Microbiological Studies of Porphyra Plants–IV

On the Relation between the Growth of the *Porphyra* Plants Cultivated in the Sea and the Bacterial Flora Accompanying the Plants

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Introduction

It was reported in one of my previous papers[1] that the *Porphyra* plants cultured in the laboratory declined in growth, sometimes abnormal cells, or eventually died when some change was given to the bacterial flora accompanying the plants.

The present paper deals with a research carried out to see whether such relations between *Porphyra* plants and bacterial flora observed in the laboratory do exist in the sea or not. The isolated microorganisms were identified to the level of their generic position.

Materials and Methods

Sampling of *Porphyra* plants: Samplings of *Porphyra* plants were made usually twice a week during a period from Oct. 19, 1970 through Feb. 16, 1971 from a culture bed set in the bay bottom off Inokuchi-cho, Hiroshima city, by picking up one or two thalli with a sterilized pincette at random from one or two portions of a *Porphyra* cultivation net, and they were placed in a test tube and brought to the laboratory within one hour.

Microorganism isolation method: The microorganisms attached to the *Porphyra* plant thalli were isolated by the two methods reported in the previous paper[2] applied singly or jointly, namely the method to streak on agar plate and the method to use homogenizer.

Medium for microorganism isolation: Various media, such as ZoBell medium, ZoBell 2216, ZoBell 2216 E, modified ZoBell 2216 E and *Porphyra* plant extract medium, all prepared by using artificial sea water as the basic component, and artificial sea water, artificial sea water plus meat extract and potassium nitrate, and artificial sea water plus peptone and potassium nitrate, were checked for their applicability. As a result, the modified ZoBell 2216 E medium was found to be goodfor culture of many isolated microorganisms, so this medium was adopted for the present research. (Table 1)

Identification of microorganisms: To identify the isolated microorganisms to the level of their generic position, the following bacteriological tests were carried out by the methods described herein.

1. **Morphology**

Morphology of bacterial colonies and cells was studied when they distinctly de-

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veloped on agar plates which has been streaked and incubated at 20°C for one day to one week.

2. **Gram stain**

Gram stain was checked by the routine method.

3. **Flagellum stain**

Flagella were examined by staining them according to the Loffler's method.

4. **Motility**

Motility of cells was checked by examining hanging-drop preparations of young cultures under the microscope.

5. **Spore stain**

Spores were examined by staining them according to the Moller's method.

6. **Oxidase test**

Oxidase test by the Kovacs'[3] method was applied to the colonies on the agar plate.

7. **Catalase test**

Isolated microorganisms were cultured on the slanting surface of agar medium, and catalase test was applied to this slant culture by pouring on it 1 ml of 3% H_2O_2 to examine whether or not gas bubbles would be generated immediately or five minutes later.

8. **Carbohydrate utilization**

Microorganisms were tested for their ability to dissimilate glucose when cultured in the marine oxidation-fermentation (MOF) medium of Leifson[4], using sea water in place of NaCl and pure water. The culture lasted for 14 days, and gas production as well as carbohydrate utilization was checked every day.

The isolated microorganisms were identified according to Bergey's Manual of Determinative Bacteriology, Breed et al. (1957)[5], and also to the works by Cowan et al. (1965)[6], Shewan et al. (1960)[7], and Shinano (1965)[8].

Observation: Observations of plant thalli and microorganisms attached to them were made with three different groups of plants, namely those cultivation of which was started on Sept. 29 and on Oct. 20, and those cultivated from Oct. 20 to Nov. 30, 1970, then store in the refrigerator at around −20°C till Jan. 6, 1971, and cultivated again from Jan. 7, 1971. The appearance rate of the bacterial colonies on agar plates was shown in the order of 10% because the borders of colonies in some cases were not clear.

Results and Discussion

The results of morphological observations of the cultivated thalli are summarized in Tables 2, 3 and 4, and the results of microbiological observations are shown in Tables 5, 6, 7 and 8. The isolated microorganisms identified to the level of genus and their characters are shown in Tables 9 and 10. Microorganisms isolated and identified to the level of genus were all bacteria, of which *Staphylococcus* and *Aeromonas* were also found on the *Porphyra* thalli cultured in vitro.

In the plant group cultivated from Sept. 29, 1970, many of the plant cells were found to have turned pale in colour and died 3 days after the *Vibrio* 1 had become predominant. Calluses were found to appear on the thalli sampled on and after Nov. 2, and in parallel with the *Aeromonas* 3 and the *Vibrio* 1 increased in number. In the

plant group cultivated from Oct. 20, 1970, the faded and dead cells, as well as the *Vivrio* 1 were not observed to increase throughout the cultivation period. Calluses were observed abundantly on the thalli sampled on Nov. 9 and 12, when the *Aeromonas* 3 was predominant. In the plant group cultivated from Jan. 7, 1971, by setting refrigerated nets in the sea, the thalli appeared to be unhealthy in color and they became pale by Jan. 17. So it was impossible to observe any correlation between the *Porphyra* plant and the appearance made by microorganisms. Calluses were not observed though there was an increase of the *Aeromonas* 3 on the thalli sampled on Jan. 21 and 26, 1971. The *Vibrio* 1 seemed to have had some effect on the production of the pale and dead cells. Whether the Aeromonas 3 has effect on the production of calluses or not is still uncertain. When calluses was observed, the *Vibrio* 1 increased in some case.

Table 1. Component of the medium used for microorganism isolation in the present research

Chemically defined medium	100 ml
Meat extract	0.2 g
Peptone	0.5 g
Potassium nitrate	0.05 g
Agar	1.5 g

Table 2. Observation of the *Porphyra* thalli cultivated from sept. 29, 1970.

Date	Thallus length*	Morphological remarks.
Oct. 23	5 mm	Thalli contorted.
Oct. 27	1 cm	Thalli contorted or dwarf.
Oct. 30	3 cm	Thalli contorted or dwarf.
Nov. 2	5～6 cm	Thalli contorted or dwarf, not healthy. Red spots were seen on the plants. Groups of clumped cells and red or faded cells were seen.
Nov. 5	8～10 cm	Thalli pale coloured. Clumped cells were scattered. Groups of red or pale cells were scattered. Cells with a shrinked chromatophore were seen.
Nov. 9	12～15 cm	Many clumped cells were seen. Groups of red or faded cells were seen. Enlarged cells and cells containing many granules were observed. Reproductive cells were formed. Bacteria were observed increasing.
Nov. 12	15～20 cm	Thalli stopped growing. Clumped cells were observed increasing. Groups of red or faded cells were seen increasing, and enlarged cells and cells containing granules decreasing. Thalli looked unhealthier than the preceding ones.
Nov. 16	30～35 cm	Clumped cells were observed. Red and faded spots were seen on the thallus surface. Thalli shirnked and with decay of tips. Groups of faded cells were greater in number than in the preceding thalli.

* The upper limits of the thallus length are shown excepting those of extremely long thalli.

Table 3. Observation of the *Porphyra* thalli cultivated from Oct. 20, 1970.

Date	Thallus length	Morphological remarks
Nov. 9	5 mm	Thalli contorted. Many clumped cells were seen. A few groups of pale cells were seen.
Nov. 12	1 cm	Clumped cells were seen. A few groups of red and pale cells were observed.
Nov. 16	1～1.5 cm	Thallus tips decaying as a result of dense growth of germlings. Clumped cells were seen. Groups of red and pale cells were seen. Holes were fomred in a line.
Nov. 20	2 cm	Thalli in dense growth. Clumped cells were observed increasing. Remarkable red dots on the thallus surface were seen. Groups of pale cells, enlarged cells, and cells containing granules were seen. Groups of red cells were formed in mosaics. Bacteria were observed increasing.
Nov. 24	5 cm	Thalli were seen to have grown well, and in good colour. Enlarged cells were seen.
Nov. 27	5～6 cm	Thalli were seen to have grown slowly, but in good colour. Clumped cells and red dots on the surface were seen. Groups of pale and red cells were seen.
Dec. 1	6～7 cm	Thalli growing slowly. Groups of red and pale cells were seen. Cells with a large vacuole and plasmolyzed cells were seen. Pythium sp. was abundant on thallus surface.
Dec. 7	10 cm	Thalli stopped growing, decaying at tips and along margins. Red dots or the symptoms of the disease called "Akagusare" were seen. Clumped cells were observed decreasing. Groups of red, pale, and green cells were seen.

Table 4. Observation of the *Porphyra* thalli cultivated from Oct. 20 to Nov. 30, 1970, then stored in the refrigerator at around −20°C for about one month, and cultivated again from Jan. 7, 1971

Date	Morphological remarks
Jan. 14	Thalli were soft and ready to decay. Groups of red and pale cells were seen abundantly. Groups of green cells, cells containing granules, and cells with a large vacuole were observed.
Jan. 18	Thalli were seen to have grown, but contorted, soft and ready to decay. Groups of red, pale, and green cells, cells with granules, and cells with a large vacuole were seen. Reproductive cells were formed.
Jan. 21	Thalli were pale, soft, and ready to decay. Groups of pale cells were decreasing. Pythium sp. was abundant on thallus surface. Reproductive cells were formed all over the thallus.
Jan. 26	Thalli stopped growing, pale in colour, and decaying at tips and along margins. Pythium sp. was abundant on thallus surface. Reproductive cells were formed all over the thallus.

Table 5. Microorganisms isolated from the *Porphyra* thalli cultivated from Sept. 29, 1970 by streaking on agar plate.

Baterial starin \ Date of sampling	Oct. 23	Oct. 27	Oct. 30	Nov. 2	Nov. 5	Nov. 9	Nov. 12	Nov. 16
①	○*	○	○	10**				
②	○	○	○	○				
③	○	○	○	○	10			
④				○	○			
⑤	○		○	10	20	30	30	30
⑥		○	40	10	10	30	30	40
⑦			40				○	
⑧	30	40		50	30	20	20	10
⑨	40	30			30			○
⑩		○	○					
⑪	○							
⑫						○		
⑬								

*○ indicates microorganism colonies which did not make their appearance clearly
** The figure shows the percentage of bacterial colonies appeared.

Table 6. Microorganisms isolated from the *Porphyra* thalli cultivated from Oct. 20, 1970 by streaking on agar plate.

Bacterial strain \ Date of sampling	Nov. 9	Nov. 12	Nov. 16	Nov. 20	Nov. 24	Nov. 27	Dec. 1	Dec. 7
①					○	○	○	○
②		20		○				
③	20			○	○	○	○	
④								
⑤	20	10	○	20		○	○	○
⑥	20		○		○	○	○	○
⑦								
⑧	40	40	○	20	○	○	○	○
⑨		20	○	20	○	○	○	○
⑩								○
⑪								
⑫					○		○	
⑬				20		○		

Table 7. Microorganisms isolated from the *Porphyra* thalli cultivated from Oct. 20, 1970 by using a homogenizer

Bacterial strain \ Date of sampling	Nov. 9	Nov. 12	Nov. 16	Nov. 20	Nov. 24	Nov. 27	Dec. 1	Dec. 7
①			○		30		10	○
②	○	○		○				
③	○	10		○			○	
④						10		
⑤		60	○	30		40	10	○
⑥			○				10	○
⑦								
⑧			○				10	○
⑨	20		○				10	○
⑩	20	20		30	30	30	20	
⑪	20							
⑫	20				○	○		
⑬								

Table 8. Microorganisms isolated from the *Porphyra* thalli cultivated again from Jan. 7, 1971 by streaking on agar plate.

Bacterial strain \ Date of sampling	Jan. 14	Jan. 18	Jan. 21	Jan. 26
①	20	10	10	10
②		○		
③			○	○
④			○	○
⑤	○	○	30	50
⑥	20	10	20	10
⑦	20	20	10	10
⑧	20	30	10	10
⑨		10	10	○
⑩	○	○		○
⑪	○			
⑫				
⑬				

Table 9. Morphological and biochemical characteristics of microorganisms isolated from *Porphyra* thalli in *Porphyra* culture year, 1970.

Micro-organism	Morphology	Gram stain	Flagella	Moti-lity	Spore	Oxi-dase	Cata-lase	O-Ftest gas produc-tion	H_2S produc-tion
strain ①	Circular, entire, R. $0.5\times1.4\ \mu$	−	Pe	+	None	−	+	F.G	+
strain ②	Circular, entire, red R. $0.7\times1.7\ \mu$	−	Pe	+	None	−	+	F.G	+
strain ③	Circular, entire, yellow R. $0.5\times1.4\ \mu$	−	Po	+	None	−	+	O.	−
strain ⑤	Circular, entire R. $0.65\times2.25\ \mu$	−	Po	+	None	+	+	F.G	+
strain ⑥	Circular, irregular R. $0.8\times2.5\ \mu$	−	Po	+	None	+	+	F.	+
strain ⑧	Circular, entire R. $0.5\times2.0\ \mu$	−	Po	+	None	+	+	F.	+
strain ⑩	Circular, entire light yellow, S. $1.1\ \mu$	+	None	−	None	−	+	F.	+
strain ⑪	Circular, entire R. $0.8\times1.5\ \mu$	−	None	+	None	+	+	F.G	−

G. gas production; F. fermentative; O. oxidative; Pe. peritrichous; Po. polar

Table 10. Identification to the level of genus of microorgamisms isolated from *Porphyra* thalli in *Porphyra* culture year, 1970

Microorganisms	Genus
strain ①	Enterobateriaceae 1
strain ②	Enterobacter 1
strain ③	Xanthomonas 1
strain ⑤	Aeromonas 3
strain ⑥	Vibrio 1
strain ⑧	Vibrio 2
strain ⑩	Staphylococcus 2
strain ⑪	Beggiatoa 1

Bacillus 1 which had effect on the production of pale and dead cells on *Porphyra* thalli in the laboratory experiments was not isolated in the present investigation. From this fact it may be said that the relationships between the *Porphyra* thalli and the accompanying bacteria in situ differ from those found in vitro. Or there may exist a synbiotic relation between plant and bacteria which includes similar relations observed in laboratory and in nature, but further studies are needed to reach a conclusion. During the present investigation, pale and dead cells on *Porphyra* thalli were observed to increase in several occasions, but they never developed so much as causing industrial damages, so no conclusive remark can be made whether or not the *Vibrio* 1 is a cause of the *Porphyra* disease "White rot".[9]

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Summary

1. This paper reports a result of the study carried out to see if there exists in the sea the synbiotic relations between the *Porphyra* thalli and the accompanying bacteria which were observed in the laboratory.

2. As *Bacillus* 1 was not isolated in this study, the synbiotic relations between *Porphyra* and microorganisms are supposed to be more complicated than observed in vitro.

3. Production of pale and dead cells on *Porphyra* thalli is considered to be related with the effect of *Vibrio* 1.

4. The bacteria isolated from Porphyra thallus and identified to the level of genus are *Enterobacteriaceae* 1, *Enterobacter* 1, *Xanthomonas* 1, *Aeromonas* 3, *Vibrio* 1, *Vibrio* 2, *Staphylococcus* 2 and *Beggiatoa* 1.

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