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On the Symbiotic Relationship between *Porphyra* Species and Attached Bacteria, and a Bacterial Pathogen in White Rot

Jun-ichi TSUKIDATE

Abstract

The thesis deals with the symbiotic relationship between *Porphyra* species and attached bacteria in conjunction with the white rot which has caused the most serious damage to the industry of *Porphyra* cultivation in Japan. This study made clear for the first time the fact that a bacterium identified as *Beneckea* sp. was one of pathogens in the white rot of *Porphyra*. The host-parasitic interaction in the outbreak of the white rot was revealed after pathological and physiological studies had been made on a bacterial pathogen, *Beneckea* sp. The following results were obtained in the present study and the basic knowledges were provided to foster the healthy lavers in the *Porphyra* farming, resulting in the increasing yields in its industry.

1) A bacterial flora was investigated on the healthy thalli of *Porphyra* grown both in laboratory and on the rocks or the nets in the sea. The composition of microflora was determined according to the scheme of Shewan *et al.* *Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* were isolated from the healthy *Porphyra* thalli *in vitro*. Of these, *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* were often isolated. *Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas*, *Aeromonas* and yeast were isolated from the healthy *Porphyra* thalli grown on the rocks or the nets in the sea. Of these, *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* were often isolated. Therefore, it was concluded that *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* were dominant parasitic bacteria on *Porphyra* thalli.

2) An epidemiological survey of the pathogenic bacteria was periodically carried out with the *Porphyra* grown on the rocks or the nets in the sea in 1973, 1974 and 1975 fiscal year of the *Porphyra* cultivation. In any fiscal year, *Beneckea* sp. was isolated from *Porphyra* thalli whenever red and white cells increased in number on its thallus surface, resulting in the deterioration of lavers and the subsequent occurrence of the white rot.

3) In order to prove causality, healthy *Porphyra* thalli which were cultured *in vitro* were

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suspended in the medium inoculated with *Beneckea* sp.. After bacterial inoculation, the host always deteriorated and caused the white rot. Beneckea sp. was reisolated from the above deteriolated thalli at a ratio of 95% and 100% in one and other four cases out of five experiments, respectively.

4) Investigation on the growth of *Beneckea* sp. and associated bacteria which were isolated from the host plants, *Porphyra* species, indicated that *Beneckea* sp. grew well at 20℃ and with some amino acids like aspartic acid and glutamic acid. In addition, it was found that there existed different nutritional requirement between *Beneckea* sp. and other parasitic bacteria.

5) From the view point of nutritional relationship between *Porphyra* and attached bacteria, extracellular products of *Porphyra* were analized and identified as organic matters and amino acids using labelled carbon and a Beckman 121 amino acid analyzer, respectively. It is the first time that amino acids are detected in the culture medium composed mostly of inorganic nutrients at the end of incubation of *Porphyra*.

6) It was indicated that the growth of bacteria attached to *Porphyra* thalli were stimulated by providing amino acids. The release of carbon dioxide was also ascertained in the culture medium of bacteria using labelling carbon. However, sorts of utilized amino acids and amount of released carbon dioxide were different among bacteria at generic level.

7) *Porphyra* was tested for its ability to grow in axenic culture when organic nutrients and carbon dioxide were provided. As a result, no growth of *Porphyra* was observed in culture medium containing any of the following substances: amino acids, vitamines, nucleic acid and plant hormons. On the contrary, carbon dioxide, in the presence of twice as much as those of artificial seawater used in the present study, supported the normal growth of *Porphyra*.

8) In summary, *Porphyra* grew well in the presence of carbon dioxide released by attached bacteria and amino acids as extracellular products of *Porphyra* stimulated the growth of bacteria. Above results indicate that the symbiotic relationship exists between *Porphyra* and attached bacteria. *Porphyra* became unhealthy when oceanographic and meteorological conditions change and may exudate amino acids which are readily available for *Beneckea* sp. It seems justifiable therefore that this nutrirional interaction leads to the vigorous growth of *Beneckea* sp., resulting in causing the white rot.

Contents

Chapter 1. General introduction

Porphyra tenera KJELLMAN is the seaweed most commonly used for food in Japan. *Porphyra* farming has been best accomplished by some of the most sophisticated techniques used in seaweed mariculture. Recently the annual crop has attained a level of 10 billion sheets of dried laver. However, *Porphyra* farming is influenced by meteorological and oceanographic conditions, and productivity is still far from constant. In addition, diseases have caused a deterioration in quality as well as low production. *Porphyra* is cultured in limited areas of protected bays, so microorganisms like diatoms, fungi and bacteria are more apt to attach to *Porphyra* than to seaweeds on exposed coasts. A problem in *Porphyra* farming is also pollution by industrial and domestic wastes.

Many researchers have attempted to solve the problems of disease in *Porphyra* farming by studies of epidemiology, physiology and bacteriolgy. Infectious and non-infectious diseases occur in both macrothallus and conchocelis stages. SUTO *et al.* (1972) gave a general review of diseases in *Porphyra* farming in Japan. According to them, red rot, chytrid blight and tumour were classified as infectious diseases, but bud blight, chill blight, diatom felt and white blight were regarded as non-infectious diseases.

Red rot is caused by *Pythium porphyrae* n. sp. (TAKAHASHI 1970, TAKAHASHI *et al.* 1970). The first symptom is a bluish green colour of the thallus. Finally a red ring appears around part of the bluish green area (ARASAKI, 1974, SASAKI and SATO, 1969). Data on the pathogen life cycle, host-parasite interaction, pathogenesis and epidemiology have been accumulated for red rot (ARASAKI 1947, SASAKI and SATO 1969). TSURUGA and NITTA (1960) reported a relationship between the pathogen, seawater temperature, salinity, and nutritional value of the thallus. ARASAKI (1962) took measures to prevent the spread of the disease according information on these relationships. Biochemical characteristics of the pathogen were investigated by ARASAKI *et al.* (1968), SASAKI and SAKURAI (1972), KATO *et al.* (1973a, b). FUJITA and ZENITANI (1976, 1977a, b, c). and FUJITA (1978).

The chytrid blight is caused by an unidentified Phycomycetes. The infected thalli become dark red. As the disease becomes serious, the colour of the plant changes to greenish white beginning at the upper end of the thallus. (Kumanoto Prefecture Laver Research Laboratory, 1969; Saga Prefecture Aquaculture Research Experimental Station (1969). ARASAKI (1960) and MIGITA (1969) investigated the morphology of the pathogen and the parasitic ecology in the host plant and showed the mechanism by which the pathogen invaded the cells. They attempted but failed to grow the pathogenic fungus in artificial media. ARASAKI *et al.* (1960) and MIGITA (1969) described the symptoms of the chytrid blight in infected *Porphyra* collected

by fishermem and the staffs of several prefectural fisheries experimental stations. The chytrid blight occurs widely in *Porphyra* farms infected by red rot.

The shot hole disease was first reported by SUTO and UMEBAYASHI (1954). Pinholes made by mechanical stimuli such as fine sands are apt to become as large as 1-4mm in diameter when the salinity is reduced. This disease is characterized by perforations in wellgrown thalli in November and December. It often occurs near river mouths when sands are discharged by flood waters right after a heavy rainfall. NOZAWA and NOZAWA (1955, 1957) succeeded in causing the shot hole disease experimentally. In farms where the salinty was reduced as much as 4-8%, they found disordered plants with the same symptoms as plants in their laboratory. Typical symptoms developed on plants growing at salinities of 6-8%. Symptoms of the shot hole disease were described by ARASAKI (1956) as follows. Small red spots spread over the thallus surface and become small holes afterwards; although the diseased thalli do not decay, their growth occasionally stops; when the salinity of the ambient sea becomes normal, the plants grow again; in advanced stages of the disease, parts of the plant become reddish purple and greenish, and groups of dead cells dot the thallus surface; ultimately holes form when the dead cells are lost.

Tumors are the result of abnormal cell divisions enhanced by deleterious chemical substances contained in industrial effluents. The symptoms of this disease are crape like upheavals induced by proliferating vegetative cells. The thalli damaged by tumors stop growing and become yellowish. The occurrence of tumors in a *Porphyra* farm was reported by FUJIYAMA (1957a, b) for the first time. He investigated the anatomy, cytology and physiology of the tumrs on *Porphyra tenera* and assumed that unknown microsubstances originating from the industrial effluents were responsible for the tumors. The substances causing tumors were studied by KAWABE and MIZUNO (1963), KAWABE *et al.* (1972), ISHIO *et al.* (1972), WATANABE and KATO (1972). KATO and WATANABE (1972) measured the amounts of DNA in the diseased cells, and KATO (1972) described the finestructure of these cells.

A bud blight develops often on thalli which are less than 1-2 cm long and believed to be physiological disorder. This disease occurs at farms which are offshore and sometimes also at the mouths of bays, and may spread extensively to the whole cultivation area. Smaller buds are more susceptible to the disease than larger buds, and even several-celled germlings are often damaged. The symptoms of this disease are dullness and a reddish discoloration of a part of on the entire thallus, a distortion at the tip of the bud and a weakening of its base. The damaged plants are stunted or stop growing and finally wear away. Even when the plants heal they remain distorted and contain abnormal cells and chromatophores. The causes of this disease and the cultural manipulation needed to control it were studied by YAMAZAKI *et al.* (1956), AKASAKA (1956), TAMURA (1956) and ARASAKI (1956).

The chill blight is a physiological disorder occurring when thalli are exposed to cold wind for long times during ebb tides. Damaged parts of thalli become reddish brown at first and at more advanced stages they become polygonal or dendroid. Microscope examination shows that the cells are reddish violet in color and that the plastids shrink, resulting in discoloration. Finally the cells loose their contents and appear as large cells with vacuoles (AKASAKA *et al.* 1961).

when a diatom falt densely covers the thallus surfaces, *Porphyra* decays and stops growing (SUTO *et al.* 1972). OKAMURA (1909) reported that the diatoms were responsible for dense floras of bacteria living in the mucus substances secreted by attached species of *Synedra* and *Licmophora* on the surfaces of *Porphyra*. In fact, the existence of a mucus-like substance can be ascertained on rotted areas of *Porphyra* containing dead cells. *Synedra* grows rapidly in a low salinity, and *Licmophora* in a high salinity. The explosive increases of both diatoms have often caused serious demage to *Porphyra* farming. One example of the low yield resulting from attack by *Synedra* was reported from Korea long ago (Chosen Sotokufu Suishi 1934). Recently, the destructive damage caused by *Licmophora* was reported in the western part of the inland sea (Yamaguchi Prefectural Naikai Fishery Experimental station 1972). In the latter case, the blooming of *Licmophora* occurred three months earlier than usual (November and December) and was thought to be due to abnormal stagnant water. General speaking, a diatom felt is apt to develop on unhealthy thalli growing in unfavourable environments (SUTO *et al.* 1972).

The white blight is also a well known physiological disorder. The diseased thalli rot after they turn whitish or reddish. There are many references to white blight in early and advanced stages and the serious economic damages caused by the disease in various areas of Japan (Fukuoka Prefecture Ariake Fisheries Experimental Station 1968, Fisheries Agency 1967; Miyagi Prefecture Fisheries Experimental Station 1968, Kesenuma Branch of Miyagi Prefecture Fisheries Experimental Station 1968, Chiba Prefecture Naiwan Fisheries Experimental Station 1968). Pathological studies were made on *Porphyra* affected by this disease by WATANABE and KATO (1970), KATO *et al.* (1970) and KATO and WATANABE (1971). The initiation mechanism of white blight was studied by WATANABE *et al.* (1968) and YAMASHITA (1969).

Of all these diseases in *Porphyra* farming, red rot and white blight are of the greatest econmic importance and occur extensively somewhere in Japan every year, resulting in serious problems in the laver industry. Though the casual organism in red rot is known to be *Pythium porphyrae*, white blight is considered to be caused by several unusual environmental factors such as too little exposure to the air and lack of sunlight, and protective measures have not been fully established yet, though some attempts have been made to reduce the damage.

The present thesis constitutes a series of the author's studies of physio-nutrition (TSUKIDATE 1968, 1971a, 1974b), pathology and bacteriology (TSUKIDATE 1970a, c, 1971b, c, 1973a, b, 1974a, 1977b) and especially deals with the symbiotic relationship between *Porphyra* species and attached bacteria in conjunction with the white rot disease (TSUKIDATE 1977a).

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Chapter 2. History

The first report of white blight causing serious damage to the *Porphyra* industry in Japan was made under the name of "Hakufu-byo" by ARASAKI (1947) in which he described detailed symptoms on the basis of an epidemiological study on the Pacific coast of central Japan. According to him, the diseased thalli discolor to reddish or whitish from their apices downwards and ultimately detach from "Hibi", (floating nets for cultivation) as a result of shrinkage and deterioration leading to weakened holdfasts. In the cells the starlike chromatophores deform and the protoplasts discolor to violet or whitish. The white blight occurs from late November to late December and becoming serious especially when the water temperature remains high and rains persist. The disease develops mainly on well grown thalli in the lower parts of "Hibi", while small thalli in the upper parts are usually healthy.

The Miyagi prefecture Fisheries Experimental Station reported *Porphyra* with symptoms similar to "Hakufu-byo" or "Shirogusare-byo" (ARASAKI 1947, 1956) but differing in the season of its occurrence and the environmental factors inducing the disease. They also called it "Shirogusare-byo", and found that it occurred around the neap tide during October and

November. The meteorological conditions responsible for the disease are considered to be: (1) wind calm during low tide; (2) air temperature dropping below dew-point; (3) low air temperature and strong wind or (4) an increase of seawater pH under the environmental conditions mentioned in (1)to (3) (Fisheries Agency 1965).

Data on another type of "Shirogusare-byo" was given by Chiba Prefecture Naiwan Fisheries Experimental Station (1947, 1956) and the Research Department of the Fisheries Agency (1965, 1967). The disease starts to develop immediately after the elongation of *Porphyra in* November. In this case, the characteristic feature is the deterioration of buds composed of 2 or 3 cells growing on the "Hibi". The symptoms of damaged thalli are dullness, a discoloration to reddish brown, a decrease in thickness together with a reddish coloration when seen in transmitted light. Diseased cells can be also observed following: (1) the affected cells are grouped; the spaces between cells broaden; chromtophores are deformed; the cells are full of granules and the cell contents become whitish and indistinct; (2) the cells enlarge (3) large vacuoles as in malnourished cells and (4) the violet dead cells are in a line or in a patch.

The disease with the above symptoms occurred in Saga Prefecture in 1967, in Fukuoka Prefecture in 1967 and 1968 and in Kumamoto Preferture in 1969. This was reported in 1968 and 1970 by the Fisheries Experimental Stations belonging to the corresponding Prefecture.

From data provided by the fisheries research stations located around *Porphyra* farms, SUTO *et al.* (1972)checked carefully symptoms of the white blight and classified the disease into 2 groups. One of them is the physiological disorder which Arasaki described in 1947 and 1956 while the other disease was considered to be caused by bacteria. The latter disease has become called white rot and develops on laver irrespective of size and growing depth. To reduce damage of the white blight, fishermen can try to improve environmental factors by controling the levl of "Hibi", but the white rot has become serious in spite of such protective measures. This suggests that white rot is of bacterial origin.

So far two diseases in which the causual organisms are bacteria are known in *Porphyra* farming in Japan. One is Ôhan-byo, a disease of the conchocelis stage. This disease was reported by Nozawa and NOZAWA (1959), KATO (1967) and TANIGUCHI (1970, 1977a, b) but its detailed description has not yet been given. "Ryokuhan-byo", or green spot rot, is the other disease of the leafy stage. NAKAO *et al.* (1972) isolated *Pseudomonas* and *Vibrio* from diseased *Porphyra* and succeeded in causing green spot rot in healthy thalli by inoculation *in vitro*. FUJITA *et al.* (1972) made further bacteriological studies of *Porphyra* and isolated *Beneckea and Agarbacterium* from infected thalli in addition to the two bacteria mentioned above. After experiments *in vitro* these newly isolated bacteria were shown to be the organisms causing the red discoloration. TOMINAGA (1970) isolated bacteria from 9 species and 2 subspecies

belonging to 8 genera from infected *Porphyra* collected in 6 prefectures in Kyûshû and on the Pacific coast of Japan during 1970 and 1971, but could not find any pathogens among them. However, there have been no bacteriological studies of the white rot so far.

The existance of microflora on *Porphyra* has been known since Sieburth's study in 1976, but until quite recently no work has been done on relationships between host and attached bacteria for the purpose of reducing white rot in *Porphyra* farming.

On the contrary, there are several reports on the nutrient requirements of marine algae in axenic culture. PROVASOLI (1950) indicated that membraneous *Ulva lactuca* grown in axenic culture became filamentous. TATEWAKI and PROVASOLI (1963) found that 3 species of Antithamnion did not grow well in axenic culture but the addition of vitamin B_{12} accelerated their growth. FRIES (1963, 1966, 1970) and FRIES and PETTERSSON (1968) investigated the effect of some nutritious chemical elements on the growth of several species of red algae in axenic culture. TSUKIDATE (1970) stated that the membraneous plant of *Grinnellia americana* grew well in axenic culture using seawater from the mouth of Tokyo Bay, but became dendroid in axenic culture using seawater from the bottom of Tokyo Bay. However, the dendroid condition became membranous by the addition of bacteria isolated from other well grown membranous of same alga.

The above facts indicate that marine algae grow well only in the presence of bacteria attached to the host.

Chapter 3. Bacterial flora of healthy *Porphyra*

A high population of bacteria exists in the surface mucilage of *Porphyra* (SIEBURTH 1976). Therefore, it is very difficult to isolate epiphytic bacteria perfectly from the *Porphyra* thallus. However, an understanding of the bacterial flora associated with helthy thalli of *Porphyra* will help in searching for pathogens causing white rot. For this reason, this chapter deals with the bacteria isolated from wild, stocked and cultivated thalli of *Porphyra*.

Materials and methods

1) Algal materials

2 species of *Porphyra* were used for the experiments. Groups of 10 plants of *Porphyra leucosticta* Thuret were collected 6 times from February 17, 1971 to October 30, 1973 and cultured *in vitro*. Groups of 12 plants of *Porphyra* yezoensis Ueda growing on rocks and "Hibi", near the Nansei Regional Fisheries Research Laboratory, Ôno, Hiroshima Prefecture were collected 11 times from February 5, 1972, to February 8, 1978.

2) Bacteriological medium

Table 1. Composition of artificial sea water used.

NaCl	2.4 g
$MgSO_4$ $7H_2O$	0.8 g
KCl	70 mg
$CaCl_2$ $2H_2O$	55 mg
$NaHCO_3$	16.8 mg
$NaNO_3$	20 mg
Na_2 glycero PO_4	2.5 mg
Metal solution	1 mℓ
Pure water	100 mℓ
Metal solution 1 mℓ =	
H_3BO_3	0.2 mg
$FeCl_3$ $6H_2O$	0.048 mg
$MnCl_2$ $4H_2O$	0.144 mg
$CoCl_2$ $6H_2O$	0.04 mg
$ZnCl_2$	0.01 mg
Na_2EDTA	1 mg

Table 2. Composition of medium forisolation and preservation of bacteria.

Stock solution	100 mℓ
Meat extract	0.2 g
Peptone	0.5 g
Pota ssium nitrate	0.05 g
Agar	1.5 g
Stock solution 100mℓ =	
NaCl	2.4 g
$MgSO_4$ $7H_2O$	0.8 g
KCl	70 mg
$CaCl_2$ $2H_2O$	55 mg
$NaHCO_3$	16.8 mg
$NaNO_3$	20 mg
Na_1glycero PO_4	2.5 mg
Metal solution	1 mℓ
Pure water	100 mℓ
Metal solution 1 mℓ =	
H_3BO_3	0.2 mg
$FeCl_3$ $6H_2O$	0.048 mg
$MnCl_2$ $4H_2O$	0.144 mg
$CoCl_2$ $6H_2O$	0.04 mg
$ZnCl_2$	0.01 mg
Na_2EDTA	1 mg

The medium used for isolation was modified from ZoBELL's medium (ZoBELL 1946) and was prepared using artificial seawater (Table 1). The artificial seawater employed was modified from a madium used by SUTO (1960). Sodium glycerophosphate was used instead of disodium phosphate to avoid precipitation after sterilization and copper was eliminated because a sufficient amount was supplied from sodium chloride and magnesium sulphate contained in the orginal artificial seawater.

3) Isolation and enumeration of bacteria

A sample of *Porphyra* was homogenized for 1 minute in a homogenizer cup with 10 ml of the sterilized artificial seawater at 3,000 r.p.m. Serial 10-fold dilutions were made of the homogenates with the sterilized artificlal seawater and a 0.05 ml or 0.1 ml homogenate taken from each dilution was spread on a plate of the isolation medium. The plates were incubated at 20℃ for 7 days. Colonies were counted with the naked eye and the bacterial populations were represented as the cell number per square centimeter. To obtain pure cultures, the whole colonies, which were estimated as one hundred in number, were picked up from the best plates.

4) Identification

Each isolate was identified to genus on the basis of cell morphology and biochemical tests according to SHEWAN's scheme (SHEWAN *et al.* 1960b).

5) Cell morphology

After each isolate was incubated at 20℃ for 1-7 days, cell size and shape, gram staining characteristics, flagellation and motility were investigated according to a routine method.

6) Biochemical tests

Oxidase tests were carried out according to Kovacs's method (KOVACS 1956) and carbohydrate utilization was tested according to the method used by LEIFSON (1963).

Besides the above tests, favorable temperatures for growth, VP test, MR test, indol production, hydrosulphide production, decrboxylation of aminoacids, hydrolysis of carbohydrate and nitrate reduction were studied.

Results

As shown in Table 3, the dominant genera of bacteria on the healthy thalli of *Porphyra* growing *in vitro* were *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* as well as small amounts of *Micrococcus*. On the other hand, the microorganisms associated with healthy thalli of *Porphyra* on rocks and "Hibi", mostly belonged to *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas*, and small amounts of *Micrococcus*, *Aeromonas* and yeast (Table 4). *Aeromonas* was the only genus isolated from healthy thalli but not *in vitro* thalli. Yeast was found exclusively on thalli from the sea. *Flavobacterium/Cytophaga* seemed to attach the young thalli more often than the aged ones.

Table 3 Generic distribution of microorganisms isolated from the healthy thalli of *Porphyra* cultured in laboratory.

Sampling date	*Porphyra* thalli No.	*Micrococcus*	*Achromobacter*	*Flavobacterium/Cytophaga*	*Pseudomonas*	No. of the isolate	Length and width of *Porphyra* thalli
Feb. 17, '71	1	%	38		62	117	4 cm×6 cm
Mar. 11, '71	2	13	38	47	2	117	2 cm×3 cm
	3	16	36	43	5	100	2 cm×3 cm
	4	2	7	87	4	45	2 cm×3 cm
May 24, '71	5			75	25	12	1 cm×2 cm
Mar. 25, '72	6		65	17	18	99	0.5cm×6.5cm
Mar. 30, '73	7	16	35	53	1	140	0.1cm×1.5cm
Oct. 30, '73	8		11	78	11	93	2mm long
	9		38	54	8	122	3mm long
	10		29	42	29	149	0.1cm×1 cm

Table 4 Generic distribution of microorganis ms and yeast isolated from the healthy thalli of *Porphyra* growing on nets or the rocks in the sea.

Sampling date	*Porphyra* thalli No.	*Micrococcus*	*Achromobacter*	*Flavobacterium/Cytophaga*	*Pseudomonas*	*Aeromonas*	Yeast	No. of the isolate	length and width of *Porphyra* thalli
		%							
Feb. 5, '72	1		71	16	6	3	4	115	0.5cm×0.5cm
Feb. 8, '72	2		65	25	1	5	4	107	0.5cm×1 cm
Feb. 22, '72	3		26	9	17	5	43	135	1 cm×1 cm
Mar. 3, '72	4		19	72	2		7	104	0.5cm×0.5cm
Mar. 7, '72	5		17	78	2	1	2	108	1 cm×2 cm
Dec. 4, '73	6		6	8	76		10	138	5mm long
Dec. 18, '73	7		9	47	43		1	116	1 cm×1 cm
Oct. 30, '75	8		3	63	33		1	45	1.5mm×1.5mm
Nov. 22, '75	9		33	28	39			36	1 cm×1 cm
Nov. 25, '75	10		8	8	84			48	1.5cm×0.5cm
Feb. 8, '78	11	1	10	65	24			111	2 cm×2 cm
	12		14	65	21			186	2 cm×2 cm

Discussion

As *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* were always detected in the epiphytic bacterial flora on *Porphyra* investigated in this study, it is likely that these 3 genera are dominant members of the bacterial flora on *Porphyra* thalli whether from the sea or the laboratory. *Aeromonas* was never isolated from the thalli grown *in vitro*, but was found on thalli grown in the sea. This might be due to repetitive inoculations to renew culture media *in vitro*. However, this is not yet clear. *Micrococcus* was present mainly on thalli grown *in vitro*, while yeast was isolated from those from the sea.

TOMINAGA (1973) investigated the bacterial flora of diseased thalli of *Porphyra* collected at farms all over Japan. He identified 9 species and 2 subspecies belonging to 8 genera, *Micrococcus lactis*, *M. roseus*, *Staphylococcus spidermidis*, *Achromobacter pestifer*, *A. parvulus*, *Flavobacterium fucatum*, *Agarbacterium uliginosum*, *Pseudomonas halestorga*, *P. membranoformis*, *Aeromonas hydrophila* subsp. *anaerognes*, *Serraeia macrescens* subsp. *Kiliensis*. In a study on epiphytic microorganisms isolated from *Polysiphonia*, *Ascophyllum* and the ambient sea, CHAN and MACMANAS (1969) reported the genera *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Achromobacter* or *Alcaligenes*, *Escherichia*, *Staphylococcus*, *Sarcina* and a pink yeast *Rhodotorula*. MURCHELANO and BROWN (1969) indicated that bacteria isolated from *Monochrysis lutheri* and *Isochyrsis galbana*, planktonic algae growing in the laboratory, belonged to the genera *Pseudomonas*, *Flavobacterium*, *Achrombacter*, *Vibrio*, *Bacillus* and *Corynebacterium*.

BERLAND *et al.* (1969) also reported that epiphytic bacteria associated with 6 species of marine planktonic algae cultured in the laboratory belonged to *Pseudomonas*, *Flavobacterium*, *Achromobacter*, *Vibrio*, *Agarbacterium*, *Xanthomonas*, *Micrococcus* and *Staphylococcus*. KONG and CHAN (1979) isolated the genera *Pseudomonas*, *Vibrio*, *Alteromonas*, *Xanthomonas*, *Achromobacter*, *Flavobacterium* and *Micrococcus* from marine algae including 3 species of green algae, 2 species of brown algae and 2 species of red algae.

The papers cited above show that the major bacterial genera associated with marine algae are *Achromobacter*, *Flavobacterium* and *Pseudomonas*. Therefore it is assumed that these 3 genera are the most common epiphytic bacteria growing on the marine algae along the coast of Japan.

Chapter 4. Isolation and identification of pathogenic bacteria causing white rot.

In the course of an ecological study of *Porphyra* growing on "Hibi", in Nagai, Kanagawa Prefecture from 1966 to 1968, the author observed white blight. The disease did not occur every year even though meteorological and oceanographic conditions were quite similar and therefore the role of microocganisms in pathogenesis was considered. As shown in chapter 3, many bacteria thrive on healthy thalli of *Porphyra*. It is therefore indispensable that physiological and microbiological studies are made on infected *Porphyra* on the farms in order to understand pathogen and host-parasite interaction. For this purpose, the present chapter deals with a study from December, 1973 to Nobember, 1975 on variations in the bacterial floras on diseased thalli and the relationships between the disease and the presence of a bacterium which seems to be pathogenic.

Materials and methods

1) Algal materials

Porphyra yezoensis Ueda was collected from rocks in 1973 and 1974 and from "Hibi" near the laboratory in 1975. The alga were brought back to the laboratory immediately after collection and observed with the naked eye and under the microscope to check the viability and the degree of the disease.

2) Bacteriological media

In this chapter except a part of Identification and Biological tests was carried out according to the method described in chapter 2 unless stated otherwise.

3) Isolation and enumeration of bacteria

4) Identification

For identification of some material strains, the type strain of *Beneckea campbellii* ATCC-

25920 which had been been preserved as a stock culture at Laboratory of Bacteriology Faculty of Fisheries, Hokkaido University was employed. Other parts were carried out according to methods described in the preceding chapter.

5) Cell morphology

6) Biological tests

GC content in DNA, hydrolysis of chitin and resistance to salinity were tested on some bacterial strains according to BAUMANN *et al.* (1971a, 1973) and ALLEN *et al.* (1971) as well as SHEWAM *et al.* (1960b). DNA was extracted and refined by Marmur's method (cf. SAITO 1966) and the content of GC was expressed as mole percentage from DNA heat-denaturation curves. Other parts were carried out according to methods described in the preceding chapter.

Results

The number of bacterial strains isolated was 2950 in the culture season of 1973 and 2470 in the culture season of 1974 and 1980 in the culture season of 1975. Each bacterial isolate was identified to genus level and the composition of the microorganisms was shown as a percentage of the total number (Table 5). Most strains were assigned to *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas* and yeast but a few strains could not be identified by the scheme of SHEWAN *et al.* (1960b). The unidentified strains were found at the 0.4% on the thalli of *Porphyra* collected on January 17, 1974, and abundant white and dead host cells were observed only on that day. These unidentified strains were 22.1% of the epiphytic bacterial flora on the healthy thalli of *Porphyra* but abundant white and dead host cells were not found find on the farm until 3 days later. The occurrence of the bacterial strains in question was as high as 20% and 30.7% on November 6 and November 13, 1975, respectively, but abundant white and dead host cells were detected only on thalli collected on November 10, 13 and 15 (Table 5). In other words, many white and dead host cells were detected up to the 4 days after the unidentified bacterial strains were isolated from the thalli.

The biochemical tests were made on the 3 unidentified strains assigned to *Beneckea* and *Beneckea campbellii* ATCC-25920 and the results (as well as their morphological characters) are shown in Tables 6 and 7.

Table 5. Generic distribution of microorganisms isolated from *Porphyra* thalli which were collected during December, 1973 and November, 1975.

Sampling date	*Achromobacter*	*Flavobacterium*/ *Cytophaga*	*Pseudomonas*	Unidentified	Yeast	No. of the isolate	Viable counts	Remarks of *Porphyra* cell
Dec. 4, '73	% 6.0	8.4	75.8	0	9.8	298	2×10^4/cm²	Healthy
Dec. 11, '73	0	53.0	27.7	0	19.3	393	6×10^3/cm²	Healthy
Dec. 18, '73	8.4	47.4	43.2	0	1.0	500	4×10^4/cm²	Healthy
Dec. 21, '73	0.8	16.4	82.8	0	0	244	2×10^4/cm²	Healthy
Dec. 25, '73	9.5	30.3	60.2	0	0	628	4×10^4/cm²	Healthy
Dec. 27, '73	0	18.3	71.1	0	10.6	142	1×10^4/cm²	Healthy
Jan. 5, '74	2.8	29.7	67.7	0	0	155	8×10^3/cm²	FC, GCC
Jan. 9, '74	7.4	15.7	69.4	0	7.5	134	8×10^3/cm²	FC
Jan. 17, '74	21.4	35.6	42.4	0.5	0	205	1×10^5/cm²	FC, WDC
Jan. 22, '74	25.2	14.7	55.4	0	14.7	251	1×10^4/cm²	FC
Nov. 22, '74	0	100	0	0	0	331	2×10^4/cm²	Healthy
Nov. 28, '74	89.3	10.7	0	0	0	373	2×10^4/cm²	Healthy
Dec. 5, '74	44.5	40.7	0	0	14.8	27	2×10^3/cm²	Healthy
Dec. 11, '74	36.4	63.6	0	0	0	132	2×10^4/cm²	Healthy
Dec. 19, '74	100	0	0	0	0	98	2×10^2/cm²	Healthy
Dec. 27, '74	75.0	25.0	0	0	0	144	8×10^2/cm²	Healthy
Jan. 6, '75	75.4	24.6	0	0	0	578	2×10^4/cm²	Healthy
Jan. 13, '75	58.8	20.6	20.6	0	0	126	2×10^3/cm²	Healthy
Jan. 18, '75	51.9	48.1	0	0	0	52	4×10^4/cm²	EV
Jan. 20, '74	11.5	8.6	52.0	22.1	5.8	173	1×10^4/cm²	Healthy
Jan. 23, '75	0	0	100	0	0	313	2×10^4/cm²	WDC
Jan. 28, '75	0	3.2	96.8	0	0	94	6×10^3/cm²	FC
Jan. 31, '75	34.5	10.3	34.5	0	20.7	29	2×10^3/cm²	Healthy
Oct. 30, '75	2.6	62.5	32.3	0	2.6	269	8×10^3/cm²	Healthy
Nov. 3, '75	0	41.0	59.0	0	0	100	4×10^3/cm²	Healthy
Nov. 6, '75	5.1	35.2	39.7	20.0	0	176	4×10^3/cm²	Healthy
Nov. 10, '75	2.6	67.9	29.5	0	0	190	8×10^3/cm²	WDC
Nov. 13, '75	20.6	26.3	22.4	30.7	0	281	1×10^4/cm²	WDC
Nov. 15, '75	24.9	75.1	0	0	0	217	2×10^5/cm²	WDC
Nov. 18, '75	40.4	31.2	28.4	0	0	109	4×10^4/cm²	Healthy
Nov. 22, '75	33.0	28.0	39.0	0	0	164	8×10^5/cm²	Healthy
Nov. 25, '75	8.3	8.4	83.3	0	0	174	4×10^5/cm²	Healthy

FC: faded chromotophores; GCC: groups of clumped cells;
WDC: white and dead cells; EV: enlarged vacuole.

Table 6. Morphological and biochemical characteristics of *Beneckea* isolated in 1973 and *Beneckea campbellii* ATCC-25920.

Characteristics	*Beneckea* isolated in 1973	*Beneckea* campbe ii ATCC-25920
Cells form	Moderate straight round	Moderate straight round
Gram stain	−	−
Motility	+	+
Flagella	Peritrichous	Peritrichous
Pigmentation	−	−
Kovacs oxidase	+	+
Catalase	+	+
Acid by (MOF) from		
Glucose	F	F
Maltose	+	+
Mannitol	+	+
Ribose	+	+
Sorbitol	+	+
Sucrose	+	−
Lactose	+	−
Arabinose	+	−
Fructose	+	+
Galactose	+	+
Xylose	+	−
Raffinose	+	+
Trehalose	+	+
Mannose	+	+
VP test	−	−
MR test	+	+
Indol	+	+
Hydro ysis		
Starch	+	+
Chitin	+	+
Gelatin	+	+
Casein	+	+
Tween 80	+	+
Alginate	−	−
Cellulose	−	−
Agar	−	−
Gelatin liquefaction	+	+
NO_3 reduction	+	+
NO_2 reduction	+	+
TMAO	−	+
Litmus milk	Peptonization	Peptonization
Decarboxylation		
Lysine	−	+
Ornithine	−	−
Arginine	−	−
Sensitivity to O/129	±	±
H_2S production	+	+
Phosphatase	+	+
Growth at 10% NaCl	+	−
Sea water require ment	H	H
Growth at		
0℃	−	−
5℃	−	−
30℃	+	+
37℃	+	+
40℃	+	−
Gluconate oxidation	−	−
NH_3 from peptone	+	+
Citrate	+	+
GC moles %	44. 2	46. 4

Table 7. Morphological and biochemical characteristics of *Beneckea* sp. isolated in 1974 and 1975.

Characteristics	Iso ated bacteria	
	Beneckea sp. isolated in 1974 38[a]	*Beneckea*sp. iso ated in 1975 35
Cells form	Moderate straight round	Moderate straight round
Gram stain	−	−
Motility	+	+
Flagella	Peritrichous 35[b]	Peritrichous 33
Pigmentation	−	−
Kovacs oxidase	+	+
Catalase	+	+
Acid by (MOF) from		
Glucose	F	F
Maltose	+	+
Xylose	+	+
VP test	−	−
MR test	+	+
Indol	+	+
Hydrolysis		
Starch	+	+
Chitin	+	+
Gelatin	+	+
Casein	+	+
Tween 80	+	+
Alginate	−	−
NO_3 reduction	+	+
Decarboxylation		
Lysine	−	−
Ornithine	−	−
Arginine	−	−
Sensitivity to O/129	+	+
H_2S production	+	+
Growth at 10% NaCl	+	+
Sea water requirement	H	H
Growth at		
0℃	−	−
5℃	−	−
15℃	+	+
20℃	+	+
25℃	+	+
37℃	+	+
40℃	+	+
GC moles %	45.9+1.1	45.7+0.7

a : Number of strains used; b : Number of strains having peritrichous flagella;
+ : Positive; − : Negative

Discussion

The unidentified bacterial isolate was assigned to *Beneckea* establishod by BAUMANN *et al.* (1971a), because of the morphological and biochemical following properties; gram negative, positive motility, peritrichous on solid media, monotrichous in liquid media, positive to oxidase, requirement of NaCl for growth, fermentation in glucose without gas production, hydrolysis of chitin and GC content in DNA in the range of 45 to 48% (Table 6). On the other hand, 3 strains of the genus *Beneckea* isolated in January, 1974, and January and November, 1975, had in common the properties shown in Tables 5 and 6, and seemed to belong to the same taxon.

7 species belonging to *Beneckea* were originally described by BAUMANN *et al.* (1971a) but *Beneckea* sp. inquestion differed from them in properties enumerated below.

Beneckea sp. differs from *B. parahaemolytica* in the utilization of carbohydrates including sucrose and xylose, from *B. neptuna* and *B. nereida* in the decarboxylation of arginine and growth at 40°C, from *B. alginolytica* in the utilization of xylose, from *B. pelagia* in flagellation, utilization of xylose and growth at 40°C and from *B. netriegens* in the utilization of xylose.

The properties examined in the present study were not enough to compare with the more detailed description by BAUMANN *et al.* (1971a). A further comparison was made with the type strains of 7 species in *Beneckea* and 1 species of *Vibrio*, all of which were treated as marine *Vibrio* by LEE *et al.* (1978). *Beneckea* sp. isolated in the present study was different from 4 type strains, Beneckea *parahaemolytica* ATCC-17802, *B. neptuna* ATCC-25919, *B. campbellii* ATCC-25920 and *B. alginolytica* ATCC-17749, in the utilization of the carbohydrates cellulose, lactose, raffinose, arabinose, sucrose sorbitol and xylose, as well as in the TMAO test, growth at 10% NaCl and growth at 15° and 37°C. It was also different from *B. pelagica* ATCC-25916 in the utilization of the carbohydrates lactose, mannose, raffinose, sorbitol and xylose, and in the TMAO test and growth at 15° and 37°C, from B. natriegens ATCC-14048 in the utilization of the carbohydrates cellulose, lactose, mannose, sorbitol and xylose, in the catalase test, TMAO test and indol production. It differed from *B. nerida* ATCC-25917 in the utilizaiton of the carbohydrates lactose, mannitol, mannose, sorbitol and xylose, and in the catalase test and TMAO test. It differed from *Vibrio fisheri* in the utilization of the carbohydrates cellulose, lactose, mannitol, mannose, raffinose, sorbitol and xylose, and in the TMAO test, MR test, indol production, growth at 10% NaCl and growth at 30°C and 37°C. *Beneckea* sp. was compared to *B. campbelli* as described by BAUMANN *et al.* (1973) and, more precisely, with *B. campbellii* ATCC-25920 preserved in the Labortory of Bacteriology. The properties of both species included hydrolys of starch, chitin, gelatin, casein, tween 80 and alginate, decarboxylation of lysine, ornithine and arginine, test oxidase, catalase, VP test, MR test, indol production and growth at temperatures between 0° to 37°C. They differed in the utilization

of carbohydrates including sucrose, arabinose and xylose, decarboxylation of lysine, growth at 10% NaCl and growth at 40°C.

Beneckea sp. could not be assigned to any known species and appears to be hitherto unreported although more studies are necessary.

The relationships of white rot to environmental factors, such as the differences between atmospheric and seawater temperatures and desiccation at low tide, will be discussed below.

According to KATADA (1967), *Porphyra* utilizes large amounts of nutrients, especially when the seawater temperature drops gradually at the beginning of the growing season. He speculated that a shortage of dissolved nutrients such as nitrogen, phosphorus and carbonic acid for normal metabolism. On the other hand, fishermen say that *Porphyra* is more apt to be infected with the disease when the differences between atmospheric and seawater tempratures are big during the low tides at dawn. FUJIKAWA (1937) found that growth was optimal when exposed to air for 2-4 hours per day.

The author checked exactly the days corresponding with 4 hours or more exposure at dawn, the occurrence of abundant white and dead cells of *Porphyra* and a difference of at least 8°C between atmospheric and seawater temperatures (Table 8, Fig. 1). During the experimental time only 4 combined these features. A discrepancy of 9.8°C was found on January 12, 1974, 5 days before the occurrence of white rot, 9.5°C on January 13. 1975, 7 days before a serious outbreak of the disease and 9.5°C on November 4, 1975, 2 or 9 days before the presence of many white and dead host cells. In other words, *Beneckea* sp. was found in the *Porphyra* farm on the 2nd to 9th days after difference of 8°C or more between atmospheric and eawater stemperatures was deteced.

Table 8. The occurrence of discrepancy more than 8°C in atmospheric and sea water temperatures when *Porphyra* tha i expose to the air more than 4 hours per day in 1974 and 1975.

Date	Temperature difference
Jan. 7, 1974	9.1℃
Jan. 12, 1974	9.8℃
Jan. 19, 1974	9.0℃
Jan. 20, 1974	7.9℃
Jan. 27, 1974	10.1℃
Jan. 12, 1975	8.9℃
Jan. 13, 1975	9.5℃
Jan. 28, 1975	4.0℃
Jan. 29, 1975	9.0℃
Jan. 31, 1975	9.3℃
Nov. 4, 1975	8.4℃
Nov. 22, 1975	9.2℃
Nov. 23, 1975	9.4℃

Moreover, the symptom of the disease observed in the present study was identical to that described as the white rot by SUTO *et al.* (1972) (Plates 1,2).

From the data mentioned above, it is suspected that *Beneckea* sp. is one of pathogens of white rot.

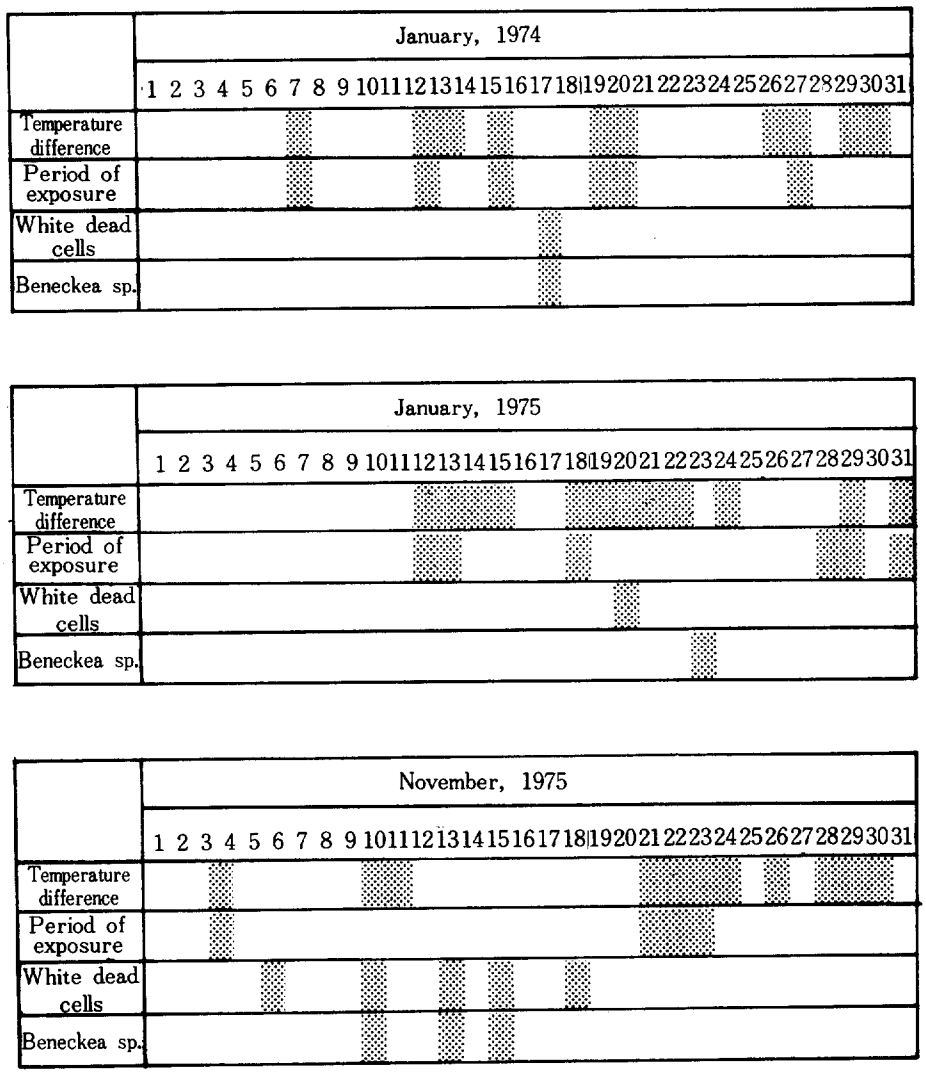


Fig. 1 The relation of maximum difference between atmospheric and seawater temperature, the period of exposure, the appearance of *Beneckea* sp. and white dead cells.

Chapter 5. Infection experiment of white rot

As mentioned in chapter 4, *Beneckea* sp. is considered one of pathogens causing white rot. In order to better understand the pathogenicity, *Beneckea* sp. was inoculated into *Porphyra*.

Materials and methods

1) Bacterial materials

Beneckea sp. was isolated on January 17 1974, from *Porphyra yezoensis* growing on inshore

rocks near the Nansei Regional Fisheries Research Laboratory, Ôno, Hiroshima Prefecture. Other bacteria including *Achromobacter* sp., *Flavobacterium/ytophaga* group and *Pseudomonas* sp. had been also isolated from *Porphyra leucosticta* on February 14, 1970, and all were incubated as a stock culture until the initiation of the experiment.

2) Algal materials

Porphyra yezoensis and *P. leucosticta* had been cultured in a 500 ml flask with aeration in artificial seawater at a light intensity of 3,000 lux and 17°C for the bud stage and at 10°C when a length of 1-3cm or more had been attained. The cultures were not axenic but the thalli of *Porphyra* were rinsed several times with the streilized artificial seawater before use.

3) Inoculation

Bacteria ranging from 10^3 to 10^4 per strain were suspended in 125ml Erlenmyer flasks containing 50ml of liquid medium (cf. Table 1) after being incubated for 1-5 days. 2 or 3 pieces of *Porphyra* were infected with bacteria by culturing for 1-2 weeks at 17°C and at 3,000 lux without aeration. Pathological observations on the thalli were made several times during the infectivity experiment.

4) Isolation and identification of bacteria from the infected *Porphyra*

Bacteria were isolated again from the infected *Porphyra* and were identified according to the methods described in chapter 3.

Results

The infectivity experiments were made 5 times using 4 species of bacteria isolated.

Experiment 1 (Table 9)

Thalli of *Porphyra* 1mm long were infected with *Beneckea* sp. and 4 days later bacteria were isolated from the infected thalli. The bacterial flora on uninfected *Porphyra* used as a control consisted of *Achromobacter* sp. (44%), *Flavobacterium/Cytophaga* group (16%) and *Pseudomonas* sp. (40%). Cells of infected *Porphyra* were all white and dead and only *Beneckea* sp. was isolated.

Table 9. Sign of white rot-like deterioration of *Porphyra* thalli caused artificially by bacteria.

Inoculated bacteria	Cell condition	Reisclated bacteria and their relative composition
None	Hea thy	*Achromobacter* sp. 44% *Flavobacterium/Cytophaga* group 16% *Pseudomonas* sp. 40%
Beneckea sp.	All are white and collapsing dead cells	*Beneckea* sp. 100%

Observation was made on the fourth day after inoculation of bacteria. The length of *Porphyra* thalli used in this experiment was 1 mm.

Experiment 2 (Table 10)

Thalli of *Porphyra* 0.2-0.3mm long were infected with *Beneckea* sp. and 8 days later bacteria were isolated from the plants. Infected *Porphyra* discolored to whitish and deteriorated. Only *Beneckea* sp. was found on infected *Porphyra*, whereas *Porphyra* used as control was very healthy.

Table 10. Sign of white rot-like deterioration of *Porphyra* thalli caused artificially by bacteria.

Inoculated bacteria	Cell condition	Reisolated bacterium and their relative composition
None	Healthy	
Beneckea sp.	All are white and collapsing dead cells	*Beneckea* sp. 100%

Observation was made on the eighth day after inoculation of bacteria. The length of *Porphyra* thalli used in this experiment was 0.2-0.3 mm.

Experiment 3 (Table 11)

Thalli of *Porphyra* 2mm long were infected with *Beneckea* sp. and 3 days later bacteria were isolated from the thalli. Cells of infected *Porphyra* were red or white, and moribund or dead. The *Porphyra* used as a control bore *Achromobacter* sp. (57%), *Flavobacterium/Cytophaga* group (12%) and *Pseudomonas* sp. (31%)whereas infected *Porphyra* had only *Beneckea* sp.

Table 11. Sign of white rot-like deterioration of *Porphyra* thalli caused artificially by bacteria.

Inoculated bacteria	Cell condition	Reisolated bacteria and their relative composition
None	Healthy	*Achromobacter* sp. 57% *Flayobacterium/Cytophaga* group 12% *Pseudomonas* sp. 31%
Beneckea sp.	Cells with a large vacuole Red or white Collapsing cells	*Beneckea* sp. 100%

Observation was made on the third day after inoculation of bacteria. The length of *Porphyra* thalli used in this experiment was 2 mm.

Experiment 4 (Table 12)

Thalli of *Porphyra* 1cm long were inoculated with *Achromobacter* sp. *Pseudomonas* sp. and *Beneckea* sp. and bacteria were isolated 6 days later.

The thalli infected with *Achromobacter* sp. were healthy and the bacteria isolated were *Achromobacter* sp. (60%), *Flavobacterium/Cytophaga* group (13%) and *Pseudomonas* sp. (27%). The thalli infected with *Pseudomonas* sp. were not healthy and most host cells were red or white, and moribund or dead. The bacteria isolated were *Micrococcus* sp. (6%), *Achromo-*

bacter sp. (4%) and *Pseudomonas* sp. (90%). The thalli infected with *Beneckea* sp. were whitish and the host cells were red of white, and moribund or dead. The bactecial flora on infected thalli consisted of *Micrococcus* sp. (4%), *Achromobacter* sp. (1%) and *Beneckea* sp. (95%).

Table 12. Sign of white rot-like deterioration of *Porphyra* thalli caused artificially by bacteria.

Inoculated bacteria	Cell condition	Reisolated bacteria and their relative composition
Achromobacter sp.	Healthy but cells	*Achromobacter* sp. 60% *Flavobacterium/Cytophaga* group 13% *Pseudomonas* sp. 27%
Pseudomonas sp.	Red or white collapsing cells	*Micrococcus* sp. 6% *Achromobacter* sp. 4% *Pseudomonas* sp. 90%
Beneckea sp.	Red or white collapsing cells	*Micrococcus* sp. 4% *Achromobacter* sp. 1% *Beneckea* sp. 95%

Observation was made on the sixth day afterinoculation of bacteria. The length of *Porphyra* thalli used in this experiment was 1 cm.

Experiment 5 (Table 13)

Thalli of *Porphyra* 1cm long were inoculated with *Achromobacter* sp., *Flavobacterium/Cytophaga* group, *Pseudomonas* sp. and *Beneckea* sp. and bacteria were isolated from the infected thalli 3 days later.

Table 13. Sign of white rot-like deterioration of *Porphyra* thalli caused artificially by bacteria.

Inoculated bacteria	Cell condition	Reisolated bacteria and their relative composition
Achromobacter sp.	Healthy	*Achromobacter* sp. 100%
Flavobacterium/Cytophaga group	Healthy	*Achromobacter* sp. 44% *Flavobacterium/Cytophaga* group 56%
Pseudomonas sp.	Healthy	*Achromobacter* sp. 40% *Pseudomonas* sp. 60%
Beneckea sp.	White and dead cells	*Beneckea* sp. 100%

Observation was made on the third day after inoculation of bacteria. The length of *Porphyra* thalli used in this experiment was 1 cm.

The thalli inoculated with *Achromobacter* sp. were healthy and only *Achromobacter* sp. was found on the hosts. The thalli infected with *Flavobacterium/Cytophaga* group were also healthy but isolated were *Achromobacter* sp. (44%) and *Flavobacterium/Cytophaga* group (56%). The thalli infected with *Pseudomonas* sp. were healthy and the bacteria consisted of *Achromobacter* sp. (40%) and *Pseudomonas* sp. (60%). The thalli infected with *Beneckea*

sp. deteriorated and host cells were red or white, and moribund or dead. No other bacteria except *Beneckea* sp. were isolated from the infected *Porphyra*.

Discussion

5 repeated experiments in which *Porphyra* were infected with *Beneckea* sp. revealed that the plants deteriorated lost their original color within 3 days, and died. *Porphyra* infected with *Beneckea* sp. was characterized by the sole occurrence of *Beneckea* sp. in 4 experiments, but including also *Micrococcus* sp. (4%) and *Achromobacter* sp. (1%)in a 5th experiment. The symptoms of the disease induced by *Beneckea* sp. were identical with those of the white rot described by Suto *et al.* (1972). There still remains some doubt about the bacterial medium being suitable for the growth of *Porphyra*. However, *Porphyra* used as a control grew well in the medium in question.

When inoculated with *Achromobacter* sp. or *Flavobacterium/Cytophaga*, *Porphyra* grew healthily, while *Porphyra* infected with *Pseudomonas* sp. grew well or lost the original cell color, which became red and white. When infected with *Pseudomonas* sp. that species occurred as much as 90% of the bacteria on the diseased *Porphyra*, but it occurred only 60% when the thalli were healthy. The reasons for the differences in bacterial populations was not clear.

When *Achromobacter* sp. was inoculated, 2 kinds of bacterial populations were present on *Porphyra*. One had a predominance of *Achromobacter* sp. and the other contained *Flavobacterium/Cytophaga* and *Pseudomonas* sp. in addition to *Achromobacter* sp. There may have been antagonistic relationships among these bacteria.

Chapter 6. Influence of nutrients, temperature and sodium chloride on bacterial growth

It is well known that bacteria isolated from marine algae utilize amino acids, organic acids, carbohydrates and other organic compounds for their growth (BERLAND *et al.* 1970). This study was carried out to learn the effects of amino acids, carbohydrates, temperature and sodium chloride on the growth of bacteria isolated from *Porphyra*.

Materials and methods

1) Bacterial materials

Micrococcus: 22 strains were isolated on February 22, 1980 from *Porphyra yezoensis* grown *in vitro*.

Achromobacter: 49 strains were isolated on February 22, 1980 from P. yezoensis grown *in vitro* and 26 strains on February 14, 1980 from *P. yezoensis* growing on inshore rocks near

the Nansei Regional Fisheries Research Laboratory, Ôno, Hiroshima Prefecture.

Flavobcterium/Cytophaga: 74 strains were isolated on February 22, 1980 from *P. yezoensis* grown *in vitro* and 121 strains on February 14, 1980 from *P. yezoensis* growing on inshore rocks near the Nansei Regional Fisheries Research Laboratory.

Pseudomonas: 43 strains were isolated on February 22, 1980, from *P. yezoensis* grown *in vitro* and 39 strains on February 14, 1980, from *P. yezoensis* growing on inshore rocks near the Nansei Regional Fisheries Research Laboratory.

Beneckea sp.: 1 strain was isolated on January 17,1974 from *P. yezoensis* growing on inshore rocks near the Nansei Regional Fisheries Research Laboratory and 6 strains on November 6, 1975 from *P. yezcensis* growing on "Hibi" near the Nansei Regional Fisheries Research Laboratory.

2) Bacterial media and culture conditions.

In the experiment on nutritional requirements, the amino acids used were alanine, valine, arginine, lysine, aspartic acid, glutamic acid, cystine, methionine, leucine, isoleucine, serine and phenylalanine; The carbohydrates tested were glucose, maltose, manitol, sucrose, lactose, arabinose and xylose. The incubations were kept under aerobic condition at 20°C for a week. For responses to temperature, incubation was at aerobic conditions for a week using the basic medium (Table 14), and 8 different temperatures: O°, 5°, 15°, 20°, 25°, 30°, 37°, and 40°C. The sodium chloride tolerance experiments were carried out according to methods described by HIDAKA and SAKAI (1968), but the incubations were kept at 20°C for a week. Bacterial strains, ranging from 10^3 to 10^4/ml, were used for the experiments on the 2nd and 5th day after inoculation to a fresh medium, except for the experiment of sodium chloride tolerance, where a 6fold dilution of the basal medium (Table 15), containing 0.05% polypeptone and 0.01% yeast extract, was used for the culture before the initiation of the experiment. Growth was measured by counting the number of colonies or observing the density with the naked eye.

Table 14. Composition of basal medium used.

Stock solution (75%)	150 mℓ
Yeast extract	0.1 g
Polypeptone	0.5 g
Agar	1.5 g
Stock solution	100mℓ=
NaCl	3 g
KCl	0.07 g
$MgSO_4$ $7H_2O$	0.26 g
$MgCl_2$ $6H_2O$	0.5 g
$CuSO_4$ $2H_2O$	0.1 g

Table 15. Composition of Herbst's artificial sea water.

NaCl	3 g
KCl	0.07 g
$MgSO_4$ $7H_2O$	0.26 g
$MgCl_2$ $6H_2O$	0.5 g
$CaSO_4$ $2H_2O$	0.1 g
Pure water	100 mℓ

Results

A. Amino acid requirements

The results obtained are shown in Tables 16 and 17.

1) The strains of *Micrococcus* sp. grew well in a medium with glutamic acid, alanine and arginine.

2) Most strains of *Achromobacter* sp. grew well by adding alanine, valine and cystine. The strains originating from the laboratory utilized well glutamic acid, while it was not

Table 16. Influence of amino acids on growth of bacteria.

Bacteria / Amino acids	*Micrococcus* 22[a]	*Achromobacter* 49	*Flavobacterium/ Cytophaga* 74	*Pseudomonas* 43
Alanine	20[b]	42	40	+
Valine	−	45	34	40
Arginine	17	23	31	+
Lysine	−	28	29	23
Aspartic acid	−	25	30	+
Glutamic acid	+	40	+	+
Cystine	−	41	35	37
Methionine	−	24	35	17

+ : Growth; − : No growth; a : Number of strains used;
b : Numbers indicate number of positive strains.

Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra leucosticta* cultured in laboratory.

Table 17. Influence of amino acids on growth of bacteria.

Bacteria / Amino acids	*Achromobacter* 26[a]	*Falvobacterium/ Cytophaga* 121	*Pseudomonas* 39	*Beneckea* 7
Alanine	+	65	+	6
Valine	+	56	36	5
Arginine	13[b]	59	38	−
Lysine	14	63	18	6
Aspartic acid	11	66	35	+
Glutamic acid	8	119	+	+
Cystine	+	55	15	4
Methionine	14	63	15	5
Leucine	12	−	−	−
Isoleucine	12	−	−	−
Serine	−	9	−	−
Phenylalanine	−	8	−	−

+ : Growth; − : No growth; a : Number of strains used.
b : Numbers indicate number of positive strains;

Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra yezoensis* grown on nets or the rocks in the sea.

certain if the strains originating from the sea utilized this amino acid or not.

3) All strains of *Flavobacterium/Cytophaga* grew well using only glutamic acid.

4) Most strains of *Pseudomonas* also grew well using alanine, valine, arginine, lysine, aspartic acid, glutamic acid and cystine.

5) The growth of most strains of *Beneckea* sp. was good in supplemental alanine, valine, lysine, cystine and methionine, but glutamic and aspartic acid resulted in the good growth of all strains, of *Beneckea* sp.

B. Carbohydrate requirements

The results obtained are shown in Tables 18 and 19.

1) Most strains of *Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* grew well in the presence of glucose, maltose and manitol, but failed to grow with

Table 18. Influence of carbohydrates on growth of bacteria.

Bacteria / Carbohydrates	*Micrococcus* 22[a]	*Achromobacter* 49	*Flavobacterium/ Cytophaga* 74	*Pseudomonas* 43
Glucose	+	+	+	+
Maltose	+	46	73	+
Manitol	+	+	70	+
Sucrose	15[b]	40	45	34
Lactose	14	25	40	30
Arabinose	11	39	48	31
Xylose	−	5	41	39

+ : Growth; − : No growth; a : Number of strains used;
b : Numbers indicate number of positive strains.

Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra leucosticta* cultured in laboratory.

Table 19. Influence of carbohydrates on growth of bacteria.

Bacteria / Carbohydrates	*Achromobacter* 26[a]	*Flavobacterium/ Cytophaga* 121	*Pseudomonas* 39	*Beneckea* 7
Glucose	+	+	+	+
Maltose	+	+	+	+
Manitol	+	120	+	+
Sucrose	20[b]	57	25	+
Lactose	14	56	21	+
Arabinose	21	65	20	+
Xylose	2	56	29	+

+ : Growth; − : No growth; a : Number of strains used;
b : Numbers indicate number of positive strains,

Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra yezoensis* grown on nets or the rocks in the sea.

the addition of sucrose, lactose, arabinose and xylose.

2) The growth of all strains of *Beneckea* sp. was supported by all carbohydrates examined.

C. The effects of temperature

The results obtained are shown in Tables 20 and 21.

All bacterial strains used in the experiment failed to grow at 0° and 5°C, but grew at temperatures higher than 20°C except *Pseudomonas* where growth did not occur at 37°C and 40°C.

Table 20. Growth of bacteria at various temperatures.

Bacteria / Temperatures	*Micrococcus* 22[a]	*Achromobacter* 49	*Flavobacterium/ Cytophaga* 74	*Pseudomonas* 43
0℃	−	−	−	−
5℃	−	−	−	−
15℃	+	+	+	+
20℃	+	+	+	+
25℃	+	+	+	+
30℃	+	+	+	+
37℃	+	+	+	−
40℃	+	+	−	−

+ : Growth; − : No growth; a : Number of strains used.

Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra leucosticta* cultured in laboratory.

Table 21. Growth of bacteria at various temperatures.

Bacteria / Temperatures	*Achromobacter* 26[a]	*Flavobacterium/ Cytophaga* 121	*Pseudomonas* 39	*Beneckea* 7
0℃	−	−	−	−
5℃	−	−	−	−
15℃	+	+	+	+
20℃	+	+	+	+
25℃	+	+	+	+
30℃	+	+	+	+
37℃	+	+	−	+
40℃			−	+

+ : Growth; − : No growth; a : Number of strains used.

Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra yezoensis* grown on nets or the rocks in the sea.

D. Sodium chloride tolerance

The results obtained are shown in Tables 22 and 23.

1) The strains of *Micrococcus* and *Flavobacterium/Cytophaga* grew well in redistitilled

water containing 0.05% polypeptone and 0.01 yeast extract and was considered to be of terrestrial origin.

2) All strains of *Achromobacter* and *Pseudomonas* were marine bacteria which required monovalent and divalent ions, as their growth was observed only in Herbst's artificial seawater (c f. Table 15) used as a basal medium.

3) All strains of *Beneckea* sp. grew well at 2 concentrations of sodium chloride as well as in Herbst's artificial seawater and are, therefore, halophilic.

Table 22. Influence of sodium chloride and sea water on growth of bacteria.

Bacteria / Salts	*Micrococcus* 22[a]	*Achromobacter* 49	*Flavobacterium/ Cytophaga* 74	*Pseudomonas* 43
DW[c]	+	−	49	−
0.5% NaCl	+	−	71	−
3% NaCl	3[b]	−	36	−
75% ASW[d]	+	+	+	+

+ : Growth; − : No growth; a : Number of strains used;
b : Numbers indicate number of positive strains; c : Distilled water; d : Artificial sea water.
Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra leucosticta* cultured in laboratory.

Table 23. Influence of sodium chloride and sea water on growth of bacteria.

Bacteria / Salts	*Achromobacter* 26[a]	*Flavobacterium/ Cytophaga* 121	*Pseudomonas* 39	*Beneckea* 7
DW[c]	−	62[b]	−	−
0.5% NaCl	−	116	−	+
3% NaCl	−	56	−	+
75% ASW[d]	+	+	+	+

+ : Growth; − : No growth; a : Number of strains used;
b : Numbers indicate number of positive strains; c : Distilled water; d : Artificial sea water.
Observations were made on the seventh day after incubation of bacteria which were isolated from *Porphyra yezoensis* grown on nets or the rocks in the sea.

Discussion

Beneckea sp. which was considered to be one of pathogens causing white rot, grew well in media containing alanine, asparatic acid and glutamic acid. On the other hand, 3 predominant genera of bacteria isolated from healthy *Porphyra, Achromobacter, Flavobacterium/Cytophaga* and *Pseudomonas* required all amino acids employed in the experiment for their growth.

All bacteria, especially strains of *Beneckea* sp. grew well at 20°C or more.

Marine bacteria were indicated as utilizing metabolites released by phytoplankton (ITURRIAGA and HOPPE 1977). WILLIAM *et al.* (1977) also showed the utilization of amino acids in seawater by marine bacteria. KONG and CHAN (1979) tested the nutrient requirements of bacteria isolated from several algae and found that the isolates utilized alanine, arginine and lysine among 119 organic compounds tested. CHAN and MCMANUS (1969) also reported that 22 out of 25 strains of bacteria isolated from 2 species of red algae required amino acids for their growth. These data are not suitable for comparison with the results from the present study, as organisms employed are different, but it seems likely that nutrients utilized by bacteria are different among bacterial taxa.

Chapter 7. Carbon dioxide produced by epiphytic bacteria

ALLEN (1971) postulated that epiphytic bacteria released carbon dioxide, as a result of his research on the relationships between macrophytes and bacteria in lakes.

In chapter 6, it was shown that bacteria associated with *Porphyra* required several kinds of acids for growth. Therefore it is reasonable to assume that bacteria decompose amino acids, leading to the production of carbon dioxide. The present experiment was carried out to study the release of carbon dioxide by bacteria associated with *Porphyra* after decomposing amino acids.

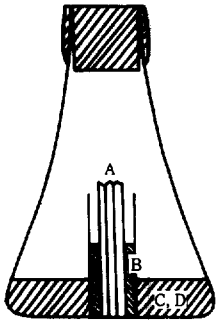
Materials and methods

1) Bacterial materials

1 strain of *Micrococcus* sp., 2 strains of *Flavobacterium/Cytophaga* group and 2 strains of *Pseudomonas* sp. were isolated on December 4, 1976 from *Porphyra yezoensis* on rocks near the Nansei Regional Fisheries Research Laboratory, located at Ôno, Hiroshima Prefecture; 1 strain of *Achromobacter* sp. on November 24, 1970 from *P. yezoensis* growing on "Hibi", near the Nansei Regional Fisheries Research Laboratory; 1 strain of *Beneckea* sp. on January 17, 1974 from *P. yezoensis* growing on rocks near the Nansei Regional Fisheries Research Laboratory.

2) Measurement of carbon dioxide released by attached bacteria

For measuring cabon dioxide released by attached bacteria, a special 50ml Frlenmeyer flask (YAMADA *et al.*, 1979) with a small built-in chamber was used (Fig. 2). A small amount of 1 μCi amino acid with labelled carbon (Table 24) was placed around the small chamber and mixed with 2ml of natural seawater and 1ml of bacterial suspension containing from 19^7 to



A. TOYO filter paper (No.2)

B. Hyamine 10—X

C. ^{14}C—labelled substrate

D. Media

Fig. 2. Apparatus for producing and trapping ^{14}C.

10^8 cells. Inside the chamber, 1ml of methanol in which hydroxide hyamine 10-x (P. (diisobutylcresoxyethoxyethy I) dimethylbenzylammonium hydroxide) was dissolved to give a concentration of 1 M was placed with one 3×3cm sheet of accordion-folded filter paper (Toyo No. 2). After stoppering tightly (rubber stopper) and covering with a black cloth. each flask was placed on a shaker for 1 hour at 25°C, and then 1 ml of 6N HCl was added by injection outside the chamber. The amount of labelled amino acid used was calculated from specific gravity. The radioactivity of hydroxide hyamine and filter paper was measured using a Packard 3255 TRI-CARB liquid scintilation spectrometer. The amount of carbon dioxide released by the bacteria was expressed as a percent of carbon produced to the carbon supplied at the initiation of the experiment.

Table 24. Specific activity of labelled substrates in stock solution and quantities added in the experiments.

Amino acids	Specific activity mCi / m mol	Quantities μg
Aspartic acid	220	0. 55
Threonine	232	0. 51
Serine	162	0. 59
Glutamic acid	270	0. 59
Proline	290	0. 4
Glycine	112	0. 63
Alanine	164	0. 51
Valine	280	0. 4
Isoleucine	330	0. 38
Leucine	330	0. 43
Tyrosine	486	0. 35
Phenylalanine	486	0. 29
Histidine	348	0. 44
Lysine	330	0. 59
Arginine	324	0. 68

Results

The results are shown in Tables 25 and 26. Owing to the impurity of the labelled amino acids and to the complexity of the procedure carbon dioxide was produced up to 0.1% even if the experiment did not run. Therefore, the amount of carbon dioxide was considered to

Table 25. Amount of CO_2 produced by bacteria from various amino acids contained in media.

Bacteria / Amino acids	*Micrococcus* sp.	*Flavobacterium/ Cytophaga* group	*Pseudomonas* sp.	*Pseudomonas* sp.
Aspartic acid	3.1%	1.8%	13.5%	3.6%
Threonine	0	18.9	4.5	25.2
Serine	0.2	54.0	0	45.0
Glutamic acid	1.8	1.8	4.5	2.5
Proline	0.5	8.1	85.6	0
Glycine	0.1	0.2	2.3	45.0
Alanine	2.7	3.5	4.5	2.3
Valine	0.4	1.8	19.5	1.8
Isoleucine	0.8	22.5	1.8	1.7
Leucine	5.4	25.2	1.0	10.0
Tyrosine	1.0	7.2	0	9.5
Phenylalanine	7.6	1.6	0	5.4
Histidine	0	1.3	5.9	1.4
Lysine	0	4.9	0.4	6.8
Arginine	0	21.6	2.6	14.9

All bacteria were isolated on Dec. 4, 1976 from *Porphyra yezoensis* which grow on rocks in the sea. The amount of carbon dioxide is expressed as a percentage of carbon in the derived carbon dioxide to the amount of carbon supplied to the substrate.

Table 26. Amount of CO_2 produced by bacteria from various amino acids contained in media.

Bacteria / Amino acids	*Achromobacter* sp.	*Flavobacterium/ Cytophaga* group	*Beneckea* sp.
Aspartic acid	5.9%		21.8%
Threonine	0	15.4%	7.8
Serine	1.6	42.0	16.8
Glutamic acid	33.5	0	35.7
Proline	80.2	24.7	48.5
Alanine	0.9	0	29.7
Valine	1.4		0.5
Leucine	1.1	33.9	5.4
Tyrosine	1.4	0	0.5
Phenylalanine	1.7	0	0.5
Histidine	2.4	35.0	0.6
Lysine	0	24.4	3.3
Arginine	3.4	0	4.5

Achromobacter was isolated on Nov. 24, 1970 from *Porphyra yezoensis* which grow on nets in the sea. *Flavobacterium/Cytophaga* was isolated on Jan. 20, 1975 from *P. yezoensis* which grow on rocks in the sea.
Beneckea was isolated on Jan. 17, 1974 from *P. yezoensis* which grow on rocks in the sea.
The amount of carbon dioxide is expressed as a percentage of carbon in the derived carbon dioxied to the amount of carbon supplied to the substrate.

be O when it was less than 0.1%. All bacterial strains used decomposed amino acids and produced carbon dioxide, although the amounts differed among the bacteria.

Micrococcus sp. decomposed several amino acids leading to the production of small amounts of carbon dioxide, but threonine, serine, glycine, histidine, lysine and arginine did not lead carbon dioxide production. *Flavobacterium/Cytophaga* group decomposed serine, leucine and threonine, resulting in the production of much carbon dioxide, but low amounts occurred when glutamic acid, alanine and phenylalanine were used. Different amounts of carbon dioxide was produced when histidine, lysine, arginine and isoleucine were decomposed by the above bacteria. *Pseudomonas* sp. showed high percentages of carbon dioxide from many amino acids, but the amounts often differed markedly between the 2 strains. *Beneckea* sp. produced much carbon dioxide by decomposing proline. glutamic acid aspartic acid, alanine and serine, but the production was extremely low when valine, tyrosine, phenylalanine and histidine were used.

Discussion

It became clear that *Beneckea* sp. decomposed aspartic acid and glutamic acid, resulting in a high production of carbon dioxide. This fact seems closely ralated to the increased growth of *Beneckea* sp. when these 2 amino acids were added to the media (chapter 6). It is therefore conceivable that aspartic and glutamic acids are important nutrients for *Beneckea* sp. 5 amino acids, serine, leucine, threonine, arvinine and lysine, are considered also important for growth of *Flavobacterium/Cytophaga* group which are predominant on healthy *Porphyra*; these bacteria utilized these amino acids, though the amounts of carbon dioxide produced differed among the bacterial strains. Proline was well utilized by nearly all the bacterial strains and carbon dioxide produced was as high as 80.2% in *Achromobacter* sp. 48.5% in *Beneckea* sp., 85.6% in *Pseudomonas* sp. and 24.7% in *Flavobacterium/Cytophaga* group. On the other hand, *Pseudomonas* sp. and *Achromo*monas sp. decomposed proline and produced the highest amounts of carbon dioxide of all amino acids tested, whereas the *Flavobacterium/Cytophaga* group produced the highest amount of carbon dioxide after decomposing serine. The numbers of amino acids highly decomposed were largest in *Beneckea* sp., and smallest in *Micrococcus* sp.

Chapter 8. Organic substances liberated by *Porphyra* as extracellular Products

It is well known that algae release many kinds of metabolites as extracellular product (FOGG 1962, LEFEVRE 1964, HELLEBUST 1974). Above all, the liberation of organic substances has been reported in many algae, eg., several marine algae (SIEBURTH 1969, MOEBUS and

JOHNSON 1974), 1 species belonging to the Chrysophceae (AARONSON 1971) and several marine phytoplankton (BERMAN and HOLM-HANSEN 1974). Little is known of organic substances as extracellular products in *Porphyra*. In chapter 6, bacteria associated with *Porphyra* were shown to utilize glucose and amino acids for their growth. It is probable that these organic substances originate from the host. The present study was carried out to show that *Porphyra* liberates organic substances.

Materials and methods

1) Algal material

Porphyra yezoensis was kept at −20°C until the initiation of the experiment. The experiments started after culturing *Porphyra* for 1 week and the thalli were punched to make round pieces, 8mm in diameter.

2) Measurements of organic carbon liberated by *Porphyra*

The instrument used in the experiment was the same as described in chapter 7 unless otherwise noted. A small amount of 1 μCi or 10 μCi of sodium bicarbonate with labelled carbon was placed outside a small chamber in a flask and then mixed with 10 or 50ml of artificial seawater and 5 or 7 round pieces of *Porphyra*. The artificial seawater used was modified from the medium shown in Table 1 to deplete the organic substance by adding disodium phosphate instead of sodium glycerophosphate. The specific activity of carbon-14 in bicarbonate was 57.7 mCi/m mol. Hydroxide hyamine 10-x dissolved in methanol and filter papers (see chapter 7) were placed in the small chamber. 1ml of 12N HCl was used to stop the reaction leading to the production of carbon dioxide by lowing the pH to 2 or

Table 27 List of experimental condition in the production of organic carbon.

Experiment No.	Media ml	Quantities of carbon-14 added μCi	Hyamine 10-x ml	No. of pieces of *Porphyra yezoensis* used
1	10	10	3	7
2	10	10	3	7
3	10	10	3	5
4	10	10	3	7
5	10	10	3	5
6	10	10	3	5
7	10	10	3	5
8	10	1	1	5
9	10	1	1	5
10	50	10	3	5
11	50	10	3	5
12	50	10	3	5
13	50	10	3	5

less. Measurements were made after shaking for another 1 hour to trap thoroughly the carbon dioxide produced outside a small chamber to filter papers. The organic compounds produced from the labelled inorganic carbon during photosynthesis remained in the medium outside the small chamber; the amounts were determined by measuring the radioactivity of the medium. The scintilator used was an instagel made by Packard. The radioactivity of carbon-14 absorbed by pieces of *Porphyra* was measured using an Aloka GM counter. The experimental conditions are shown in Table 27.

Results

Results obtained are shown in Table 28. The amounts of extracellular products released by *Porphyra* varied depending on plant viability, concentration of carbonate in medium, temperature and light intensity. The final values of the radioactivity of organic carbon in the medium ranged from 0.6 to 4.1% of the initial value of carbon dioxide supplied.

Table 28. Radioactivity of organic carbon found in the media at the end of the experiments.

Experiment No.	Radioactivity at the beginning μCi	Radioactivity at the end	
		dpm	μCi
1	10	434, 416	0. 195
2	10	318, 190	0. 143
3	10	271, 413	0. 122
4	10	596, 279	0. 268
5	10	136, 172	0. 061
6	10	741, 141	0. 333
7	10	407, 634	0. 183
8	1	76, 911	0. 034
9	1	45, 751	0. 020
10	10	536, 232	0. 241
11	10	691, 581	0. 311
12	10	874, 732	0. 394
13	10	919, 476	0. 414

dpm : Disruption per minutes.

Discussion

It was elucidated that *Porphyra* released organic matter produced during photosynthesis.

MOEBUS and JOHNSON (1974) reported that *Ascophyllum nodosum* discharged dissolved organic matter. AARONOSON (1971) indicated that *Ochromonas danica* released carbohydrates, nucleic acid, proteins and lipids. BERMAN and HOLM-HANSEN (1974) showed that marine plankton liberated photoassimilated carbon as dissolved organic substance into eutrophic water in amounts of 6-12% and into oligotrophic waters 17-27% of photoassimilation. They also found that

marine phytoplankton released orgnic substance only 5% of photoassimilation in logarithmic growth phase. TANAKA *et al.* (1974) demonstrated that the amounts of extracellular metabolic exuded by phytoplankton in Lake Biwa amounted to 1-11% of the total carbon of photosynthesis. They detected considerable quantities of glycollic acid in the water and suggested that this chemical was liberated by phytoplankton and might play an important role as nurishment for microorganisms.

It has been known that considerable amounts of free amino acids and peptides are liberated by planktonic algae (HELLEBUST 1974). FOGG and WESTLAKE (1955) showed that these nitrogenous substances accounted for as much as 59.6% of the total extracellular organic matter in *Chlamydomonas* sp. and 60.9% in *Anabaena* sp.. SIEBURTH (1969) detected carbon, carbohydrate, protein and polyphenol substances in metabolites discharged by several marine algae, and found that the amounts of total organic carbon liberated ranged from 54.2mg C/100g/h in *Ascophyllum nodosum* to 4.4mg C/100g/h in *Chondrus crispus*.

Table 29. Radioactivity caliculated in one piece[a] of *Porphyra yezoensis* at the end of the experiments.

Experiment No.	Radioactivity at the end cpm
2	2,532
	2,133
	2,898
	2,295
4	2,360
	3,029
	1,860
8	218
	200
	156
	198
	168
	204
13	1,628
	1,618
	1,442
	1,160

cpm : Count per minutes ; a : 50. 24mm^2.

As stated before, the minimum final value of radioactivity of organic carbon in the medium was 0.6% of the initial value of carbon dioxide supplied. This value can be changed to 0.0145 μg on the basis of specific activity. This calculated value corresponds to 0.88mg C of organic carbon extracellularly liberated for 1 hour by *Porphyra*, on the basis of 100g dry weight. From this transformed value, the final value of organic carbon in the present experiment ranged from 0.88mg C/100 g/h to 5.88mg C/100 g/h. The values obtained are smaller than those described by SIEBURTH (1969), but the seaweed taxa are different. On the other hand, the values, of ^{14}C-assimilation of *Porphyra* (Table 29) are mostly identical to data which TSUKIDATE (1968) showed using healthy *Porphyra* and the amounts of organic carbon liberated in the present experiment are therefore considered reasonable.

Chapter 9. Exudation of amino acids into the environment by *Porphra*

As descibed in Chapter 8, *Porphyra* releases organic carbon as much as 0.6-4.1% of the carbon supplied as inorganic form. Some unicellular algae discharge organic carbon in amino acids, as has been shown by FOGG and WESTLAKE (1955), FOGG (1962), HELLEBUST (1974) and JOLLEY and JONES (1977). Therefore, in the present study the media in which *Porphyra* grew were analysed for the existence of extracellular amino acids.

Materials and methods

1) Algal material

Porphyra tenera used was collected in Matsukawa-ura, Miyagi pref. 10 healthy plants 2-5cm long were grown at 3,000 lux at 10°C for 23 days, or at 20°C for 8 days in 500ml Erlenmeyer flasks in the modified artificial seawater described in the Materials and Methods of Chapter 8. At the end of the time in culture, the thalli were somewhat paler and more mature, but no white and dead cells were present. Most thalli attained a length of 10cm and a width of 1cm.

2) Amino acid analyse

After removing *Porphyra*, the media were filtered successively through 0.45μm and 0.22μm milipore filter to eliminate microoorganisms. The filtered samples were evaporated in a water bath kept at about 40°C until reduced to 200ml or one tenth, the original volume. The concentrates were passed through an ion exchange resin of Umberlite CG-120 Type 3 in a colum 1.5cm in diameter and 20cm in length after washing the resin with 30-40ml of distilled water and injecting 0.01N HCl until pH reaches 2. Each loaded column was eluted with about 100ml-200ml of a 2N ammonia solution. The fluates were completely evaporated under vacuum at about 40°C. Before passing through the resin, the concentrates were sometimes evaporated thoroughly and dissolved with 5ml of 1N HCl in 45ml of methanol.

The solutions were filtered through Toyo No. 2 filter paper and then passed through the resin. Recovery of amino acids with this preparation was 82-80% except for 40% of the cystin.

Table 30 Composition of buffer used.

Sodium citrate $2H_2O$	19.6 g
Conc. HCl	16.5mℓ
Thiodiglycol 25% solution	20 mℓ
Caprylic acid	0.1mℓ
Final volume	1,000 mℓ

3) Measurements of amino acids

Samples were calculated to a fixed amount with 30% formic acid and again evaporated. In each sample, a buffer solution (Table 30) of 0.1 ml was added

prior to analysis. A beckman 121 type amino acid analyser was used.

Results

As described in Chapter 6, bacteria attached to *Porphyra* utilized amino acids for their growth. Therefore, free amino acids analysed in the prerent study seem to be a surplus of the acids exuded by *Porphyra* over the consumption of bacteria. The amount and composition of the free amino acids liberated by *Porphyra* into the media are shown in Table 31 and Figs. 3, 4 and 5. Many amino acids were discharged into the media by *Porphyra* during the experiment. When *Porphyra* was cultured at 10°C for 23 days, amino acids produced extracellulary by 973.8mg dry weight of the plant contained the highest amount

Table 31. Amount and composition of free amino acid which *Porphyra thalli* liberated in culture medium.

Age of culture	23 days	8 days
Temperatures	10℃	20℃
Amino acids	μg / 973.8mg on dry basis	μg / 316.7mg on dry basis
Aspartic acid	319.2	168.5
Threonine	166.6	43.6
Serine	735.0	185.5
Glutamic acid	102.9	53.9
Proline	333.5	0
Glycine	360.0	180.0
Alanine	204.7	68.2
Valine	105.3	23.4
Methionine	14.9	0
Isoleucine	52.4	17.5
Leucine	64.5	30.1
Tyrosine	162.9	72.4
Phenylalanine	115.5	82.5
Histidine	31.0	25.8
Lysine	14.6	97.3
Arginine	51.0	51.0

of serine as much as 735μg and the lowest amount of methionin as much as 4.9μg. On the other hand, 316.7mg dry weight of *Porphyra* cultured at 20°C for 8 days released amino acids containing the highest amount of serine as much as 185.5μg and the lowest amount of isoleucine as much as 17.5ug. The amounts of amino acids fluctuated depending on temperature, but their relative compositions were always unchanged. Asparatic acid, glutamic acid, glycine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine were higher in amount when *Porphyra* was cultured at 20°C for 8 days than at 10°C for 23 days (Table 31, Figs, 3, 4 and 5).

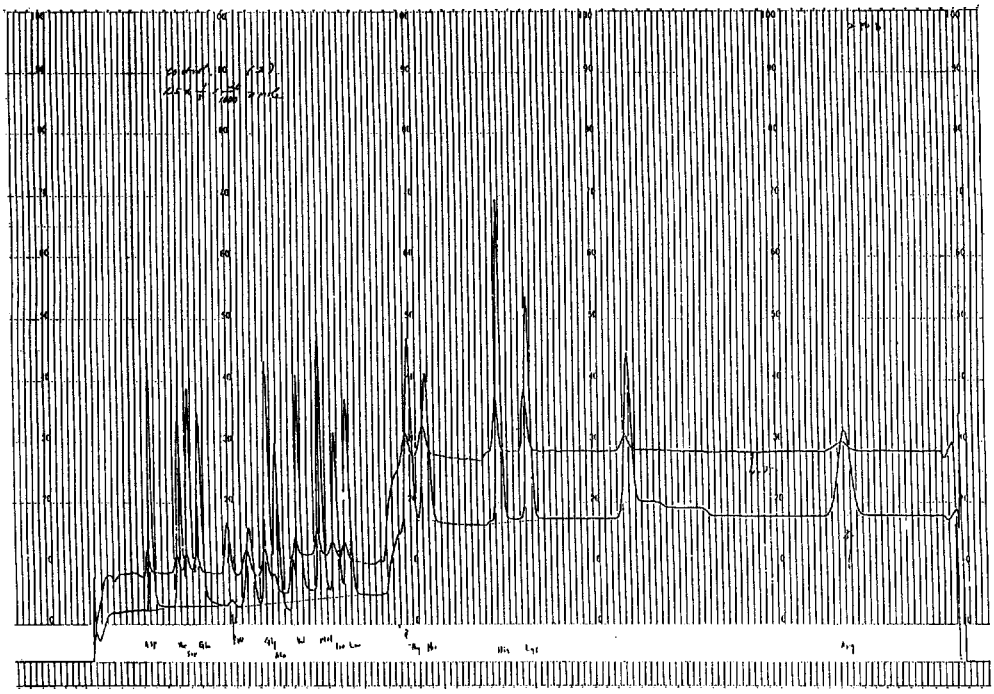


Fig. 3. Chromatogram of amino acids mixture (5 n mole).

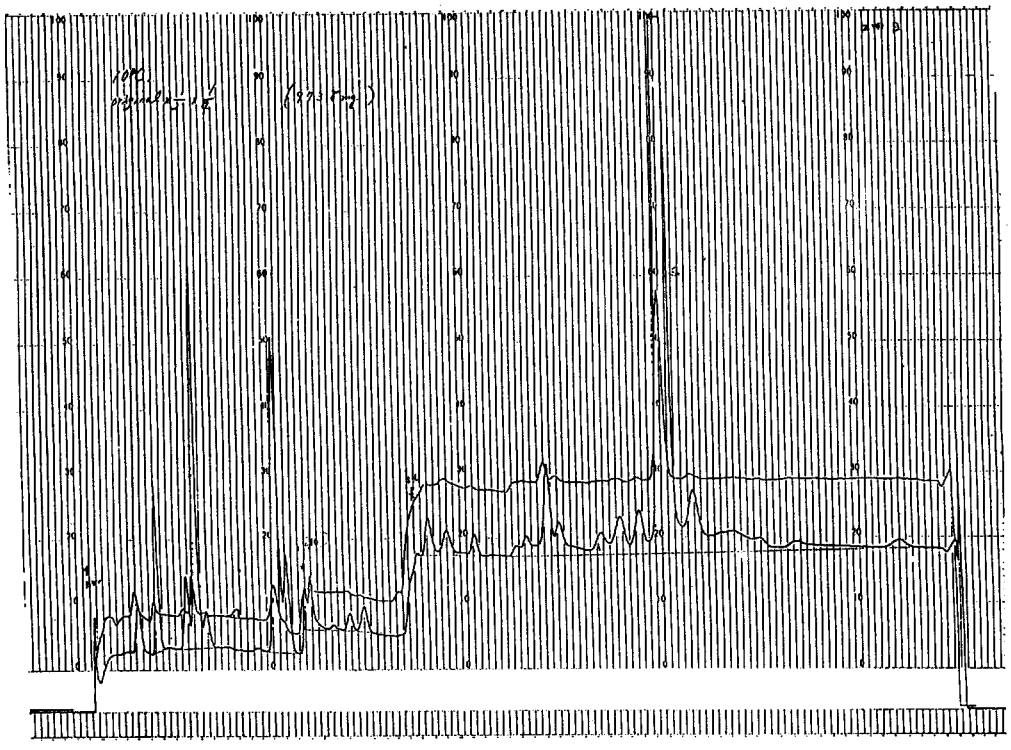


Fig. 4. Chromatogram of amino acids in the medium in which *Porphyra* species grow at 10°C.

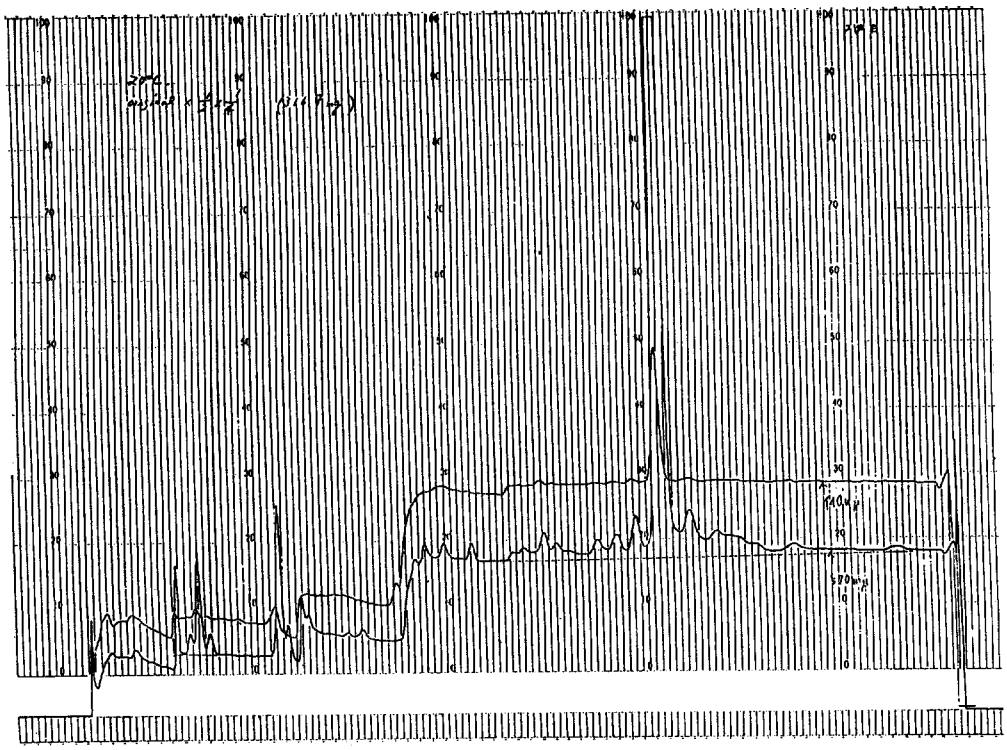


FIg. 5. Chromatogram of amino acids in the medium in which *Porphyra* species grow at 20°C.

Discussion

FOGG and WESTLAKE (1955) reported that several unicellular fresh water algae discharged some amino acids into ambient water. FOGG (1962) also found that some amino acids existed in extracellular metabolites liberated by algae. However, amino acids were not fractionated in above two references. The present study is the first to show that *Porphyra* liberates amino acids and that they are fractioned in detail. Amino acids secreted by *Porphyra* include almost all amino acids which have been known so far. The quantities of the amino acids discharged changed depending on enviromental conditions and increased at higher temperature like 20°C which was too high and unfavorable for *Porphyra* to grow. It is interesting to note that asparatic and glutamic acids, amino acids favoring the growth of *Beneckea* sp., one of the pathogens causing white rot, are contained in the extracellular amino acids secreted at higher temperature by *Porphyra*.

Chapter 10. Effect of nutrients on the growth of *Porphra* in bacteria-free culture

It is known that a deficiency of organic substances slows growth of *Porphyra*, apparently as a result of physiological upsets (Author's unpublished data). On the other hand, bacteria attached to *Porphyra* utilize amino acids for their growth and discharge carbon dioxide, as described in Chapters 6 and 7. Therefore, in the present study *Porphyra* was grown in axenic cultures so as certain the requirement of organic nutrients and the effects of carbonic acid substances for its growth. Carbonic acid substances includes the following four chemicals in the present study: dissolved carbon dioxide, carbonate ion, bicarbonate ion and carbonic acid.

Bacteria-free cultures of unicellular algae have been grown by LEWIN (1959), MCDANIEL *et al.* (1961), BROWN and BISHOFF (1962) SOLI (1964), PRINGSHEIM (1964), TOKUDA (1966) and STEIN (1973). Multicellular algae have been grown in axenic culture by Provasoli on *Ulva* (1958) by TATEWAKI and PROVASOLI on three species of *Antithamnion* (1963) and by FRIES and PETTERSSON on several red algae (1968).

Materials and methods

1) Algal materials

The following three species were employed in the present study.

a) *Porphyra leucosticta*, harvested from a stock culture maintained in a round-bottom 500ml flask with aeration using artificial seawater (Table 1).

b) *Porphyra yezoensis*, collected from population growing on inshore rocks near the Nansei Regional Fisheries Research Laboratory.

c) *Porphyra tentra*, collected at Matsukawa-ura, Miyagi pref.

2) Purification

The attainment of bacteria-free culture of *Porphyra* was difficult, because of microorganisms in its mucilagenous surface layer. Surface sterilization was utilized using exclusively a mixture of the anti-biotics. The thalli were cut into 25mm^2 pleces. The pieces were dipped and dragged for 30-35cm through artificial seawater (Table 1) solidified with 1% agar. Then the pieces were buried for two weeks in a petri plate containing 50ml artificial seawater mixed with penicillin G in a dose of 40,000 units and streptomycin sulfate in a dose of 20mg (pot.) solidified with 1% agar.

Another method of eliminating microorganisms was also used. Pieces of thallus was placed for overnight in 50ml seawater containing 20,000 unit of penicillin G, 10mg (Pot.) of strepto-

Table 32. Component of the enrichment used.

Pure water	100 mℓ
$NaNO_3$	350 mg
Na_2glycero PO_4	50 mg
$FeCl\ 6H_2O$	12.1mg
Metal solution	25 mℓ
Vitamin B_{12}	10 γ
TRIS	500 mg
Metal solution 1 mℓ =	
H_3BO_3	0.2 mg
$FeCl_3\ 6H_2O$	0.048mg
$MnCl_2\ 4H_2O$	0.144mg
$CoCl_2\ 6H_2O$	0.04 mg
$ZnCl_2$	0.01 mg
Na_2 EDTA	1 mg

mycin sulfate and 2ml of ES medium (Table 32), after the "dip and drag" treatment. These procedures were repeated several times to obtain perfect sterilization.

Axenic culture of juvenile *Porphyra* plants 1mm long were obtained by soaking for two days in a test tube containing 30ml of artificial seawater mixed with 200,000 units of penicillin G and 1g (Pot.) of streptomycin sulfate, and was shaked violently in the test tube to eliminate attached microorganisms. After this procedure was repeated three times, the thallus was removed from the test tube to a Petri dishes containing bacterial media and was cultured for 5-7 days. Then the thallus free from bacteria was transferred into a sterilized media. The last mentioned technique was most successful to obtain clean thalli.

3) Sterility test

The sterility test media used were ST_3 (TATEWAKI and PROVASOLI 1963) and E_6 (PROVASOLI *et al.* 1956) and the establishment of axenic culture was examined one month later.

4) Culture of *Porphyra*

In experiments 1, 2, 3 and 4, a piece of sterilized 25mm² *Porphyra* was cultured in a 20ml L test tube containing 10ml of medium using a culture apparatus of Monad. The cultures were maintained at 16 ±0.5°C under a 9 : 15 LD cycle. The light intensity was 5,000 lux at the surface of the test tube. In experiments 5 and 6, a sterilized thalli 1mm long were each cultured in 125ml, Erlenmeyer flasks containing 50ml of media using a shaking culture apparatus. The cultures were maintained at 17± 2°C under a 9 : 15 LD cycle. The light intensity was 3,000 lux at the surface of the flask. A modified artificial seawater described in Materials and Methods of Chapter 8 was used as a control.

Besides the above experiments, the nutritive requirement for the growth of *Porphyra* was examined using the following substances; Vitamines: thiamine, riboflavin, pyridoxine, pyridoxamine, cyanocobalamine, orotic acid, ascorbic acid, pantothenate, nicotinic acid, inositol, thymine, folic acid, putrescine, p-aminobenzoic acid, folinic acid, choline, biotin, amino acids: aspartic acid, glutamic acid, alanine, valine, arginine, lysine, leucine, isoleucine, serine, thyrosine, phenylalanine, threonine, tryptophane, histidine, proline, glycine, cystine, taurine, purine pyrimidine: guanine, cytosine, adenine, uracil, phytohormones: indol acetic acid, gibberellin.

At the end of experiment, plants were observed for colour, shape and the state of the growth and their length was measured.

Results

The effect of organic substances on the growth of *Porphyra* were not detected, though its growth seemed to be promoted by some amino acids. Therefore, it is uncertain whether organic substances used in the present experiment were required for the growth of *Porphyra*.

Results obtained are shown in Tables 33-38. In experiment 1 (Table 33), the media

Table 33. Growth of *Porphyra* in axenic culture using artificial sea water containing Tris (Experiment 1).

Addition mg/100mℓ	Remarks on growth	
	14 days	30 days
None	Reddish but growing	Reddish but growing
Tris 50	Normal and growing	Normal and growing

were replaced once 2 weeks later. The growth of *Porphyra* was better in the medium containing tris (hydroxymethyl) aminomethane (Tris) than the control. In experiment 2 (Table 34), media were not replaced. At the end of 19 days, *Porphyra* grew better and had

Table 34. Growth of *Porphyra* in axenic culture using artificial sea water containing Tris (Experiment 2).

Addition mg/100mℓ	Remarks on growth		
	9 days	16 days	19 days
None	Reddish but growing	Reddish but growing	Pale 5 mm long
Tris 50	Normal and growing	Normal and growing faster	Normal 1cm long

more natural colour in the medium containing Tris than the control. In experiment 3 (Table 35), the media were replaced once 3 weeks later. At the end of 30 days, the growth of

Table 35. Growth of *Porphyra* in axenic culture using artificial sea water containing Tris and sodium bicarbonate (Experiment 3).

Addition mg/100mℓ	Remarks on growth		
	3 days	21 days	30 days
None	Growing	Growing	No growing but alive
Tris 50 NaHCO 16.8	Growing	Growing faster	Normal and growing 3 cm long

Table 36. Growth of *Porphyra* in axenic culture using artificial sea water containing Tris and sodium bicarbonate (Experiment 4).

Addition mg/100mℓ	Remarks on growth		
	3 days	9 days	21 days
None	Growing	Normal and growing	Growing
Tris 50 NaHCO 16.8	Growing	Normal and growing	Growing faster

Table 37. Growth of *Porphyra* in axenic culture using artificial sea water (Experiment5).

Addition mg/100mℓ	Remarks on growth	
	39 days	44 days
None	Growing	Growing 5 mm long

Table 38. Growth of *Porphyra* in axenic culture using artificial sea water (Experiment 6).

Addition mg/100mℓ	Remarks on growth		
	10 days	14 days	22 days
None	Growing	Growing	Growing 5 mm long

Porphyra was better in the medium containing Tris and carbonic acid substances which were twice the concentration of artificial seawater. In experiment 5 and 6 (Table 37 and 38), the medium was exclusively modified artificial seawater used as control in other experiments and was not replaced. At the end of 44 days, *Porphyra* did not grow well and attained a length of only 5mm in both cases.

Discussion

Though antibiotic purification of *Porphyra* was not easy to establish, it became evident that better results were obtained when juvenile thalli less than 1mm long were used. In most cases, *Flavobacterium/Cytophaga* resisted to be eliminated in the pure culture. On the other hand, from the author's unpublished data, it was found that *Flavobacterium/Cytophaga* became undetected in bacteria flora on *Porphyra* within two weeks and *Pseudomonas* or *Vibrio* predomonated instead when casamino acid was added to the medium. Therefore it might be better to sterilize aseptically *Porphyra* after changing composition of bacterial flora.

Porphyra attained merely to the length of 3 cm at its maximum, as the culture vessel was not big enough for its growth. In addition, *Porphyra* retarded to grow and became pale when enough carbonic acid substances were not provided. However it could be said that *Porphyra* grows healthy in the bacteria-free culture provided the culture vessel is shaked and the medium is replaced during the experiment. Shaking is assumed to play an important role in supplying carbonic acid substances to *Porphyra*. The carbonic acid substances are also thought to be one of factors indispensable for the growth of *Porphyra* in the bacteria-free culture.

From the above results, it might be concluded that *Porphyra* grows well in the presence of carbon dioxide released by the attached bacteria.

Chapter 11. General discussion

SUTO *et al.* (1972) reviewed generally the diseases in *Porphyra* farming, describing the infectious white wasting disease as white rot. In order to detect the pathogens of white rot, the attached bacteria were isolated from healthy *Porphyra* and their biological and biochemical characteristics were investigated. Each isolate was identified to generic level according to the scheme described by Shewan *et al.* (1960a). The microflora on healthy *Porphyra* plants which had been cultured *in vitro* for several years consisted of *Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* and these bacteria, except *Micrococcus*, were often detected. On the other hand, *Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas*, *Aeromonas* and yeast were isolated from healthy *Porphyra* growing on rocks or "Hibi". The common genera which were detected on naturally growing *Porphyra* were also the same as described on *Porphyra* growing *in vitro*. Accordingly, it can be concluded that *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas* were the dominant genera of bacteria associated with healthy *Porphyra*.

Beneckea sp. was isolated from diseased *Porphyra* having the symptoms of white rot. The cells of diseased *Porphyra* became white and died, and the plants finally sloughed off from "Hibi". After examining the relationships between environmental factors and the occurrence of white rot, it was found that *Beneckea* sp. thrived on *Porphyra* when the thalli were exposed for more than 4 hours at dawn and a discrepancy of 8°C or more existed between the atmospheric and seawater temperatures. It is not clear whether this big discrepancy corresponds with the rise of seawater temperature or not. These factors seemingly responsible for outbreaks of white rot were considered to be a part of many primary causes. When *Beneckea* sp. were inoculated to healthy *Porphyra*, the cells became homogeneous and finally deteriorated, showing the symptom of white rot. *Beneckea* sp. was always isolated again from infected *Porphyra* in which white rot was caused experimentally. Therfore, *Beneckea* sp. was considered to be one of pathogens causing white rot.

The bacteria associated with *Porphyra* were tested for their nutrient requirement. Generally speaking, all bacteria attached to *Porphyra* utilized amino acids for their growth. Especially *Achromobacter*, *Flavobacterium/Cytophaga* and *Pseudomonas*, common members of bacterial flora on healthy *Porphyra*, used many amino acids, but *Beneckea* sp. grew well exclusively in the presence of alanine, aspartic acid and glutamic acid. As for the effects of temperature, *Beneckea* sp. grew well over 20°C, whereas *Flavobacterium/Cytophaga* grew slowly at this temperature. From the sodium chloride requirement, it was conceivable that *Micrococcus* and *Flavobacterium/Cytophaga* were of terrestrial origin, *Achromobacter* and *Pseudomonas* were marine bacteria and *Beneckea* sp. was halophilic. Considering the results obtained above,

Beneckea sp. seemed different from other attached bacteria studied in its nutrient requirements and the way it adapted to environmental factors.

It has been known that planktonic unicellular algae discharge organic substances but the fact that *Porphyra* also does so was here reported for the first time. The organic carbon produced by *Porphyra* during photosynthesis amounted as much as 0.6-4.1% of the inorganic carbon supplied.

Porphyra also released many kinds of amino acids to a medium composed exclusively of inorganic nutrients and the liberation of the mine amino acids, aspartic acid, glutamic acid, glycine, leucine, tyrosine, pheylalanine, histidine, lysine and arginine, was stimulated as temperature rose. Amino acids excreted by *Porphyra* were utilized by bacteria, leading to the production of carbon dioxide. However, the types of utilized amino acids and the amounts of released carbon dioxide differed among the bacteria. *Beneckea* sp. utilized well proline, glutamic acid, aspartic aicd, alanine and serine, whereas *Flavobacterium/Cytophaga* used only serine, leucine and threonine. *Porphyra* did not grow by providing any amino aicds, nucleic acids, and phytohormones when grown in axenic culture. On the contrary, carbon dioxide in amounts twice as high as in the artificial seawater used in the present study supported normal growth of *Porphyra*. It is clear from the above result that axenic *Porphyra* needs more carbon dioxide than when cultured in a medium contaminated with bacteria.

Porphyra grew well in the presence of carbon dioxide released by the attached bacteria and the extracellular amino acids of *Porphyra* also stimulated the growth of the bacteria. In other words, *Porphyra* provides amino acids to the attached bacteria and the bacteria produce carbon dioxide which stimulates the growth of *Porphyra*.

JOLLY and JONES (1977) reported a symbiotic relationship between *Navicula* and *Flavobacterium*. They speculated that the metabolite produced by the bacterium might work as a chelator to nourish the growth of the diatom. ALLEN (1977) considered that there was a mutual exchange of nutrients between aquatic plants and the attached bacteria with the result increased productivity in the lake. He also speculated that the amount of nutrients taken in by the diatoms attached to the aquatic plants increased with a greater availability of carbon dioxide produced by bacteria. The literature cited above suggests that a symbiotic relationship occurs exclusively between unicellular algae and the attached bacteria; there is so far no direct proof. However, now the first time a symbiotic relationship is indicated between *Porphyra* and the attached bacteria in relation to the liberation of amino acids and the production of carbon dioxide.

The outbreak of white blight disease is said to be triggered by unfavorable environmental factors as follows: (1) the seawater temperature becomes higher than usual, especially higher at the days between 50 and 70 from the beginning of cultivation in the sea (KATADA 1967);

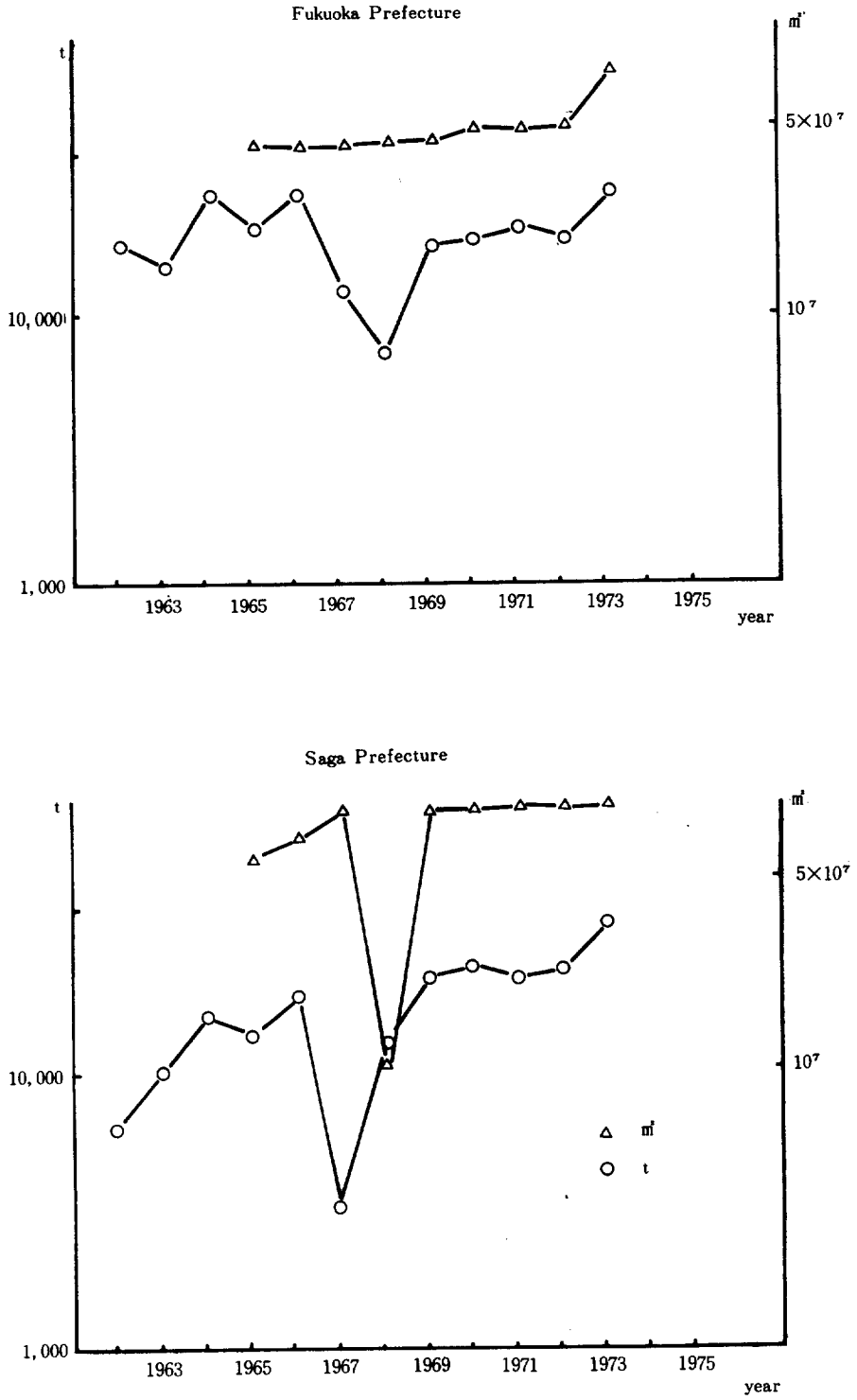


Fig. 6. Nori farming area and annual production.

(2) a shortage of nutrients like carbonic acid substances for the normal metabolism of *Porphyra* (KATADA 1967) and (3) when the pH increases due to stagnating seawater, resulting in the low metabolism of *Porphyra* (WATANABE *et al.* 1968).

As mentioned above, *Porphyra* utilizes carbon dioxide produced by bacteria; it needed more carbonic acid substance when grown in axenic culture. It was also shown that *Beneckea* sp decomposed aspartic and glutamic acids which were liberated by *Porphyra* at high temperature. Therefore, *Beneckea* sp. probably thrives when these 2 amino acids exist on the surfaces of *Porphyra* at higher seawater temperatures. In summary, *Porphyra* becomes unhealthy when oceanographic and meteorological conditions change and the plants exude abnormally amino acids which are readily available for *Beneckea* sp. ; the result is white rot.

The white wasting-like disease contains 2 disorders, the white blight and the white rot. When the 2 diseases occur together, much damage occurs in the *Porphyra* industry. Remarkable drop in the annual crops were shown in Saga Prefecture in 1967 and in Fukuoka Prefecture in 1967 and 1968. It is assumed that the drops were caused by bacteria and physiological disorders (Fig. 6).

Another disease caused by bacteria was described by ANDO and INOUE (1961) on *Laminaria japonica* var. *ochotensis*, in Wakkanai, northern Hokkaido. Experiments included the inoculation of *Vibrio* sp. which had been isolated from diseased *Laminaria*. A deteriorated spot, 1 mm in diameter, produced after 1 day, developed into a hole after incubation of inoculated *Laminaria* for another 2 days. They concluded that the disease was caused by alginase produced as the bacteria multiplied on *Laminaria*.

Seaweed diseases have been known to be caused by biological and physiological factors, but there has been little information about their pathology (ANDREWS 1967). There remain many unsolved problems of host-parasite interactions, except in some diseases of *Porphyra*, such as the relationships between *Porphyra* and *Pythium Porphyrae* in the red rot.

The present study shows for the first time that white rot disease of *Porphyra*, is probably caused by *Beneckea* sp.

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Explanation of Plates I—III

Plate I

Red cells found in the thalli grown on the rocks or the nets in the sea.

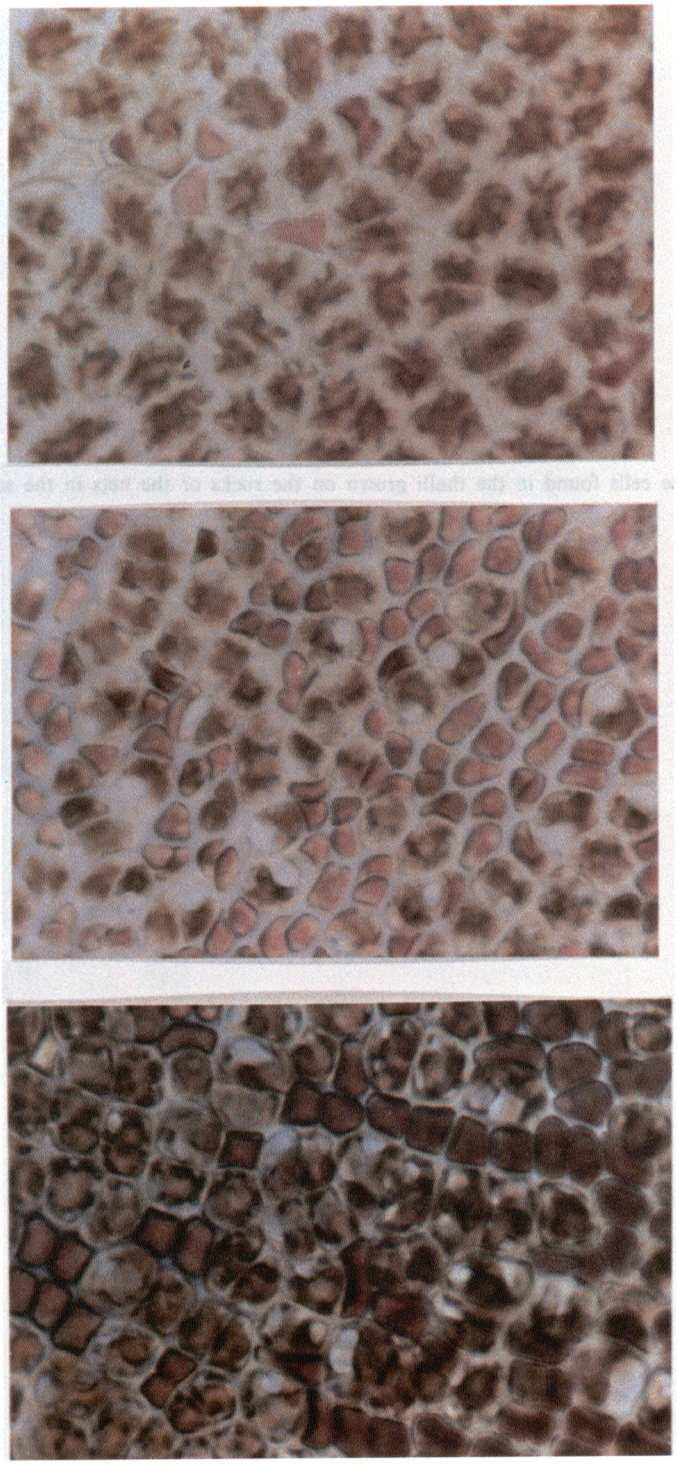


Plate II

White cells found in the thalli grown on the rocks or the nets in the sea.

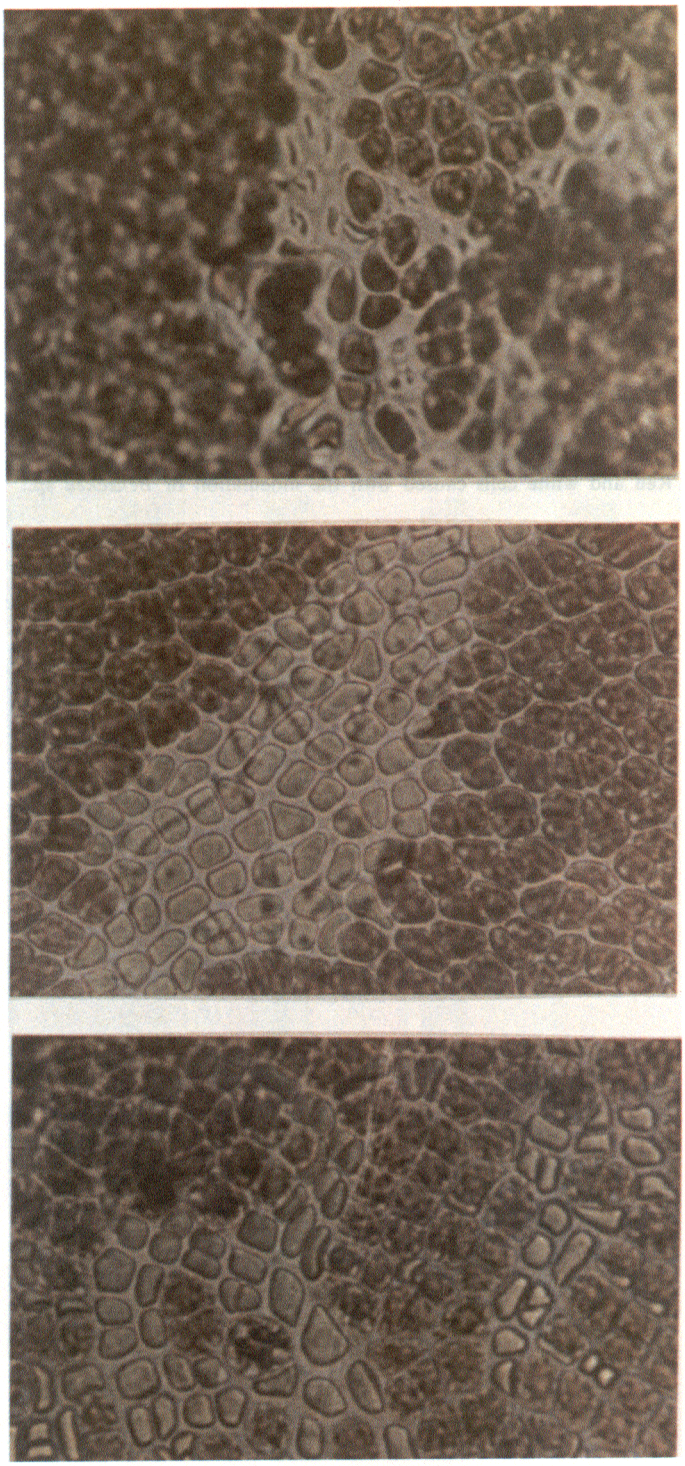
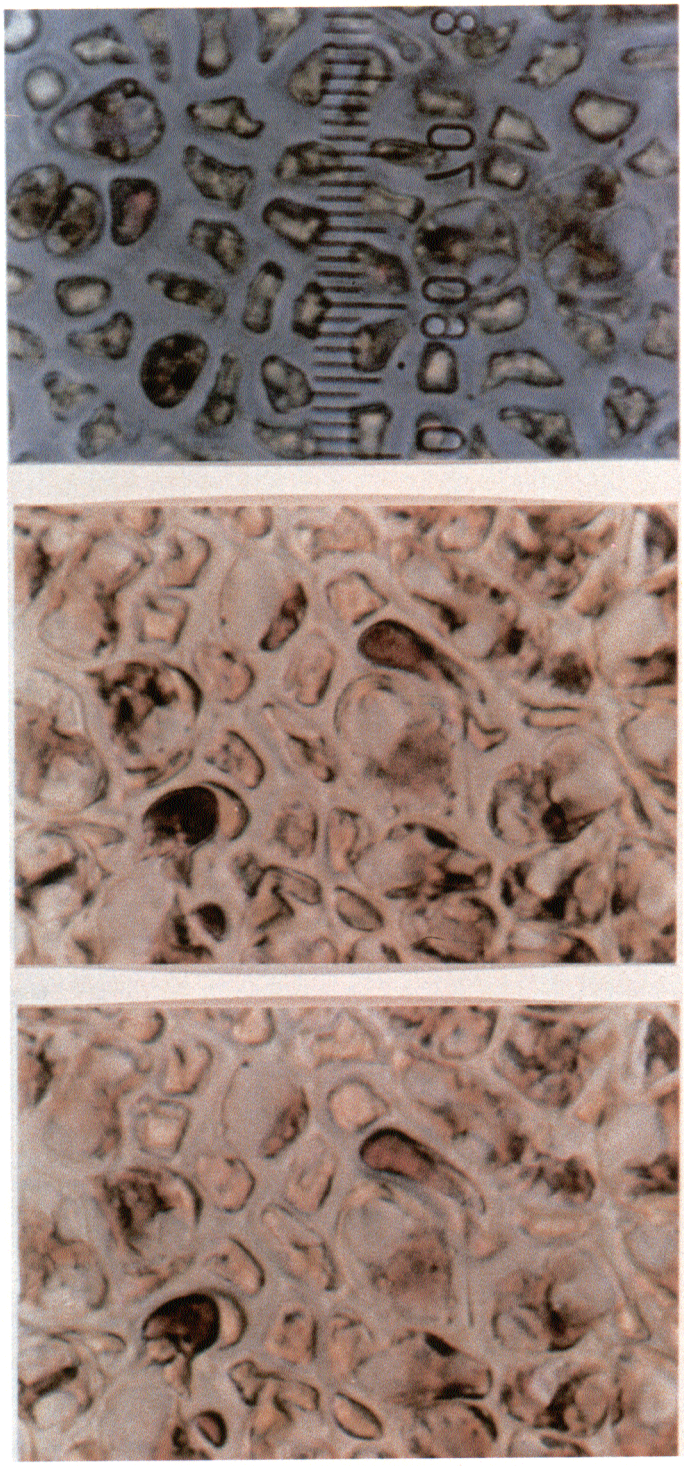


Plate III

Red and white cells caused with the inoculation of *Beneckea* sp..



ノリと着生細菌の相互作用及び白ぐされ病原因菌について

月 舘 潤 一

本研究は，わが国において養殖ノリに発生する病害のうち，産業的被害の最も大きい「白ぐされ病」の原因を調べるために行われた。その結果，ノリと細菌との間に相互関係の存在することが明らかとなり，又，細菌が本病の原因に関与していることも明らかとなった。これにより，原因菌が判明し，病気発生の機構が明らかになり，養殖の過程で生理異状の起きないように，養殖技術を適正に管理するなどの防除対策を立てる基礎資料を得た。本研究で得られた結論は以下のとおりである。

1) ノリ葉体の表層には，粘液層があり，その中に無数の細菌が存在することから，まず健全なノリ葉体上の菌叢を調べたところ，実験室で培養しているノリでは *Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas* から成り，このうち *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas* が常時みられた。又，天然の岩に自生しているノリ及び，養殖しているノリからは，*Micrococcus*, *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas*, *Aeromonas* 及び yeast が分離され，このうち *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas* が常時存在していた。従って，*Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas* がノリの着生細菌と考えられる。

2) 1973, 1974, 1975年にわたるノリ養殖年度において，ノリ病害調査を行なった。ノリには，前述のごとく着生細菌が存在するので，病葉だけでなく，健全葉体から病葉体になるまでの，全過程を通じて，定期的に葉体を調べ，細菌の分離を行った。その結果，いずれの年度においても，ノリ葉体上で *Beneckea* 属の細菌が菌叢の大部分を占めると，ノリの細胞は白色化して死滅するものが増加し，症状が進むと葉体の流失が見られた。

この *Beneckea* 属の細菌は，1973, 1974, 及び1975年のいずれの年度の分離菌株も諸性状が調べられた範囲では一致しており，これらの性状は均一で同一種と推定された。又，Type species の *Beneckea campbellii* ATCC-25920とは，かなり類似していたが，サッカローズ，ラクトーズ，アラビノーズ，キシローズの分解活性が異なり，TMAO，リジンの脱炭酸，10%食塩加培地での発育能，40℃における発育などの点で明らかに異なり *Beneckea campbellii* とは同定し得なかった。

更に Baumann ら (1971) 及び Lee ら (1978) の *Beneckea* 属菌株とくらべても性状は細部で異なり，既報のいずれの菌株とも一致しないため *Beneckea* sp. とした。

3) *Beneckea* sp. の病原性を調べるため，室内実験で，発症実験を行なった。*Beneckea* sp. を接種すると，ノリ葉体はすべて「白ぐされ病」の病徴を呈し，葉体は崩壊して原形が失われ，ついには消失するに致った。その際，病葉体から *Beneckea* sp. の再分離を試みたが，5回のうち4回は出現率が100%であり，残り1回は95%であった。このように，*Beneckea* sp. の病原性が明らかとなり，「白ぐされ病」の原因菌のひとつであることが確認された。

4) ノリと着生細菌の相互関係を明らかにするために *Beneckea* sp. 及び健全なノリ葉体から分離された細菌の形態的，生物学的，生化学的性状を調べた。その結果，*Beneckea* sp. は好塩菌であり，20℃でよく増殖し，着生細菌の *Achromobacter*, *Flavobacterium/Cytophaga*, *Pseudomonas* と比較して，アミノ酸に対する要求が異なっており，特に，アスパラギン酸，グルタミン酸を利用することが判明した。

5) 着生細菌はアミノ酸を利用して増殖するが，その結果，炭酸ガスを放出することが推定され，放射性物質のアミノ酸を用いて調べた。その結果，供試した7株の細菌は *Beneckea* sp. も含めて，すべてアミノ酸を利用し，分解して炭酸ガスを放出したが，細菌の種類により利用するアミノ酸が異なり炭酸ガスの放出量も異なった。そして，*Beneckea* sp. は，プロリン，グルタミン酸，アスパラギン酸，アラニン，セリンをよく分解した。

6) 藻類は代謝産物を細胞外に放出することが知られているが，ノリも生長の過程で，有機物質を細胞外に放出することを，放射性同位元素の炭素を用いて明らかにした。更にノリの着生細菌は，アミノ酸を利用し増殖しているという前述の結果から，ノリを培養した培養液中のアミノ酸を分析したところ，ノリは生長の過程で16種類のアミノ酸を細胞外に放出することが判明した。しかも水温をあげると，放出されるアミノ酸の組成には変化はないが，放出量が変り，アスパラギン酸，グルタミン酸，グリシン，ロイシン，チロシン，フェニールアラニン，

ヒスティディン，リジン，アルギニンが多くなることが明らかとなった。

7) 有機物質の欠乏は生理異状の原因となる。又，着生細菌はアミノ酸を利用して，炭酸ガスを生成しているので，ノリを無菌にして各種栄養塩の吸収を調べ，炭酸の影響も調べた。そこで，無菌培養を行ない，各種の有機物質を培養液に添加したが，生長はよくなかった。しかし，炭酸量を増加して培養したところ，生長が混菌培養と同程度になり，無菌ではノリの生長に炭酸ガスの補給が必要であることを明らかにした。このことから着生細菌はノリに炭酸ガスを補給していることが確認された。

8) 以上の諸結果から，ノリと着生細菌との間には次のような関係のあることが考えられる。即ち，ノリは着生細菌にアミノ酸を補給し，着生細菌はこのアミノ酸を利用して増殖し，その際，細菌は炭酸ガスを放出してノリの生長を促進する。又，細菌性の「白ぐされ病」が，発生する機構は以下のように考えられる。ノリと着生細菌との関係が，気象，海況の諸条件の変化で乱れて，ノリの放出するアミノ酸の量が異なり，アスパラギン酸及びグルタミン酸が増加すると，***Beneckea*** **sp.** が出現し，「白ぐされ病」が発生する。