Microbiological Studies of *Porphyra* Plants–III.*

Abnormality on the Growth of *Porphyra* Plants by the Disturbance of the Bacterial Flora Accompanying the Plant

Jun-ichi TSUKIDATE

As reported previously[1),2)], strains 1, 2, 3, 4, 5, 6, and 7 became attached to or adhered to the surface or penetrated the cells of the healthy growing *Porphyra* plants all or part of the way. Then it is assumed that when the *Porphyra* plant grows normally and healthy, accompanying bacteria balance each other, and if these bacteria lose their balance, the plant begins to grow abnormally. In order to demonstrate this hypothesis, culture experiments were conducted by inoculating isolated bacteria[2)] singly into the medium, into which the healthy plant was been growing. Results are reported here which were verified by repeating the experiment seven times that the plant was grown abnormally with the different bacterial floras.

Material and Method

Material. A material used in this experiment was *Porphyra leucosticta* THURET which had been grown in the laboratory. A size of material was 5 mm long and 2–4 cm long. 5 mm long thalli were used intact while 2–4 cm long thalli were cut into about 5 mm square with a razor.

Elimination of Bacteria. The material employed in this experiment was washed in sterilized sea water and then streaked on 1% agar plate with an artificial sea water to disturb the balance of bacteria on or in the cell. The following procedure was practised to get the materials less contaminated. At first a thallus or a part of the thallus was washed in sterile sea water in the test tube by shaking with a cap on it. The purpose of this procedure is to eliminate bacteria physically from the material. After some shaking the material was taken up with a sterile pipet into another test tube and did the same thing again.

Next the plant was brought onto the 1% agar plate with the artificial sea water in a Petri dish and was streaked with a sterile pipet. This procedure is also for eliminating bacteria physically from the material. Yet the material was not free of bacteria with these procedures, that is, some bacteria still grew on the plant. Bacteria other than strains 1, 2, 3, 4, 5, 6 and 7 are considered to be washed off from the facts previously reported[2)]. However all isolated strains were not necessarily attached to, or adhered to the plant or penetrated the cells.

The material obtained like this was kept for one day in the constant temperature

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and light intensity case for checking the effect of the bacteria elimination procedure. The material free from any effect of the procedure was used in the experiment.

Inoculation and Culture. The experiments were carried out at 16±0.5°C with the illumination by a fluorescent light of 3,000 lux at the surface of water of the Monad style culture apparatus. L type test tubes with 10 ml of the artificial sea water of *Porphyra* was attached to the culture apparatus. One thallus or a part of the thallus was grown in the test tube. Inoculation of bacteria was from the slanted stock culture and was five days old. The stock cultures were incubated at 21–24°C with the isolation medium. One picking of the bacteria with a platinum rod was transfered into the test tube and mixed with the medium. Observation was made on the 6th to 30th day after the experiment started. The medium was sterilized on a 1 kg/cm^2 for 25 minutes in an autoclave. All tools employed were also sterilized for an hour in an oven sterilizer at about 150°C.

Strain 5 did not grow well on the isolation medium now used. As it is difficult to get new culture satisfactorily, strain 5 was excluded from the present experiment. On account of this, bacteria inoculated in the culture experiments were strains 1, 2, 3, 4, 6 and 7. The morphological and biochemical characteristics of these bacteria are given in Tables 1 and 2.

Table 1. Morphological characteristics of marine organisms isolated from an alga, P. leucosticta

Organisms	Gram stain	Flagellation	Form	Colonial appearance
Strain 1	Positive		Spherical cells	Circular, white
Strain 2	Positive	Peritrichous	Rod shaped-cells	Circular, translucent, colourless
Strain 3	Positive	Peritrichous	Coccobacillary forms	Circular, translucent, white, becoming dim at margin
Strain 4	Negative	Peritrichous	Rod shaped-cells	Circular, brown yellow
Strain 6	Negative	Polar monotrichous	Rod shaped-cells	Circular, translucent, white
Strain 7	Negative	Polar monotrichous	Rod shaped-cells	Circular, dirty white, becoming dim at margin

Table 2. Biochemical characteristics of marine organisms isolated from an alga, P. leucosticta

Organisms	Oxidase test	Carbohydrate utilization
Strain 1	Negative	
Strain 2	Negative	
Strain 3	Negative	
Strain 4	Positive	
Strain 6	Positive	Fermentative, much gas
Strain 7	Positive	Fermentative, much gas

Observation. After the colour, shape and growth of the plant were observed macroscopically, the morphology of the cell was observed microscopically. The growth was

only checked if it was different from the original sample. The observation of bacteria was made both with the naked eye and through a microscope. The macroscopical observation was performed with the dilution method to examine the bacteria on the plate of the medium. The appearance rate was calculated into per cent though it might not be an indication of a real meaning because each bacteria has its own size. The microscopical observation was made of the plant and arount the plant, by picking up the plant and placing it on the slide and viewing it with one to two different visual fields. The appearance rate was produced roughly in per cent just to get an idea. Strains 3 and 4 were difficult to know microscopically from other strains so that it is reasonable to consider that these strains might sometimes be admitted as strains 2, 6 or 7. However strains 3 and 4 never appeared in the dilution method except when they were inoculated. It was then thought that they grew slowly or they played a small role in the bacterial flora.

Results

Experiments of using 5 mm long thallus. The experiments were repeated four times. Results are shown in Tables 3–5. Experiment 1 and 2 were run Apr. 28 to May 28. Experiment 3 was run Jun. 5 to Jun. 12. Experiment 4 was run Jun. 6 to Jun. 12.

When strain 1 was inoculated into the medium, the plant was grown normally and healthy with the exception of Exp. 3. The observation of the bacterial flora was as follows. In Exp. 1, strain 1 appeared 52% and strain 2, 24% in the dilution method. In Exp. 2, strain 1 appeared the most in the dilution method, an appearance rate being nearly 100% and it was also strain 1 that was mostly observed microscopically. In Exp. 4, strain 1 turned up the most, both in the macroscopical and microscopical observations, although strains 2, 6 and 7 were also seen microscopically.

On the contrary in Exp. 3 in which white and dead cells were found at the base or the margin of the plant, the appearance rate of strain 1 was 41%, strain 2, 36%, strain 6, 10% and strain 7, 13% in the dilution method.

The plant started to form more white and dead cells or became total white in colour when strain 2 was inoculated. A bacterial flora observation showed both macroscopically and microscopically that the appearance rate of strain 2 was nearly 100% in Exps. 1, 3 and 4.

The plant was grown normally and healthy in some cases, grown abnormally in others when strain 3 or 4 was inoculated. In cases where the plant was grown normally and healthy, strain 1 appeared the most while the plant formed more white cells or became white totally, strain 2 appeared more than strains 3, 4, 6 and 7 with strain 1 decreasing less than 50%, both macroscopically and microscopically.

The plant were not grown well and produced a callus when strain 6 was inoculated. However the callus was not made in Exp. 4 although strain 6 was observed 40–50% microscopically. In Exps. 1, 2 and 3, the appearance rate of strain 6 was nearly 100% both macroscopically and microscopically.

When strain 7 was inoculated, the plant was grown normally and healthy, started to form more white and dead cells, turned to make the callus, or seemingly was spoiled

Table 3. Results of Experiment 1

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	6 days	Healthy			
	30 days	Tip decaying	New plants healthy Dead cells on both original and new plants	Strain 1, 52% Strain 2, 24% Strain 6, Strain 7, 24%	Strain 1, about 50% Strains 2, 6 and 7 also observed equally
Strain 2	6 days	Disappearing			
	30 days		Original plant not observed New plants all dead	Strain 2, 99%	Mostly strain 2 Strain 1 also observed
Strain 3	6 days	Disappearing			
	30 days		Original plant not observed New plants healthy though a few dead cells observed	Strain 1, 69% Strain 2, 4% Strain 3, 11% Strain 6, Strain 7, 16%	Ratio of appearence of strain 1 and strain 7, 3:1 Strain 2 also observed
Strain 4	6 days	Disappearing			
	30 days		Original plant not observed New plants healthy though a few dead cells observed	Strain 1, 61% Strain 2, 15% Strain 4, 13% Strain 7, 11%	Ratio of appearance of strain 1 and strain 2, 3:1
Strain 6	6 days	Disappearing			
	30 days		All new plants produced callus Some dead cells also observed	Strain 6, 99%	Mostly strain 6 Strain 1 and strain 2 also observed
Strain 7	6 days	Healthy but red coloured			
	30 days	Healthy and growing well	New plants mostly healthy Large vacuole A few dead alls	Strain 1, 51% Strain 7, 49%	Strain 1 and strain 7 observed equally

Table 4. Results of Experiment 2

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	6 days	Healthy			
	30 days	Healthy and growing	New plants healthy	Strain 1, 97% Strain 2, 3%	Strain 1 only observed
Strain 2	6 days	Decaying			
	30 days	Disappearing			
Strain 3	6 days	Tip decaying			
	30 days	Decaying	Many dead new plants observed though some healthy	Strain 1, 46% Strain 2, 30% Strain 3, 24%	Strain 1 and strain 2 observed equally
Strain 4	6 days	Tip decaying			
	30 days	Decaying	Many dead new plants observed though some healthy	Strain 1, 41% Strain 2, 36% Strain 4, 20%	Strain 1 and strain 2 observed equally
Strain 6	6 days	Tip decaying			
	30 days	Growing	Small new plants with calluses Bigger new plants contorted though many healthy	Strain 6, 99%	Mostly strain 6 Strain 1 and strain 2 also observed
Strain 7	6 days	Tip decaying			
	30 days	Growing	Most new plants healthy A few cells with decreased chromatophore	Strain 1, 59% Strain 7, 41%	Strain 1 and strain 7 observed equally

depending on when the experiments started. The inconsistent results might be due to the change of the bacterial floras. Although a few white cells or the cells having large vacuoles were found in Exps. 1 and 2, the plant on the whole was grown normally and healthy and the bacterial flora in the dilution method showed that strain 1 appeared 51% in Exp. 1 and 59% in Exp. 2. When it appeared that the plant was spoiled in Exp. 3, the bacterial flora both macrosopically and microscopically showed that strain 7 appeared nearly 100%. The calluses were found in Exp. 4 but bacteria came up in the dilution method were strain 6, 56% strain 7, 44%.

Table 5. Results of Experiment 3

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	7 days	Colour paling	A few dead cells observed at base and margin	Strain 1, 40% Strain 2, 37% Strain 6, 10% Strain 7, 13%	Strain 1, about 50% Strains 2, 6 and 7 also observed equally
Strain 2	7 days	Colour paling	A few dead (white and red) cells observed at base and margin	Strain 2, 99%	Strain 2 only observed
Strain 3	7 days	Colour paling	A few dead cells observed at base and margin	Strain 1, 66% Strain 2, 7% Strain 3, 14% Strain 6, 13% Strain 7,	Strain 1; about 60-70% Strains 2, 6 and 7 also observed equally
Strain 4	7 days	Colour paling	A few dead cells observed at base	Strain 1, 64% Strain 2, 15% Strain 4, 19% Strain 7, 2%	Strain 1; about 60-70% Strains 2, 6 and 7 also observed equally
Strain 6	7 days	Colour paling	A few dead cells observed at base and middle Callus observed	Strain 6, 99%	Mostly strain 6 Strain 1 also observed
Strain 7	7 days	Colour paling	A few dead cells observed at base and middle Many cells seemingly were spoiled	Strain 7, 99%	Mostly strain 7 Strain 1 also observed

Experiments of using 2-4 cm long thallus. The experiments were repeated three times. Results are shown in Tables 6-9. Experiment 5 was run Jun. 12 to Jun. 26. Experiment 6 and 7 were run Jun. 23 to Jun. 29.

When strain 1 was inoculated into the medium, the plant was grown normally and healthy as well as the experiments of using the 5 mm long thalli. The bacterial flora could not be observed in Exp. 5 as the thallus disappeared. In Exp. 6, both the macroscopical and microscopical observation showed strain 1 appearing nearly 100%. In Exp.

Table 6. Results of Experiment 4

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	6 days	Healthy	Healthy Monospores and new plants observed	Strain 1, 99%	Mostly strain 1 Strains 2, 6 and 7 also observed
Strain 2	6 days	Colour paling	Many dead cells observed at base Monospores and new plants observed	Stairn 2, 99%	Mostly strain 2 Strain 1 also observed
Strain 3	6 days	Colour paling	Many dead cells observed at base Monospores and new plants observed	Strain 2, 99%	Strain 2 only observed
Strain 4	6 days	Colour paling	Many dead cells observed at base Monospores and new plants observed	Strain 2, 99%	Strain 2 only observed
Strain 6	6 days	Colour paling	A few dead cells observed at margin Monospores and new plants observed	Strain 2, 23% Strain 6, Strain 7, 73%	Strains 6 and 7 observed equally Strains 1 and 2 also observed
Strain 7	6 days	Colour paling	A few dead cells observed at base Monospores and new plants observed Callus observed	Strain 6, 56% Strain 7, 44%	Strains 6 and 7 observed equally

7, the appearance rate of strain 1 was also nearly 100%.

When strain 2 was inoculated in Exp. 5, the thalli disappeared and monospores were not observed. Even if it were due to the effect of the elimination procedure, the results of disappearing thalli were thought to be caused by the inoculation of strain 2 because

Table 7. Results of Experiment 5

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	8 days	Disappearing			
	14 days		Original plant not observed New plants healthy		
Strain 2	8 days	Disappearing			
	14 days		Both original and new plants not observed		
Strain 3	8 days	Disappearing			
	14 days		Both original and new plants not observed		
Strain 4	8 days	Decaying			
	14 days		Original plant not observed New plants healthy		
Strain 6	8 days	Disappearing			
	14 days		Original plant not observed New plants healthy		Ratio of appearance of strain 1 and strain 6, 4 : 6 or 3 : 7
Strain 7	8 days	Tip decaying			
	14 days		New plants healthy	Strain 1, 3% Strain 2, 2% Strain 6, Strain 7, 95%	Mostly strain 7 Strains 1, 2 and 6 also observed

monospores and new plants were observed when strains 1, 3 and 6 were inoculated respectively though the thalli disappeared. The bacterial flora were not observed in Exp. 5 as thalli disappeared. When strain 2 was inoculated in Exp. 6, the plant started to form more white and dead cells and became total white in colour and their bacterial flora mostly consisted of strain 2 and a few strains 6 and 7. In Exp. 7, the abnormality of the cell was not seen though many spores were found to be shed.

When strains 3 or 4 was inoculated respectively all the plants were grown normally

Table 8. Results of Experiment 6

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	6 days	Healthy	Healthy	Strain 1, 99%	Strain 1 only observed
Strain 2	6 days	Dead	All cells white and dead		Mostly strain 2 Strains 6 and 7 also observed
Strain 3	6 days	Tip decaying	Healthy Monospores shedding		
Strain 4	6 days	Healthy	Healthy	Strain 1, 95% Strain 7, 5%	Mostly strain 1 Strains 2, 6 and 7 also observed equally
Strain 6	6 days	Healthy	Healthy	Strain 1, 99%	Strain 1 only observed
Strain 7	6 days	Tip decaying	Healthy Monospores shedding	Strain 1, 69% Strain 2, 4% Strain 7, 27%	Ratio of appearance rate of strains 1 and 7, 2:1 Strain 2 also observed

and healthy with the exception of Exp. 5 of inoculating strain 3. In case the plants were not grown, the bacterial flora was not observed as the plants disappeared.

When strain 6 was inoculated, the thallus disappeared in Exp. 5 but the new plants were grown normally and healthy. The strains appearing in the microscopical observation were strains 1 and 6 and their ratio was 4:6 or 3:7. In Exp. 6, the plant was grown normally and healthy with strain 1 accompanied instead of strain 6. In Exp. 7, the plant also was grown normally and healthy with strain 6 accompanied nearly 100% of the appearance rate. In any experiment the calluses were not produced on the plant.

When strain 7 was inoculated, the plants in all of the three experiments were grown normally and healthy. The monospores were shed in all three experiments and also were grown normally. Even though strain 7 grew very fast and caused a turbid medium, the plant was not spoiled as seen in the experiments using the 5mm long thalli.

The bacterial flora showed that strain 7 appeared nearly 100% in Exp. 5 and 7 although strains 1, 2 and 6 were also seen, and that in the Exp. 6, strain 1 appeared more than strain 6 having the ratio of 2:1.

Table 9. Results of Experiment 7

Inoculated bacteria	Date of observation	Plant observation		Bacterial observation	
		Observation with the naked eye	Observation through a microscope	Observation with the naked eye	Observation through a microscope
Strain 1	6 days	Tip decaying	Healthy Monospores shedding		Strain 1 only observed
Strain 2	6 days	Tip decaying	Healthy Monospores shedding		Mostly strain 1 Strain 2 also observed
Strain 3	6 days	Tip decaying	Healthy Monospores shedding		Mostly strain 1
Strain 4	6 days	Tip decaying	Healthy Monospores shedding		Mostly strain 1
Strain 6	6 days	Tip decaying	Healthy Monospores shedding		Mostly strain 6 Strains 1, 2 and 7 also observed
Strain 7	6 days	Tip decaying	Healthy Monospores shedding		Mostly strain 7 Strains 1, 2 and 6 also observed

Discussion

That the thalli often disappeared in the experiments were suspected to be due to the effect of the bacteria elimination procedure and not to the effect of bacteria.

Strain 1 is indispensable on the growth of *Porphyra* plant whereas strain 2 is not. On the contrary, strain 2 made the plants grow abnormally. If the population of strain 1 decreases below 50% and that of strain 2 increases more than that of strains 3, 4, 6 and 7, the white and dead cells were more observed on the plant than on the healthy growing plant. In some cases the plant became total white in colour. The plant continued to be grown well and healthy so far as strain 1 exists to accompany it over 50% of the appearance rate. Thus there seems to be an antagonism between strains 1 and 2.

In the experiments of inoculating strain 3 or 4, it was found that when the plant was grown well and healthy, strain 1 was on the plant or in the cell, and when the plant was grown abnormally having more white cells and finally died, strain 2 appeared more or the most. For this reason and together with the fact that the appearance rate of strains 3 and 4 was very small, these strains have no relation to the growth of the *Porphyra* plant.

In the experiments of using 5mm long, calluses were observed in three out of four experiments. The bacterial observation showed that strain 6 appeared nearly 100% in all three experiments. In experiment in which the calluses were not observed, the appearance rate of strain 6 was about 50% in the dilution method. Accordingly if strain 6

appears nearly 100%, the calluses are produced on the thalli of 5 mm long. On the other hand, the calluses were not observed with the inoculation of strain 6 in any experiment using 2–4 cm long thalli. The bacterial observation showed that strain 6 appeared 60–70% in Exp. 5, nearly 100%, in Exp. 6 and 7.

The calluses were not formed even if strain 6 turned up over 60–70% in the two out three experiments. It might be concluded that strain 6 does not cause the calluses on the thalli of 2–4 cm long differing from the thalli of 5 mm long. However it is too early to draw any conclusion on the formation of the calluses as the experiment was repeated only three times. Further experiments on the culture of *Porphyra* plant with the inoculation of strain 6 are being carried out.

Strain 7 grow very fast on the isolation medium now being used. Strain 7 also grow very well on the medium of *Porphyra* plant with the addition of L-tryptophane. Therefore it is expected to have a conspicuous effect on the plant irrespective of good or bad. Although the thalli were spoiled when strain 7 grew very fast, it was found unexpectedly that strain 7 had no influence on the plant.

That the strains inoculated did not always increase on the plant might be due to the change of the antagonism by some unknown factors or the physiological state of the plant at the particular time.

Further studies are needed to elucidate on whether or not strain 1 excretes or secrets micronutrients, or neutralizes toxic substances, or acts as a stabilizer of the medium near the surface of the plant, by itself or by producing chemical substances.

It must be brought to light if the strain 1 makes a bacteria-free *Porphyra* plant grow normally and well and how it does that.

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Summary

Culture experiments were conducted by inoculating isolated bacteria[2] singly into the medium, into which the healthy growing plant was been growing to demonstrate if accompanied bacteria lose their balance and the plant begins to grow abnormally. Results are summaried as follows.

1. Strain 1 is indispensable for the growth of the *Porphyra* plant, *Porphyra leucosticta* THURET. The plant continues to grow normally and healthy so far as strain 1 exists to accompany it over 50% of appearance rate.

2. When strain 2 was inoculated into the medium and the population of strain 1 decreased below 50%, and that of strain 2 increased more than that of strain 3, 4, 6 and 7, the white and dead cells more observed on the plant than on the healthy growing plant. In some cases, the plant became total white in colour.

3. Strains 3 and 4 have no relation to the growth of the *Porphyra* plant.

4. When strain 6 was inoculated into the medium and strain 6 appeared nearly 100%, the calluses were formed on the thallus of 5 mm long. However the calluses were not observed with the inoculation of strain 6 in any experiment using 2–4 cm long thalli.

Further experiments on the culture of *Porphyra* plant with the inoculation of strain 6 are needed to state the effect with any certainty.

5. Although the thalli were spoiled at times when strain 7 grew very fast, it was found unexpectedly that strain 7 had no influence on the plant.

References

1) Tsukidate, J. 1970: Bull. Nansei Reg. Fish. Res. Lab., 3, 19-22.
2) Tsukidate, J.: Bull. Jap. Soc. Sci. Fish., in contribution.

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31	Fig. 10.	1965, 1966, 1967	1967, 1968, 1969
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