractions.

Further purification was by HPLC with a 50% ethyl acetate/isooctane solvent. This fraction comes out as one single peak with an Rf of 0.45 on TLC developed with diethyl ether, and has a characteristic stain pattern with H_2SO_4/vanillin, showing a yellow core surrounded by a halo with strongly absorbs ultraviolet light. The NMR spectrum, showed that there were two compounds in the fraction and these were finally separated by reverse-phase HPLC with 75% methanol/water as eluant. The active compound, produced by all three strains of bacteria, is an oil, deep orange in color, and it is produced in moderate amounts by the strains under our culture conditions.

Characteristics of Active Compound: deep orange oil; HRMS: requires 320.11 for $C_{18}H_{24}O_5$, found 320.16; M^{+2}: 322.1712; IR ($CHCl_3$): 1710, 1645 cm^{-1}; UV (MeOH): max= 274 nm; ^1H NMR, 360 MHz ($CDCl_3$): δ 4.9+(t, 1H), 4.1(s, 3H), 4.0(s, 3H), 3.2(d, 2H), 2.4(t, 2H), 2.1(s, 3H), 2.0 (s, 3H), 1.9(t, 2H), 1.7 (d, 3H), 1.6(m, 2H); ^{13}C NMR, 200 MHz($CDCl_3$): δ208.3, 184.6, 183.8, 144.7, 144.7, 141.6, 138.9, 119.9, 61.0, 42.9, 38.9, 29.7, 25.3, 21.9, 16.0, 11.8. The structure of the isoprenoid chain was determined by ^1H NMR decoupling experiments: irradiation of proton at 4.9 ppm (C11) (Fig. 6.3) resulted in a singlet at 3.2 ppm (C10); irradiation of protons at 2.4 ppm (C15) resulted in a triplet rather than a multiplet at 1.6 ppm (C14); protons at 1.9 ppm (C13) are coupled to protons at 1.6 ppm; irradiation of protons at 1.6 ppm resulted in two singlets

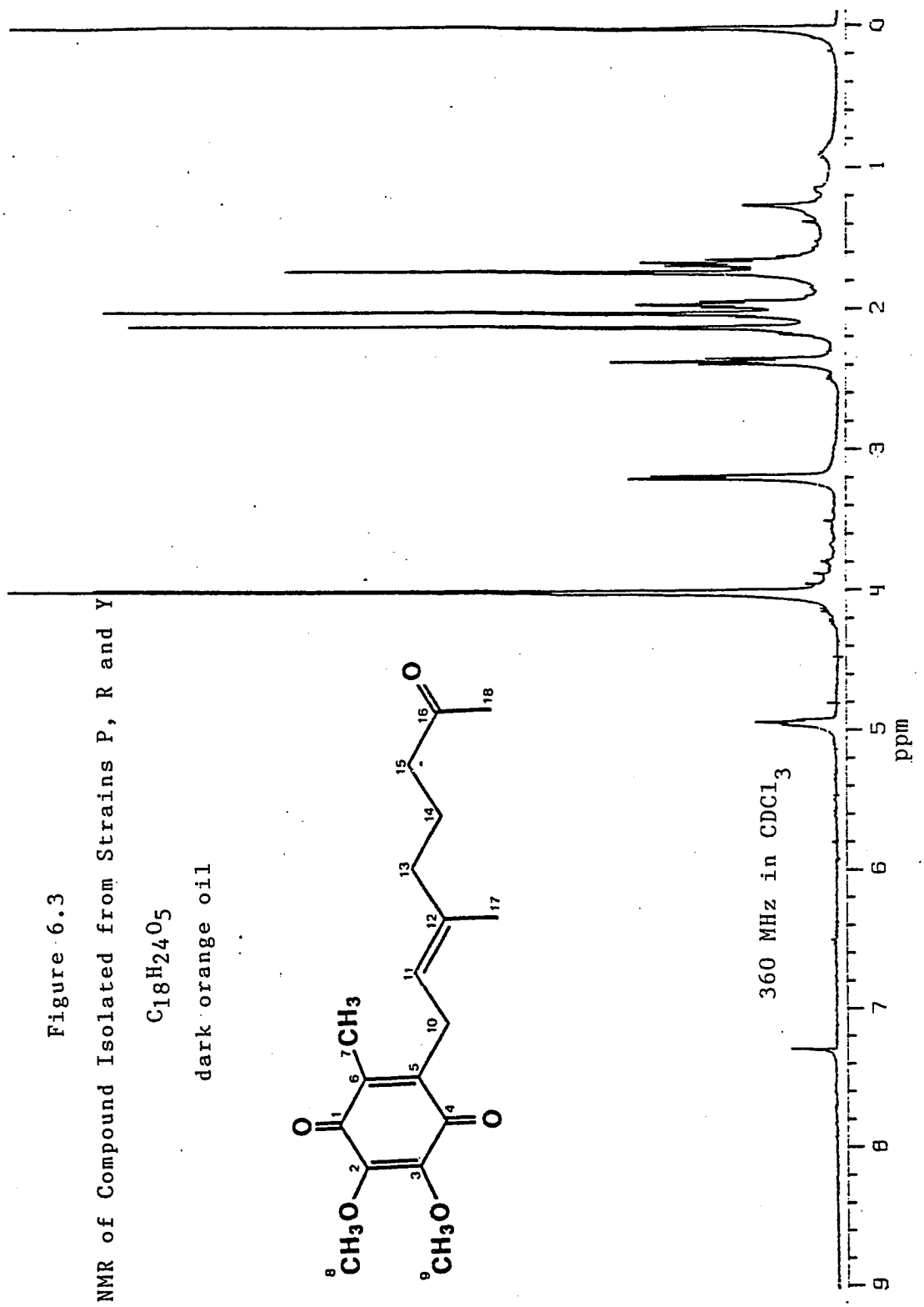


Figure 6.3

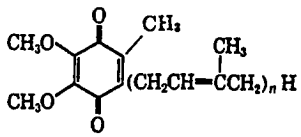
1H NMR of Compound Isolated from Strains P, R and Y

$C_{18}H_{24}O_5$

dark orange oil

at 1.9 and 2.4 ppm. Upon irradiation of protons at 3.2 ppm (C10) an NOE was observed at 2.1 ppm (C7). IR absorption at 1645 cm^{-1}, and the presence of a M+2 signal in the high resolution mass spectrum, confirmed that the ring system was a quinone. UV absorption at 274 nm and the NOE between protons at C10 and C7 allowed us to assign the positions of the methoxy and methyl substituents on the quinoid ring.

This compound is structurally related to the ubiquinones (I). It has not been isolated as a natural

(I) 

product and it is not reported in the literature either as a derivative of natural or of synthetic products. Ubiquinones play a major role in the respiratory electron transport localized mostly in the mitochondria of eukaryotic cells (Thomson, 1971; Luckner, 1984). In cell respiration, they function by attaching to the lipid portion of membranes by the hydrophobic side chain, while the quinoid ring easily undergoes oxidoreduction. Ubiquinones with polyprenyl chains shorter than 5 isoprene units occur in extremely low concentrations, however, and can usually be detected only by light spectroscopy (Tamoaka, et al., 1985; Daves et al., 1967). Thomson (1971) and Rose (1968) suggest a biosynthetic pathway for this type of compounds in microorganisms where mevalonate is the precursor of the prenylated side chain and methionine contributes the nuclear

methyl group. Studies of redox reactions of physiological quinones (Futami et al., 1978) show that ubiquinones with side chains of less than five isoprene units are extremely poor catalysts in electron transport. The biological activity of derivatives of other types of quinones is well known and several of these compounds have been isolated from marine organisms (Frincke and Faulkner, 1981; Gerwick and Fenical, 1981; Howard et al., 1979; McIntyre, et al., 1979; McEnroe and Fenical, 1978; Cimino, et al., 1972; Thomson, 1971) but there are no reports of naturally occurring biologically active ubiquinone derivatives.

Electron micrographs: Specimens for SEM were prepared as follows: fixation in 2% glutaraldehyde in filtered sea water for several days; rinsing in sterile filtered sea water six times; treatment with 1% osmium tetroxide for 1 hour on ice; rinsing in filtered sea water; dehydration with an ethyl alcohol series to Freon. The specimens were then critical-point dried under Freon. The procedure was similar to that described in Chapter V for the lobster embryos. Strands of M. lyngbyaceus were mounted on specimen stubs by first placing adhesive tape on the stubs and then lightly touching it to the filaments. Gold coating thickness was approximately 300 A.

Preparation of samples for TEM was as follows:

- Very small portions (0.5 mm diam) of six different specimens were cut off.

- Specimens were fixed separately in 3%

glutaraldehyde in sodium cacodylate/sea water buffer with a few drops of Ruthenium red solution.

- Each specimen was rinsed in buffer 3 times for 10 minutes each time.

- Samples were postfixed with 1% osmium tetroxide in seawater buffer with Ruthenium red.

- Subsequent dehydration was done with a series of 50%, 60%, 70%, 80%, 90% and pure ethyl alcohol, and finally three changes of propylene oxide.

- Resin and activator for mounting plastic (hard) were mixed according to manufacturer's (Pella) instructions and stored in freezer.

- Each fixed specimen was transferred to vials containing liquid mounting plastic. The vials were rotated every few minutes for a few hours and carried around for the next day, as there was no mechanical rotator available.

- Next day, each specimen was transferred to vials with freshly prepared mounting plastic. The vials were rotated every few minutes for three hours.

- Specimens were placed in individual wells of a silicone rubber mold, carefully removing bubbles. Small paper labels were also immersed in the resin.

- The mold was placed in a makeshift oven consisting of a can filled to 1/3 its volume with stones and placed on an electric stove with a cover made with aluminum foil.

- Back in Dr. Holland's laboratory, thin sections were cut with glass and diamond knives, mounted on copper

grids and stained with uracyl acetate and lead citrate

The micrographs were done at the analytical facilities in the UCSD Biology Department.

CHAPTER VII

CONCLUSION

The research described in the preceeding chapters represents not so much a new area in the study of nature as a new way to answer an old, unsolved question. The most difficult step in any area of microbiology is to establish the link between *in vitro* and *in vivo* facts. In 'modern' science we work based on the immutable assumption that every organism has an ecological *raison d'etre* (Alexander, 1971), and that each organism possesses a unique combination of characteristics that explains its existence in a particular environment. All biological characteristics, when analyzed at the level of their basic components, are reduced to chemical interactions, from the most recondite cellular function to complex exchanges among different members of a community. Larger organisms can be observed in nature, collected and analyzed in many different ways and, once each measurable element has been dissected, hypotheses can be formulated which can be studied. Some organisms are better known than others or are more amenable to laboratory culture or observation in nature, which make different strategies in research methodology necessry. In general, however, there is a clear philosophy of research and a fairly good concensus regarding the approach to understanding an organism's effect on its environment and vice versa. The world of microbes, on the other hand, has remained an

unsolved mystery, where the dichotomy between laboratory observations and microbial effects on the environment seems inssuperable. To quote Martin Alexander (1987):

> The point of focus for the biochemical ecologist is nature and not the laboratory, the characteristic environments where the species grows and affects its surroundings and not the artificial conditions imposed on the isolate, which is pampered or mishandled -depending on the investigator's propensities- to perform this or that reactions. The biochemical ecologist is an <u>in vivologist</u> applying chemistry to explain the functions of an organism in nature or its restrictions to given regions, not an <u>in vitroologist</u> attempting to describe the functioning of an organism in isolation. Granting that the laboratory is the chief professional home of the microbial ecologist ant that he may, in fact, deal with axenic cultures, his efforts are nonetheless directed to problems in natural habitats and to accounting for phenomena in heterogeneous communities.

Even though an organism's ecological position is indeed determined by a set of properties, it is necessary to isolate each one in order to perform systematical studies that will result in information sufficiently detailed to be manipulated in the process of exploring working hypotheses. Undoubtedly, bacteria have a variety of properties that determine their position in given habitats in relationship to all other organisms. Size, extremely short generation times, metabolic flexibility and the ability to acquire genetic material from different cells, makes it most likely that a bacterial strain's most important competitors will be other microorganisms and that the diversity of interactive strategies will be largest against bacteria and fungi sharing the same habitat. Direct observation of these

activities is so far impossible, but the tendency of bacteria to associate with larger or more organisms provides an invaluable observation platform, where these processes become magnified to the size of the host organism. It makes great adaptive sense to form an alliance with organisms that do not exert great physical demands but provide protection against a complex group of potential pathogens, thus allowing the host to concentrate its defense metabolism budget on a more manageable number of predators and competitors. Once such a relationship is identified, the bacterial processes can be studied with the usual methods by observing how the absence or modification of its bacterial associates affect the host.

All three examples discussed in this dissertation were chosen purposefully for certain characteristics that made the host organims stand out with relation to other organisms as particularly resistant to microbial encroachment or infection, and because they were found to be associated in a consistent manner to a specific population of bacteria.

Embryos of the shrimp Palaemon macrodactylus are resistant to a pathogenic fungus that quickly overcomes larvae, juveniles and even adults of the species. The embryos are most likely highly desirable substrate for microorganisms and, furthermore, they must spend approximately 17 days without any apparent protection from the numbers of microorganisms being washed over their

surface. Larvae, juveniles and adults, on the other hand, have the benefit of frequent moltings, mobility, many appendages to clean their surface, and yet they rapidly succumb to this invasive pathogen. The fact that embryos treated with penicillin lose their resistance to the pathogen and the consistent isolation of penicillin-sensitive bacteria from their surface with *in vitro* antifungal activity, provided the first clue that the extraordinary resistance to the fungal pathogen may be mediated by bacteria. Two strains of bacteria seem to be involved, one possibly slightly dominant. Both strains produce antifungal compounds under laboratory culture conditions. When the embryos are experimentally cleaned of their natural penicillin-sensitive bacterial flora and exposed to the fungal pathogen, they rapidly become diseased and die. Embryos from the same clusters, exposed to identical treatment but either reinfected with pure cultures of the active bacterial strains or the major antifungal compound, are protected from the fungus at comparable levels. Some penicillin-treated embryos were also infected by pathogenic bacteria. The active compounds isolated from both strains studied were inhibitory to a number of bacterial strains collected in the Bodega Bay area. The spectra of activity of the two strains against the test bacteria were quite different, which suggests that antimicrobial health is probably achieved in this case by a

complex interaction of the different members of the epibiotic bacterial flora.

In the case of the American lobster embryos, evidence for an exclusive host-symbiont relationship is quite strong. There is a tight correlation between the amount of coverage of the embryonic coat by one characteristic bacterial strain and the successful development of the embryos throughout their long brooding period of more than nine months. As the embryos mature, those that remain healthy are gradually covered by an extremely thick veneer of the bacteria. It is likely that these bacteria produce other inhibitory compounds or provide other types of antimicrobial protection, as the embryos are seemingly invulnerable to colonization by microorganisms, with the exception of the filamentous bacterium Leucothrix mucor.

Finally, for Microcoleus lyngbyaceus, the two elements that more strongly support the model proposed in this thesis are the obvious absence of epiphytic fungi and protozoans on the filaments and the undisputable presence of three characteristic bacterial strains in all of the specimens studied and nowhere else. These results, however, raise a number of puzzling questions beyond the scope of this thesis. For example, the production of the same compound by all three strains with antimicrobial activity seems redundant, unless the compound has also other functions so essential to the host that selection for the

establishment of the association excluded non-producers of this particular compound.

An unexpected by-product of this research has been the possible applications of the isolated compounds as therapeutic and preventive agents in microbial diseases. The fact that the first observations steered the work in the direction of organisms that are of interest to aquaculture, provided the initial impetus for the experiments performed, and serendipity added the remaining elements, when it was found that the antifungal agent most commonly used, is a human carcinogen. Researchers from five different laboratories have expressed interest in testing the compounds we isolated and they are now experimenting with several microbial pathogens of crustaceans and fish. Rapid worldwide advances in mass-cultivation techniques and equipment and the advent of genetic broodstock modification, have brought aquaculture to its apogee. Control of microbial diseases, however, remains one of the major problems and an area where little progress has been made. Ecological and health concerns and the problem of resistant strain promotion have made the control of microbial diseases in aquaculture a major issue. Industry also recognizes the need to develop products that will comply with stricter safety standards. Attitudes are clearly changing so that the former potent, wide-spectrum, long-lasting antibiotic agents will be replaced by highly specific, mildly active

and easily degradable control agents.

Eventually we may come to accept that it is in our interest to observe nature and, not only analyze its comoponents, but pay closer attention to its regulatory mechanisms. In the field of microbiology in particular, the interaction of these two approaches is essential to understanding any natural process. In fact, in the microbial world, ecology without chemistry offers no answers and chemistry without ecology poses no questions.

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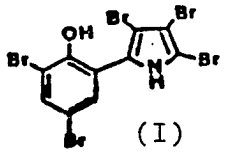
APPENDIX A

Known Antimicrobial Compounds Produced by Marine Bacteria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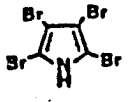
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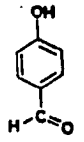
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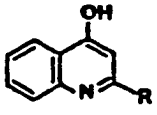
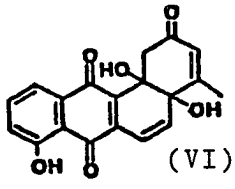

R=C_5H_{11} (IV)
R=C_7H_{13} (V)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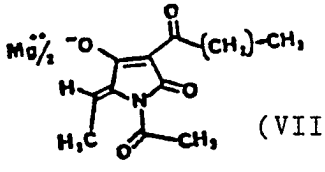
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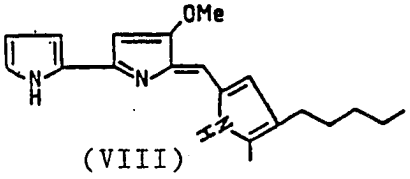
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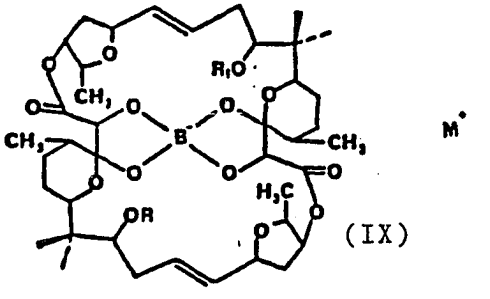
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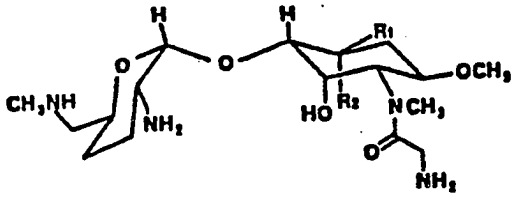
R_1=NH_2
R_2=NH_2 (X)

R = R_1= H aplasmomycin A
R = Ac, R = H aplasmomycin B
R = R_1= Ac aplasmomycin C