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Occasional pathogenic bacteria promoting *ice-ice* disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta)

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Abstract

The bacterial isolates from normal and diseased branches of *Kappaphycus alvarezii* and *Eucheuma denticulatum* in the Philippines were examined for possible role in the development of the *ice-ice* disease. The numbers of bacteria on and in *ice-iced* branches were 10–100 times greater than those from normal, healthy ones. Gram-positive bacteria predominated in almost all branch sources, but with an increasing proportion of agar-lysing bacteria in branches suffering from the *ice-ice* disease. These agar-lysing bacteria were composed of yellow and non-pigmented, spreading colonies identified to the *Cytophaga-Flavobacterium* complex and the *Vibrio* group. Among isolates which mainly appeared on *ice-iced* branches, two strains, designated as P11 (*Vibrio* sp.) and P25 (*Cytophage* sp.), which showed pathogenic activity, were obtained. These strains caused early *ice-ice* whitening of *K. alvarezii* especially when subjecting branches to environmental stress, such as reduced salinity and light intensity, suggesting that these bacteria were occasionally pathogenic. This paper offers new evidence of bacterial role in the development of so-called *ice-ice* disease among farmed species of *Kappaphycus*.

Introduction

The 'ice-ice' problem, first reported in 1974 during the start of commercial seaweed farming in the Philippines, has reportedly wiped out entire farms (data of F.R. Uyenco). Since then, only few investigations have been made with a general perception of *ice-ice* as due to unfavorable environmental conditions in the planting site (Uyenco *et al.*, 1981). Collens & Pedersen (1992) detected the release of toxic haloamines from *Eucheuma* when thalli are stressed in high light intensity and suggested that *ice-ice* could be caused by this compound. In another experiment using a re-circulating culture system, Largo *et al.* (1995a) were able to re-produce *ice-ice* symptoms similar to those observed in the Philippine seaweed farms by subjecting the seaweeds to stressful levels of temperature, light intensity and salinity.

These studies, so far, pointed only to environmental factors as the main cause of *ice-ice* and had no implications of bacteria in the development of the disease. Seaweed diseases, especially those which are associated with bacteria, are a rare phenomenon (Andrews & Goff, 1984). Few cases have been reported so far. Tsukidate (1983) found the non-pigmented bacterium, *Beneckia* sp. (= *Vibrio* sp.) to be the causative factor of the white rot disease of the Japanese 'nori' *(Porphyra leucosticta)*. Kusuda *et al.* (1992) also described a yellow bacterium, *Flavobacterium* sp., to be the cause of 'suminori' disease of the same seaweed. In Funka Bay, Hokkaido, Japan, the bacterium, *Alteromonas* sp., was suspected to be the causative agent of the red-spots disease of the cultured brown seaweed species, *Laminaria japonica* (Yumoto *et al.*, 1989a, 1989b). Recently, Weinberger *et al.* (1994) reported a bacterial cause of the so-called 'white tips disease' of the

agar-producing *Gracilaria conferta* in Israel. These diseases among cultured species were found to be associated with abnormal culture conditions, such as high temperature, high light intensity and low salinity, preceding few days before the occurrence of these diseases. Although extremes of these environmental factors were also found to trigger the development of the *ice-ice* disease in eucheumatoid algae (Largo *et al.*, 1995a), it has never been known that bacteria associated with the *ice-iced* branches of the eucheumatoid algae have some potential pathogenic activity similar to those reported in other seaweeds. This study offers evidence that implicate some epiphytic bacteria as probable causative agent in the development of the *ice-ice* disease in *Kappaphycus alvarezii* under certain growth conditions.

Materials and methods

Bacterial identification

The seaweed cultivation ground in Danajon Reefs, northeastern Bohol, the Philippines was visited on June 11, 18 and 21, 1993, coinciding the period of high *ice-ice* occurrence. Fresh materials of *Kappaphycus alvarezii* (Doty) Doty and *Eucheuma denticulatum* (Burman) Collins & Hervey, consisting of normal and sickly branches, were obtained during these dates by aseptic method. The healthy plants were divided into branch tips and midbranch portions for comparison of bacterial composition and relative abundance. About 5 g of wet algal materials were placed in sterile borosilicate bottles containing autoclaved seaweater and brought, in chilled condition, to the marine laboratory of the University of San Carlos (Cebu, Philippines).

About 0.1 g subsample of each material were immediately homogenized in 10 ml sterile seawater using a blender (Nihon Seiki Seisakusho Co., Japan) at 3000 rpm for 2–3 minutes. A dilution series of up to 1:100,000 were then prepared for each sample and 0.1 ml aliquots were spread-plated on three kinds of agar media: ZoBell 2216E (Oppenheimer & ZoBell, 1952), Knutsen's modified Yaphe medium (Knutsen, 1991) and FeTY agar media (Fukami *et al.*, 1992), for initial comparison. Numbers of colonies were counted after incubating at room temperature (25 to 28 °C) for up to 14 days. They are presented here as colony-forming units per gram (CFU g^{-1}) of wet algal sample.

Since it has been observed that *Eucheuma* planted over seagrass beds have high incidence of *ice-ice*, it is assumed that seagrass beds, and possibly other benthic vegetation nearby, are the source of some of the bacteria found in the cultured seaweeds causing the *ice-ice* disease. Some bacterial strains were therefore isolated from parallel samples of two other dominant macrophytes, a brown seaweed (*Sargassum* sp.) and the turtlegrass (*Thalassia hemprichii* (Ehrenberg) Ascherson) collected from the same area. In addition, seawater samples were collected for comparison.

Differentiable colonies were randomly picked and streaked on agar media for purification and subsequent identification. The following tests were performed for tentative identification of bacterial groups based on examination of differentiable colonies: colonial morphology and pigmentation, cell morphology, motility, Hugh & Leifson's O/F test for glucose, catalase reaction with 1% hydrogen peroxide and oxidase reaction using tetramethyl-*p*-phenylene-diamine solution. Some selected strains were also tested for growth in certain sugars, galactose and mannose, of which red algae are known to produce. The results were referred to Simidu's (1985) identification scheme and the Bergey's Determinative Bacterioloy (Holt *et al.*, 1994).

Infection of K. alvarezii by selected bacterial strains

Under normal conditions. Ten selected strains from *ice-iced* materials obtained from a Philippine seaweed farm were initially screened for their ability to cause *ice-ice* in healthy,non-axenic branches, following Koch's postulate of disease development (Andrews & Goff, 1984). All strains were pre-cultured in seawater enriched with trypticase peptone (0.5 g l^{-1}; Becton Dickinson, Maryland, USA) until cell density reached $> 10^5$ ml^{-1} in more than 24 h. The cells were then harvested by centrifugation at 8000 × *g* for 10 minutes at 4 °C during their exponential growth phase, washed twice and re-suspended with autoclaved seawater, and finally inoculated to the seaweed culture of 5-cm fragments in autoclaved seawater (pH adjusted between 7.4 and 7.8). Initial concentration of bacterial inocula was determined by epifluorescence microscopy using DAPI stain (final concentration = $10^3 - 10^5$ cells ml^{-1}). Parts of the branches showing deterioration or whitening indicative of *ice-ice* were homogenized and their bacterial association was determined as above. Branch-

Table 1. Average number of colony forming units of attached bacteria from *K. alvarezii, E. denticulatum* and two other benthic macrophytes (*Thalassia hemprichii* and *Sargassum* sp.) and seawater collected from a Philippine seaweed farm.

	Part determined	CFU g^{-1} wet algal sample
K. alvarezii		
	Healthy tip	4.9×10^4
	Healthy midbranches	4.5×10^5
	Ice-iced branches	2.4×10^6
E. denticulatum		
	Healthy tip	2.0×10^3
	Healthy midbranches	3.4×10^4
	Ice-iced branches	1.5×10^7
Sargassum sp.		
(brown alga)	Leaf	4.6×10^6
Thalassia hemprichii		
(turtle grass)	Blade	4.5×10^7
Seawater*		1.1×10^3

* CFU in ml^{-1}

es grown in normal seawater at optimal condition without bacterial inoculum served as control. Up to five replicate flasks for each diluted seawater were made for each bacterial strain plus a control set (without inoculation). All transfers of algal materials and media were done under aseptic conditions in 'clean bench' using pre-sterilized glasswares, forceps, blades, etc. Another infection experiment using sonicated and antibiotic-treated branches were conducted to compare results between bacterially-reduced branches (total axenicity or suppression of bacterial growth failed in most trials). Treatment with mixed antibiotics composed, per liter of seawater, of streptomycin (1 g), erythromycin (100 mg), kanamycin sulfate (200 mg), chloramphenicol (500 mg), neomycin (50 mg), polymixin B (36 mg), and gentamicin (10 mg), were done for 8–9 days (on rotatory shaker) prior to the experiment.

After inoculation, the algal materials, unless specified for stress conditioning as below, were maintained in autoclaved seawater inside an incubator (Sanyo, Japan), adjusted to optimum conditions of temperature (25 °C), light intensity (up to 200 μmol photon m^{-2} s^{-1}), and photoperiod (12:12 h L/D cycle). All observations were done periodically until visible *ice-ice* whitening occurred up to a maximum of 3 weeks. The conditions of the branches were graded according to their general appearance indicated by a plus (+) or minus (−) sign (see legend from Tables).

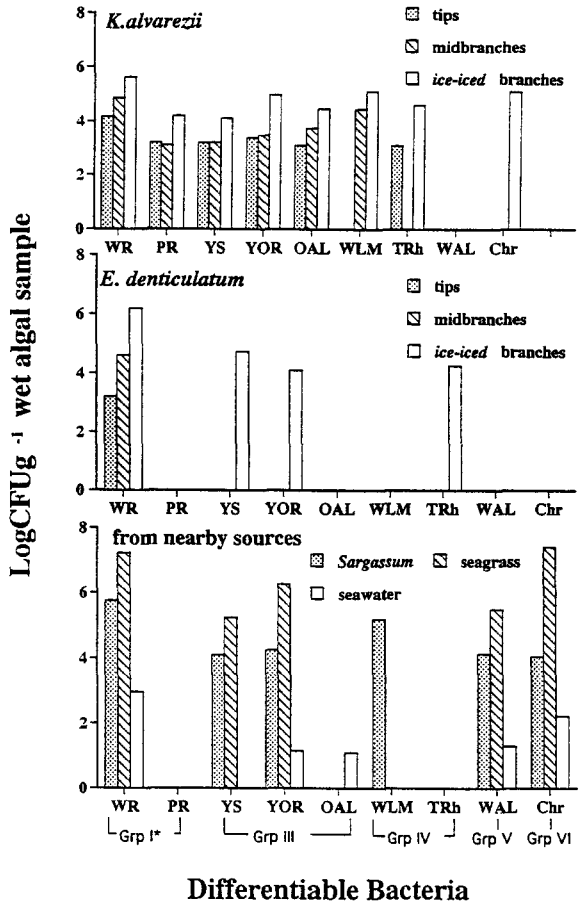

Fig. 1. Abundance of bacteria showing differentiable colonies from healthy and *ice-iced* branches of *K. alvarezii, E. denticulatum* and from other macrophytes (*Thalassia hemprichii* and *Sargassum*) nearby and seawater samples collected from a Philippine seaweed farm. Group with asterisk represents Gram-positive bacteria forming the most dominant bacteria in all isolations. See Table 2 for legend of bacterial groups.

Effect of stress conditioning. It has been shown that reduced salinity and light intensity were stressful conditions for the algae (Largo *et al.*, 1995a). The same conditions were therefore used in the preparation of stressed branches using flask culture in axenic or relatively axenic condition prepared as above for bacterial inoculation with the same selected strains. After 4 days of stress conditioning, these bacteria were inoculated as described above.

Results

Our preliminary counts varied between the three agar media used, but since there seemed to be higher counts

Table 2. Characteristics of bacterial strains isolated from *K. alvarezii* and *E. denticulatum* materials (both from *ice-iced* and healthy plants) sampled from a Philippine seaweed farm, including samples of neighboring benthic vegetation (*Sargassum* sp. and seagrass) and seawater.

Characteristics[a]	Group Ia WR	Group Ib PR	Group Ic WR	Group II St	Group IIa YS	Group IIb YOR
1. Colonial Morphology	Circular	Circular	Circular	Stellate	Spreading	Circular
2. Pigmentation	White	Pinkish	Cream white or orange	Cloudy white	Yellow/ orange	Yellow/ orange
3. Cell Morphology	Dumbbell-shaped rods	Curved rods shaped rods	Short rods	Long, straight rods	Long, straight rods	
4. Motility	−	−	+	−	−	−
5. Gram Reaction	+	+/−	+	−	−	−
6. Catalase Test	+/−	+	+	+	+/−	+/−
7. Oxidase Test	−	−	−	nd	−	+
8. Agar hydrolysis	−	−	−	−	+	−
9. Growth in carrageenan	−	−	−	+/−	+	−
10. Hugh & Leifson's O/F Test[b] for:						
glucose	O	NR,Ow	NR,Ow	O	NR,Ow	Ow
galactose	O	NR,Ow	NR,Ow	nd	NR,Ow	Ow
mannose	O	NR,Ow	NR,Ow	nd	NR,Ow	Ow
Identification	*Arthro-bacter*?	Unident.	Unident.	Unident.	*Cytophaga*	*Flavo-bacterium*
Source of isolates[c]	Et, Em, Ei, Km, Ki, sw, *Sarg.*	Kt, Km, Ki, sw	Ki, SG, *Sarg.*	Et, Km, Ki, sw	Ei, Kt, Km, Kt, Ki, *Sarg.*	Et, Ei, Km, Ki, *Sarg.* SG
Representative Strain Nos.[d]	P1, P22, P44, P67, P130	JP45, JP47, JP53, JP55, P65	P102, P104, P114, P117, P119	P2, P24, P39, P49, P65	P5, P25, P46, P68, JP42, JP46	P2, P70

[a]: WR – white, round; PR – pink, round; St – stellate; YS – yellow, spreading; YOR – yellow/orange, round; OAL – orange, agar lyzing; WLM – white, lobed margin; TRh – translucent, rhizoid; WAL – white, agar lyzing; Chr – chromogenic.
[b]: O – oxidative; Ow – weak oxidative; nd – not determined; F – fermentative.
[c]: Et – *Eucheuma* tips; Em – *Eucheuma* midthalli; Ei – *Eucheuma ice-ice*; Kt – *Kappaphycus* tips; Km – *Kappaphycus* midthalli; Ki – *Kappaphycus ice-ice*; Sarg. – *Sargassum* leaf; SG – seagrass *(Thalassia hemprichii)* blade; sw – seawater.

and diverse colonies obtained from those of the FeTY agar plates, we only show here the results from these plates. The average density of attached, culturable bacteria in healthy branches, ranged from 2×10^3 to 4.5×10^5 CFU g^{-1} of wet algal samples. Branches with *ice-ice* contained higher bacterial counts between 2.4×10^6 and 1.5×10^7 CFU g^{-1} (Table 1). The surrounding seawater contained consistently lower bacterial population (mean = 1.1×10^3 CFU ml^{-1}). Bacterial density varied at different parts of healthy branches. Branch tips have consistently lesser bacterial number than at midbranches.

A total of 135 bacterial strains have been isolated from 16 samples of *K. alvarezii* and *E. denticulatum* plus isolates from 6 samples of nearby vegetation, consisting of *Sargassum* sp. and *Thalassia hemprichii*, and from seawater samples collected from a Philippine seaweed farm (coded here as P-strains). In addi-

Table 2. Continued.

	Group IIIc OAL	Group IIId OAL	Group IVa TRh	Group IVb WLM	Group IVc TRh	Group V WAL	Group VI Chr
1.	circular	oval (1.5 cm dia.)	rhizoidal margin	lobed margin (0.4 cm dia.)	spreading	circular w/ depression on agar	circular
2.	yellow/orange	orange	translucent	milky white	greenish yellow	white	purple
3.	coccoid	long, straight rods	curved to sigmoid rods	curved to sigmoid rods	curved to sigmoid rods	helical	short rods
4.	+	–	+	+	+	+	+
5.	–	–	–	–	–	–	–
6.	+	+/–	+	+	+	nd	+?
7.	–	+/–	+	+	+	nd	+
8.	+	+	+	+	+	+	–
9.	+	–	+	+	+	–	nd
10.	NR, Ow	Ow	F	F	F, gas	F	O
11.	nd	nd	F	F	F, gas	F	nd
12.	nd	nd	F	F	F, gas	F	nd
	Unident.	*Cytophaga*	*Vibrio*	*Vibrio*	*Aeromonas*	Unident.	*Pseudo-monas-Altero-monas*
	Ei, Ki, *Sarg.*	Km, Ki	Ei, sw	Ki, *Sarg.*, SG, sw	Ei	Ki	Et, Km, Ki, sw
	P14, P16, P26, P27, P29, P30, P38, P57 P120, P124	P3A, P19, P48, P50, P73	P11, P12, P13, P15, P17	P53, P96, P97, P98, P99, P115	P54, P55	P18, JP63, JP69	P108, P109, P111

d: P strains - Philippine isolates
JP strains - isolates from *Eucheuma* and *Kappaphycus* materials transplanted to Japan

tion, 75 isolates and re-isolates from *Kappaphycus* and *Eucheuma* materials maintained in Japan during batch and continuous culture experiments have been made (coded as JP-strains). The characteristics of these bacteria are summarized in Table 2, while their abundance, classified according to groups showing differentiable colonies on agar plates, is shown in Fig. 1. The isolates from the macrophytes and from seawater samples were dominated by Gram-positive (except with Group Ib which were Gram-variable), catalase-positive, motile or non-motile, highly pleomorphic, forming coccoid to dumbbell-shape rods, forming white or pinkish, creamy colonies on agar surface. These bacteria constituted the highest population from both healthy and *ice-iced* branches ranging from 45–100% of the isolates.

Agar-digesting bacteria (groups YS, OAL, TRh, WLM and WAL in Table 2), which showed either a clear zone or depression around their colonies, appeared in greater proportion in *ice-iced* branches of both *Eucheuma* and *Kappaphycus* (Fig. 1). All of these bacteria were Gram-negative, straight to curved rods which were either motile or non-motile. The non-motile rods belong to the yellow *Cytophaga-Flavobacterium* group which figured prominently in the culture plates because of their spreading growth on the agar surface (Group III). The motile bacteria were all classified to the *Vibrio-Aeromonas* group (Group

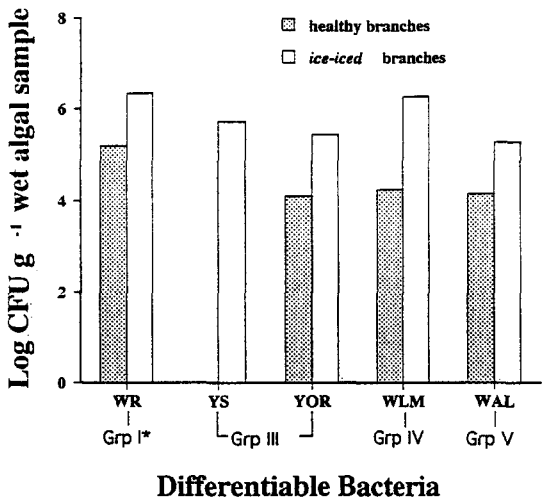

Fig. 2. Abundance of bacteria from normal and *ice-iced* branches of *K. alvarezii* maintained in the aquatron culture system. Group with asterisk represents Gram-positive bacteria dominating all bacterial groups. See Table 2 for legend of bacterial groups.

Table 3. Infection of non-axenic branches of *K. alvarezii* using bacterial strains originally isolated from *ice-iced* materials. The bacteria were pre-cultured in peptone-seawater (0.5 g l^{-1}) at initial cell density of 10^6 to 10^7 ml^{-1} (final concentration in the seawater medium = 10^4 – 10^5 ml^{-1}).

	Observations after			
	2 days	5–8 days	11–13 days	18 days
P1	–	–	–	+
P11	+	++	++	+++
P14	–	+	+	ND
P19	–	+	++	ND
P25	–	++	++	++
P50	–	–	+	++
P57	–	+	++	+++
P77	–	+	++	+++
P81b	–	–	–	–
JP6	–	+	+	+
Control	–	+	+	+

+ – generally healthy but with small pale or white patches; ++ – 50% or less of branches yellowish or white at segments; +++ – 90–100% of branches white or deteriorate; – – no apparent change or any discoloration; ND – no data.

IV) as having a fermentative action on glucose, some with production of gas (in the case of *Aeromonas*). The *Cytophaga-Flavobacterium* group and vibrios reached up to 10^5 CFU g^{-1} and became particularly evident in culture plates of samples from *ice-iced* branches of both species (Fig. 1). Although similar differentiable colonies composed the isolates from all macrophytes as well as from seawater, the vibrio strains were seldom isolated from the seawater samples obtained from the seaweed farm.

Seaweed materials transferred to southern Japan and grown in recirculating culture tanks (aquatron), also occasionally developed *ice-ice* symptoms among otherwise healthy branches. When bacteria from *ice-iced* and healthy samples were isolated, the colonial composition were similar to those of the samples collected from the seaweed farm. Figure 2 presents the strains from the aquatron-maintained branches showing differentiable colonies together with their average number. Healthy branches have bacterial densities ranging from 10^4 to 10^5 CFU g^{-1} while *ice-iced* part ranged from 10^5 to 10^7 CFU g^{-1}. The non-pigmented bacteria, composed of Gram-positive, pleomorphic forms similar to those isolated from the Philippine-grown materials were, except in a few plates, also dominant. In total, of the 13 differentiable bacterial groups isolated (Table 2), slightly over 50% were agar-digesters (Groups IIIa, IIIc, IIId, IVa–c, and V). The agar-digesting, yellow *Cytophaga-Flavobacterium* group appeared mainly in *ice-iced* branches, accompanied by an increased number of CFUs in the other groups. While these bacteria increased by 10-fold, the vibrios increased by 100-fold.

Infection of branches with bacterial strains with and without the influence of environmental stress factors

Among the 10 strains screened for infective ability on non-stressed, non-axenic branches, strains designated as P11 and P25 indicated initial pathogenic activity, showing whitening of branches earlier than others (Table 3). A repeat using antibiotic-treated branches, also showed P11 and P25 re-producing the whitening condition. When branches were stressed by reduced salinity (20‰), the effect of strains P11 and P25 were amplified by producing the whitening condition early on day 7 (Table 4). Slight paling was observed only on day 10 in cultures of control and those inoculated by apparently non-infective strains P50 and JP6. When deteriorated branches from P11-, P25- and P50-inoculated flasks were homogenized and their bacterial association enumerated, at least the same species

Table 4. Effect of inoculation of bacterial strains P11, P25, P50 and JP6 to antibiotic-treated *K. alvarezii* branches under environmental stress of reduced salinity (20‰) (Condition: temp. = 25 °C, irradiance = 200 μmol photo m^{-2} s^{-1}, photoperiod = 12:12 h L/D cycle). Trial 1. (Final concentration of inoculum in the seawater medium = 10^5 ml^{-1}).

	Bacterial inoculum								Control (no inoculum)	
	P11		P25		P50		JP6			
Flask no.>	1	2	1	2	1	2	1	2	1	2
Days of observation										
7	++	++	++	ND	+	+	+	+	+	+
10	+++	+++*	++*	ND	+	+	+	+	+	+*
15	+++	+++	++	ND	++	++	++	++	++	++

+ – generally healthy but with small pale or white patches; ++ – 50% or less of branches yellowish or white at segments; +++ – 90–100% of branches white or deteriorated; * – part of branches homogenized for re-isolation; ND – no data.

were re-isolated, exhibiting similar colonial and cellular morphology as well as reactions to biochemical tests as the inocula, in addition to associated bacteria which survived antibiotic treatment (Table 5). The number of re-isolated bacteria similar to P11 (3.8×10^5) was never higher than the associated bacteria (constituting only 1.7% of the bacterial association), while bacteria similar to P25 (2.23×10^7) and one replicate of P50 (7.23×10^7) were higher than the other associated bacteria. No bacteria appeared in agar plates from control branches, remaining in relatively healthy condition for at least 10 days but finally deteriorated on day 15 (50% of branch part became *ice-iced*) apparently due to low salinity.

A repeat of salinity-stressed branches (Table 6), showed that strain P11 enhanced deterioration of branches early on day 3 with some whitening on parts or the entire branches. After 7 days, complete whitening with this strain occurred. Control flasks (no inoculum) still underwent partial whitening due to salinity stress, but this became apparent only 7 days later. Surprisingly, branches inoculated with strain P25 remained in generally good condition, except for some small whitened patches, even until day 12. The re-isolation of bacteria yielded similar results to those of Table 5, except that bacteria similar to P11 re-isolates reached up to 10^7 CFU g^{-1} when no other contaminating bacteria were present (data not shown).

Branches stressed by reduced light intensity deteriorated gradually until day 7 while branches under non-stressed condition (control) only became slightly white in the form of small pale patches but still in generally healthy condition until day 17, except in one flask which became totally white earlier (Table 7). However, the effect of bacterial inoculation between stressed and non-stressed branches resulted in whitening after more than a week. In stressed branches, strain P11 produced the *ice-ice* whitening more intensely after day 10, and on day 17, almost all became completely white. Non-stressed branches receiving P11 inoculum were generally healthy until day 17, except for one branch which started to produce whitening on day 7, and another, only on day 17. Stressed branches inoculated with P25 produced *ice-ice* on day 17 in only 2 flasks, one became completely white, otherwise the rest were in good condition. Non-stressed branches with the same strain, produced only small, pale patches without completely becoming white on day 17. Branches with P50, under both stressed and non-stressed environment, remained in good condition all throughout the observation period.

Table 5. Re-isolates from infected branches based on experiment in Table 4.

Bacterial inoculum	Condition of homogenized sample	Reisolated bacteria (CFU g^{-1} of wet algal sample)		
		Similar strain	Associated strains	Total
P11	*Ice-iced*	3.80×10^6	2.21×10^7	2.25×10^7
P25	*Ice-iced*	2.23×10^7	2.00×10^7	4.23×10^7
P50				
Repl. 1	*Ice-iced*	7.50×10^5	4.32×10^7	4.39×10^7
Repl. 2	*Ice-iced*	7.23×10^7	2.67×10^7	9.90×10^7
Control	Healthy		0	0

Table 6. Effect of inoculation of strains P11 and P25 to antibiotic-treated *K. alvarezii* branches under environmental stress of reduced salinity (20‰) (Condition: temp. = 25 °C, irradiance = 200 μmol photo m^{-2} s^{-1}, photoperiod = 12:12 h L/D cycle.) Trial 2. (Final concentration of bacterial inoculum: P11 = 4.41 × 10^4 ml^{-1}, P25 = 1.5 × 10^3 ml^{-1}).

	Bacterial inoculum										Control				
	P11					P25					(no inoculum)				
Flask no.>	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Days of observation															
3	++	++	++	+++	++	−	−	−	−	−	+	+	+	+	+
7	+++	+++	++	+++	+++	+	−	−	−	−	+	+	++	++	++
10	+++	+++	+++	+++	+++	+	+	+	++	+	+	+	++	++	++
12	+++	+++	+++	+++	+++	+	+	+	++	+	+	+	++	++	++

+ – generally healthy but with small pale or white patches; ++ – 50% or less of branches yellowish or white at segments; +++ – 90–100% of branches white or deteriorated; − – no apparent change or discoloration.

Table 7. Effect of inoculation of strains P11, P25 and P50 to antibiotic-treated *K. alvarezii* branches under stressful and non-stressful condition of light intensity in batch culture. (Condition: temp. = 25 °C, salinity = 34‰, photoperiod = 12:12 h L/D cycle). (Final concentration of bacterial inoculum in seawater medium: P11 = 4.41 × 10^4 ml^{-1}, P25 = 1.5 × 10^3 ml^{-1}, P50 = 1.58 × 10^5 ml^{-1}).

	Bacterial inoculum																Control				
	P11					P25					P50						(no inoculum)				
Replicate no.>	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5		1	2	3	4	5
Days of observation																					
Stressed																					
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	ND	ND	−	−	−	−	−
7	−	++*	−	+	+	−	−	−	−	−	−	−	−	−	ND	ND	−	−	++	−	−
10	+	++	+	+	+	−	−	−	−	−	−	−	−	−	ND	ND	+	−	+++	−	−
12	++	++	+	+	+	−	−	−	−	−	−	−	−	−	ND	ND	+	−	+++	−	−
17	+++	+++	+++	+++	+++	−*	+++*	++	−	−	−	−	−	−	ND	ND	++	+	+++	+	+
Non-stressed																					
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	−	++*	−	−*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	−	++	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
12	−	++	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
17	−	++	−	++	++	−*	−	−	++*	+		+					+				

+ – generally healthy but with small pale or white patches; ++ – 50% or less of branches yellowish or white at segments; +++ – 90–100% of branches white or deteriorated; − – no apparent change or discoloration in branches; * – part of branches homogenized for bacterial re-isolation; ND – no data.

Re-isolation of the bacteria made from P11-inoculated materials from both deteriorated and healthy branches (Table 8) showed that bacteria similar to P11 can only be recovered in the deteriorated (*ice-iced*) branches, regardless of whether they were from stressed or non-stressed condition, and not from the apparently viable (healthy) materials. Re-isolation of P25-inoculated branches showed slightly different results, wherein similar species were recovered both from healthy and *ice-iced* branches from both stressed and non-stressed conditions. However, deteriorated branches yielded high number of bacteria similar to P25 than on branches which remained healthy (Table 8). Similar results were obtained in a replicate experiment.

Discussion

Bacteria play an important role in the condition of the plant in which they are associated with. This association is also true of the eucheumatoid algae presently

Table 8. Re-isolates from infected branches based on experiment in Table 7.

Bacterial inoculum	Condition of homogenized sample	Reisolated bacteria (CFU g^{-1} of wet algal sample)		
		Similar strain	Associated strains	Total
P11				
non-stressed	Healthy	0	5.00×10^5	5.00×10^5
	Ice-iced	1.34×10^7	0	1.34×10^7
stressed	Healthy	0	0	0
	Ice-iced	1.07×10^6	1.64×10^7	1.75×10^7
P25				
non-stressed	Healthy	7.14×10^4	3.21×10^5	3.93×10^5
	Ice-iced	4.29×10^7	0	4.29×10^7
stressed	Healthy	2.94×10^4	0	2.94×10^4
	Ice-iced	2.29×10^8	0	2.29×10^8

investigated and it is interesting to note that most of the bacteria showing differentiable colonies increased in number when the branches become *ice-iced* by a factor of 10- to 100-fold compared to healthy plants. Sieburth (1969b) and Linley *et al.* (1981) observed that stress on algae can trigger the release of more organic products, mostly in the form of mucilage by exudation, favoring growth of certain bacteria.

Eucheumatoid algae produce organic nutrients, possibly favoring the growth of certain groups. The elevated population density of vibrios and *Cytophaga-Flavobacterium* group in the sickly eucheumatoid branches indicates their preference for algal products which probably increase during stress conditions. This apparent specificity of bacterial association involving species of *Cytophaga-Flavobacterium*, and probably certain species of Vibrionaceae and pseudomonads has been implicated also in a number of studies (Chan & McManus, 1969; Quatrano & Caldwell, 1978; Shiba & Taga, 1980; Sarwar *et al.* 1983; Ramaiah & Chandramohan, 1992). The present study presents another case of the close affinity of these bacteria with algae, particularly with the eucheumatoids. Bacteria designated as P11 and P25, indicated occasional pathogenic activity in branches stressed by reduced salinity and light intensity. The recovery of only a small percentage of bacteria similar to P11 after infection, suggests that this bacterium acts as a trigger but is a weak competitor against the Gram-positive bacteria which predominated in most samples. Marine Gram-positive bacteria were observed by Nair & Simidu (1987) to have an antivibrio activity, possibly explaining why P11, a vibrio, was never higher in number when Gram-positive bacteria were present in a non-axenic culture used in the experiment.

The yellow *Cytophaga* strain P25 attacked agar on primary isolation and was commonly associated with *ice-iced* branches of *Eucheuma* and *Kappaphycus* from the seaweed farm. When used to inoculate (antibiotic-treated) branches of *K. alvarezii* under stressful condition, enhanced whitening was also observed, although not as efficient as P11. A second trial using the same strain failed to re-produce the *ice-ice*, possibly due to the loss of certain degrading enzymes after repeated transfer of this bacterium in culture media. When *ice-iced* branches in both experiments inoculated with P25 and its bacteria re-isolated, bacteria of similar characteristics were recovered in significantly higher concentrations, up to $10^6 - 10^7$ CFU g^{-1} of wet algal sample, even when other species were present. This observation suggests comparative differences between strains P11 and P25 in terms of their infective and competitive abilities against other species, mainly the Gram-positives, in that P11 triggers *ice-ice* whitening faster but is a weak competitor, while the effect of P25 is not so drastic but can continue to longer period due to its strong competitive ability. Based on the similarity of their characteristics, the re-isolated bacteria from P11- and P25-infected branches must be the same strains as the inoculated bacteria.

Although stress conditions may not lead directly to whitening, especially if the stress factor is a subtle one, these opportunistic bacteria can nevertheless promote the *ice-ice* disease in a rather shorter time. Gradual whitening of branches in the absence of bacterial inoculation, can be understood mainly as a physiological

effect caused by environmental stress and probably by naturally occurring bacteria. The overall weakening of the algae brought about by environmental stress is apparently a less drastic process and the presence of occasional pathogenic bacteria, which must be present in the natural seawater, promote the observed *ice-ice* disease. The number of lytic bacterial cells of vibrios and cytophagas building up on the surface, as the algae are weakened by environmental stress, appear to be necessary to develop an *ice-ice* condition, therefore intra-bacterial competition, probably through growth inhibition and promotion, is a determining factor for the symptoms to develop, an aspect that needs further investigation.

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