Journal of Applied Phycology **11:** 129–136, 1999.

Time-dependent attachment mechanism of bacterial pathogen during ice-ice infection in *Kappaphycus alvarezii* (Gigartinales, Rhodophyta)

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Received 1 April 1998; revised and accepted 8 December 1998

Key words: Kappaphycus/Eucheuma, ice-ice disease, infection mechanism, seaweed-bacteria interaction, *Vibrio* sp. P11

Abstract

The mechanism of infection by *Vibrio* sp. P11 promoting the ice-ice disease in *Kappaphycus alvarezii* was investigated *in vitro*. Its intensity of infection differs from that of another ice-ice promoter (*Cytophaga* sp. P25) by promoting the disease much faster. However, when secondary infection by other bacteria starts, its ability to compete with these bacteria gradually diminishes, whereas, infection by P25, although not displaying such drastic effects as P11, shows consistent competitive ability against other bacteria. Time-series infection experiments with application of polyclonal antibodies to specifically detect *Vibrio* sp. P11 revealed that this bacterium has a high affinity for the seaweed especially when the latter is stressed. It promotes the disease after a rapid increase in cell density of up to 10^7 g^{-1} (wet wt.) in the first 24 h. This bacterial cell build-up may take only 1–2 h on stressed thalli, but takes about 24 h on non-stressed thalli. Build-up is not sustainable in non-stressed thalli as high density is usually followed by a sudden decline in cell number believed to result from an algal defence against potential pathogens. Inoculation of the bacterium on thalli incubated in continuous culture system extends the time of bacterial attachment due to laminar flow and, possibly, competition by existing bacteria on the seaweed surface and in ambient seawater medium. Motility-driven cell attachment by this bacterium is suggested as an important factor for infection.

List of abbreviations: DAPI-DC, Diamidinophenylindole – Direct Count; IFA, Indirect Fluorescence Antibody; Pabs, Polyclonal Antibodies.

Introduction

Bacterial disease in seaweeds is a rare phenomenon (Rheinheimer, 1992) compared to terrestrial plants. However, reviews by Andrews (1979) and, more recently, by Correa (1997) on seaweed diseases, suggest that algal pathology as a discipline is still in its infancy and much is yet to be learned on the existence of an algal pathosystem.

The recent phenomenon reported by Littler and Littler (1994, 1995) of a bacterial pathogen they called coralline lethal orange disease (CLOD), consuming a large population of reef-building coralline red algae in the South Pacific, underscores the potential of a bacterial pathogen to wreak havoc on coral reef ecosystems. Similar threats of this magnitude could possibly happen among cultivated seaweed species as farming practices of these algae mainly rely on clonal propagation methods (e.g. *Kappaphycus/Eucheuma* and *Gracilaria*), allowing the seaweed to become susceptible to a potential pathogen (Santelices, 1992).

The ice-ice disease of the red algae *Kappaphycus/Eucheuma* initially thought of as mainly a non-infectious disease and which could be triggered by unfavourable environmental conditions such as extremes of temperature, irradiance and salinity (Largo et al., 1995b), could also be attributed to some opportunistic bacterial pathogens (Largo et al., 1995a). So far, the mechanism of the seaweed-bacteria interaction during the process of infection has not been elucidated or demonstrated.

In an attempt to determine the existence of a seaweed-bacteria pathosystem for the ice-ice disease, the growth behaviour of ice-ice-promoting bacterial strains on *Kappaphycus alvarezii* (Doty) Doty was investigated under various culture conditions, based on the premise that the ability of a bacterial pathogen to colonise the alga will depend on the prevailing environmental conditions in which the alga is grown. In this paper, we report the time-dependent influence of water movement, temperature and conditions of the seaweed host on the ice-ice infection of *K. alvarezii* by the opportunistic bacterium *Vibrio* sp. P11; this bacterial strain was also compared with another ice-ice disease promoting strain *Cytophaga* sp. P25 on *K. alvarezii.*

Materials and methods

Source of experimental seaweed materials

Healthy, epiphyte-free parts of main branches of *Kappaphycus alvarezii* were selected from a seaweed stock cultured at Usa Marine Biological Institute, Kochi University (Japan). Prior to experiments, they were washed and brushed several times in clean seawater, followed by another washing with autoclaved seawater. To remove loosely-associated bacteria, the thalli were sonicated for 10 min at 38 kHz using a washing sonicator (Iuchi Co., Japan) with temperature adjusted to 25 °C. The branches were then treated with an antibiotic mixture as previously described (Largo et al., 1995a) to inhibit, if possible, growth of naturally existing bacteria. The branches were then cut into 5 cm long pieces and distributed individually into ten 100-mL flasks containing 100 mL autoclaved seawater prior to further treatment as described below.

Infection intensities of P11 and P25 in stressed and non-stressed seaweed

To compare the intensities of infection between *Vibrio* sp. P11 and *Cytophaga* sp. P25, bacteria cultured in FeTY medium (see Largo et al., 1995a) were inoculated separately in 10 replicate flasks containing *K. alvarezii* branches; half of these flasks contained stressed branches. Stress conditioning was conducted using a salinity of 20‰ for 3–5 days after which thalli were returned to seawater (autoclaved) at normal salinity (34‰) for 3 days prior to bacterial inoculation. Approximately 4.41×10^4 cells mL^{-1} of *Vibrio* sp. P11 and 1.5×10^3 cells mL^{-1} of *Cytophaga* sp. P25 were inoculated into the flasks containing seaweed. Since this experiment was performed before we could develop an immunofluorescent probe with polyclonal antibodies against *Vibrio* sp. P11 (Largo et al., 1998), the standard agar plate method was used to enumerate the bacteria (Largo et al., 1995a). Identification of strains P11 and P25 from infected thalli was based on the morphological characteristics of the re-isolated colony-forming units (CFUs) formed after spreading samples onto 1.5% agar as previously described (Largo et al., 1995a). The alga-bacterial consortium was incubated, without shaking, at the alga's optimum culture conditions of 25 °C, 12:12 L:D cycle and 50 μmol photons m^{-2} s^{-1}. Samples of both healthy and ice-ice diseased parts from the same and/or different thalli were obtained for bacterial enumeration.

Behaviour of Vibrio *sp. P11 in stressed and non-stressed seaweed*

Vibrio sp. P11 culture was inoculated into replicate flasks containing *K. alvarezii* to a final inoculum size of 3.19×10^5 cells mL^{-1}. The flasks were then incubated as described above. Sampling for bacterial counting involved the removal of small duplicate pieces of thalli, each less than 1 g (about 4–6 mm long), every hour for the first 24 h and every 1–2 days thereafter, from cultures incubated for between 14 and 21 days. In addition, parallel ambient seawater samples of 1 mL were obtained. Bacteria were counted by epifluorescence microscopy using DAPI staining for the total number (DAPI-DC) and a specific polyclonal antibody for *Vibrio* sp. P11 counting (IFA method; see Largo et al., 1998).

Behaviour of P11 on thalli under the influence of seawater movement

Vibrio sp. P11 was in this experiment allowed to interact, in moving water, with naturally occurring bacteria from seawater supplied in a continuous culture system (Figure 1). The effect of water turbulence compared

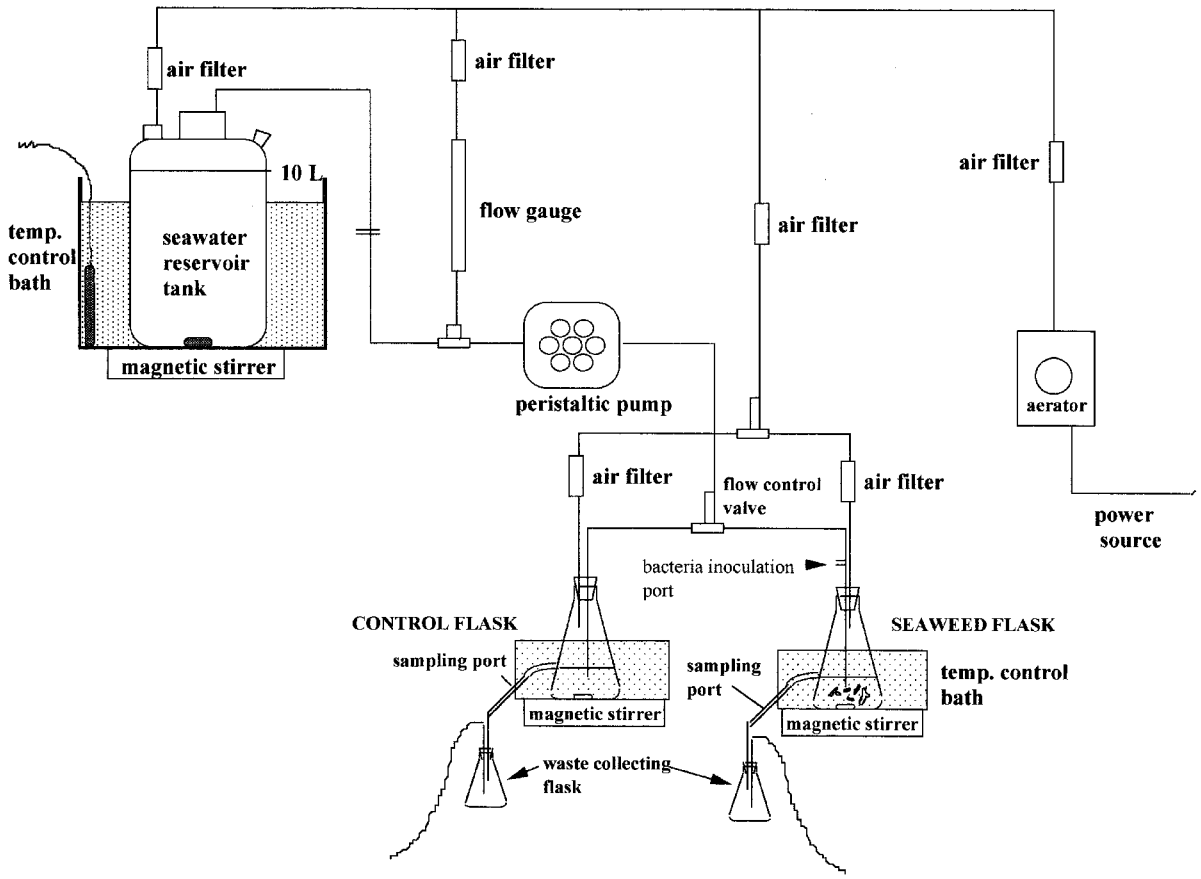

Figure 1. Diagram of the continuous culture system consisting of a seawater reservoir pulse-feeding, via a peristaltic pump, two 300-mL incubation flasks supplied with seawater at a culture volume exchange rate of 0.5–1.0 volume per day. Freshly collected seawater (from Uranouchi Inlet of Tosa Bay, southern Japan) was used in the experiment after passing it through a GF/C filter to remove grazers and large organic particles while keeping the natural bacteria intact. One of the incubation flasks contained seaweed branches cut into 5-cm pieces as described above while the other flask contained nothing except seawater, as control.

to laminar flow on bacterial attachment was determined by moving the seaweed with a magnetic stirrer at half the maximum speed of a Pasolina mini-stirrer (Iuchi Co., Japan). The results were compared to those from an identical experiment but without stirring. The desired temperature of 25 °C for the seaweed and control flasks were maintained using a heated water bath, and they were illuminated under the same conditions as described above. The seaweed was acclimatised for a 4 d period before strain P11 was inoculated. When depleted, the seawater in the reservoir was replenished with the same seawater stock as used from the start of each experiment in order to provide natural bacteria from the same population.

One of the experiments was performed to determine the effect of temperature on P11 attachment on non-stressed thalli within 24 h without stirring. Two flasks containing the seaweed were adjusted to the upper (30 °C) and lower (20 °C) temperatures of the optimal range required by the seaweed. Sampling and counting of bacteria were performed as above.

Results

Infection intensities of Vibrio *sp. P11 and* Cytophaga *sp. P25 on* K. alvarezii

The re-isolation of bacteria from both deteriorated and healthy branches of P11- and P25-inoculated cultivars is shown in Figure 2. P11-like bacteria, forming translucent and arboreous colonies, were recovered only from ice-ice diseased branches of both stressed and non-stressed thalli, and not from the apparently

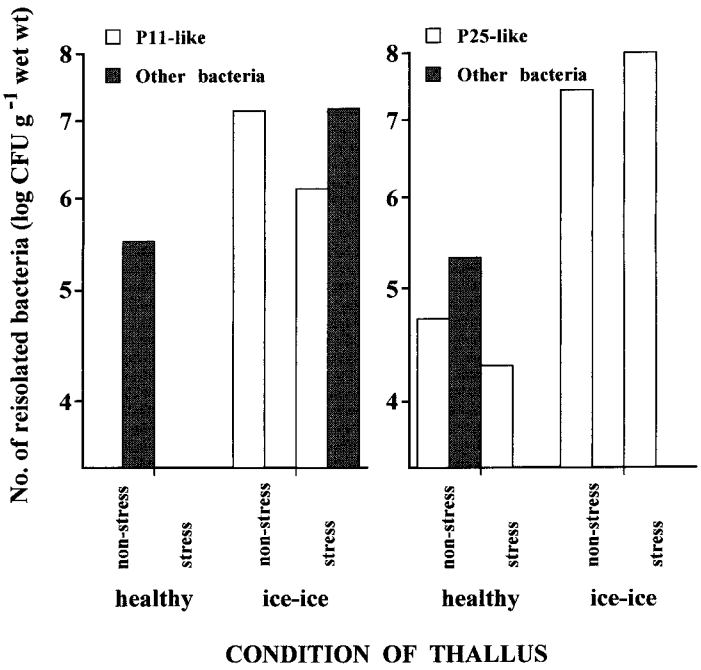

Figure 2. Re-isolated bacteria from P11- and P25-infected thalli of *K. alvarezii* in a batch culture experiment. Seaweed samples consisting of healthy and ice-ice diseased parts were obtained at the first sign of the disease.

healthy ones. Bacteria showing morphologically different CFUs from that of P11 were also found to increase in density, up to about one order of magnitude higher (1.64×10^7 CFUs g^{-1} wet wt.) than that of P11 (1.07×10^6 CFUs g^{-1} wet wt.) in stressed thalli. While other bacterial strains could be successfully suppressed by antibiotic treatment, P11-like CFUs in non-stressed thalli were high (1.34×10^7 CFUs g^{-1} wet wt.). Although P25-like CFUs were recovered from both healthy and diseased branches of both stressed and non-stressed thalli, diseased branches yielded higher numbers. These trends were similar to those obtained in a separate experiment (data not shown).

Behaviour of Vibrio *sp. P11 in stressed and non-stressed seaweed*

The fluctuation in strain P11 number counted by P11 polyclonal antibodies (PAbs) and that of total bacteria determined by DAPI-DC in batch cultures of stressed and non-stressed thalli of *K. alvarezii* are presented in Figure 3. *Vibrio* sp. P11 on both stressed and non-stressed thalli increased in cell density during the first hour of inoculation but always reached considerably higher numbers in stressed thalli than in non-stressed ones until day 12; at that time, the number of bacteria on the stressed thalli was lower than on the non-stressed thalli. P11 on stressed thalli reached cell densities of over 10^7 cells g^{-1} after 24 h but only less than 10^6 cells g^{-1} in non-stressed thalli over the same period. The early build-up of P11 cell density on stressed thalli was followed by an early onset of the ice-ice disease on the day 2 (Figure 3A, upper). However, the build-up of P11 cell density on non-stressed thalli was not immediately followed by an ice-ice condition as there was a sharp decline in its density after 3 d. Ice-ice started to develop in non-stressed thalli as the number of P11 started to increase again, reaching a high cell density on day 11; this time P11 underwent a stationary phase followed by a decline from day 14 onwards. A separate but identical experiment (data not shown) using P 11 revealed a typical declining trend in the first few days following a rapid cell increase. This phenomenon was observed only in non-stressed thalli.

Compared to P11 cells, the total bacteria numbers on stressed and non-stressed thalli did not differ widely in the first 10 to 11 h (Figure 3A). Total bacteria growth followed that of P11 in the first 6 days, but then numbers tended to increase due to the appearance of other bacterial strains in the culture.

In the ambient seawater medium (Figure 3B), a consistent increase of P11 cell density was observed

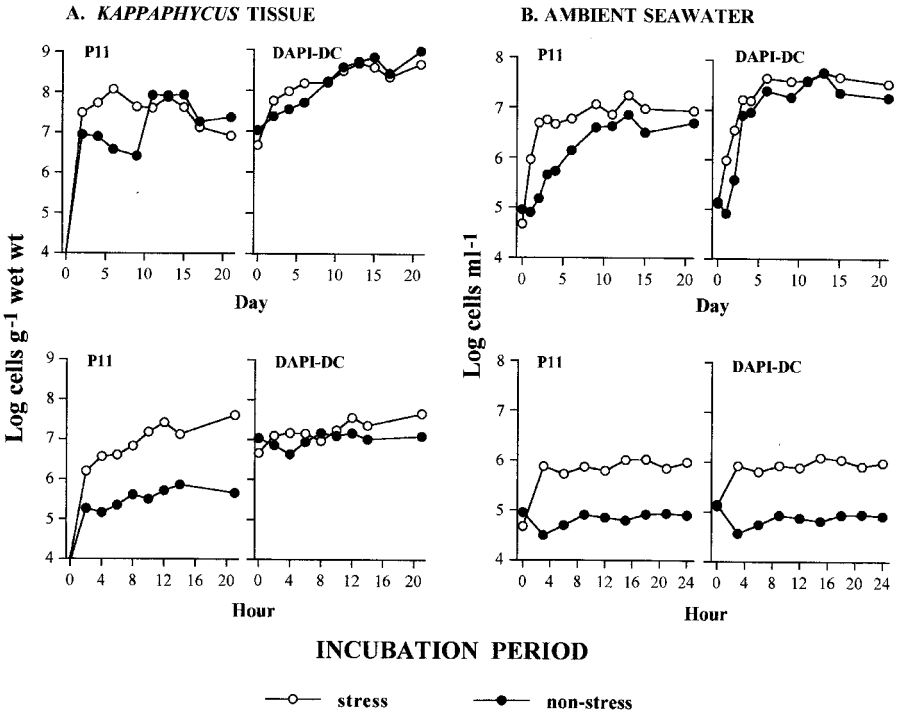

Figure 3. Behaviour of *Vibrio* sp. P11 under stressed and non-stressed condition in a batch culture system. Ice-ice developed (horizontal bars) after 2 days in stressed- and 8 days in non-stressed thalli. A: counts of bacteria from seaweed tissue. B: Counts of bacteria from ambient seawater. Each point represents the average count of duplicate subsamples. Legends apply to all panels.

in both stressed and non-stressed thalli, with stressed thalli giving consistently higher numbers. A similar pattern was observed for total bacteria.

Behaviour of P11 on seaweed thalli under the influence of seawater movement

Using a continuous culture system with stirring, strain P11 failed to attach to *K. alvarezii* tissue and a gradual disappearance of the bacterium in the ambient seawater was observed (Figure 4). When the same system was used in another experiment, but without stirring, strain P11 was able to colonise the seaweed tissue. When attachment was observed within the first 24 h, P11 cell density peaked at a value just over 10^6 cells g^{-1}. This was immediately followed by a slight decline for 2 d after which a generally increasing trend in cell density was observed up to about day 14 before equilibrium was reached. Ice-ice disease was observed to develop after about 5 d. The timing of ice-ice manifestation in the thalli was quite similar to our previous observations in batch culture, i.e. within a week (Largo et al., 1995b).

The density of P11 after inoculation in the ambient seawater of the continuous culture system with and without stirring dropped to about 50% from its initial density of $2 - 3 \times 10^5$ cells mL^{-1} after 24 h. In the stirred system, P11 gradually disappeared after 14 days but in the non-stirred system the decline occurred in the first 7 days after which it equilibrated (Figure 4B). In the system without stirring, total bacteria density from the *Kappaphycus* tissue only increased very slightly during the first 24 h but those from the culture medium declined dramatically, reflecting a more or less similar pattern to that of P11.

There seemed to be no distinct behavioural pattern of P11 in response to the two temperature regimes tested (20 and 30 °C; Figure 5). P11 appeared to be in a very unstable condition of attachment in the *Kappaphycus* tissue at both temperatures (Figure 5A) and this was accompanied by a gradual loss in the ambient seawater (Figure 5B). In the case of total bacteria, a slightly higher density was observed at 30 °C than at 20 °C in both seaweed tissue and ambient seawater, with no marked trend during the 24 h observation period.

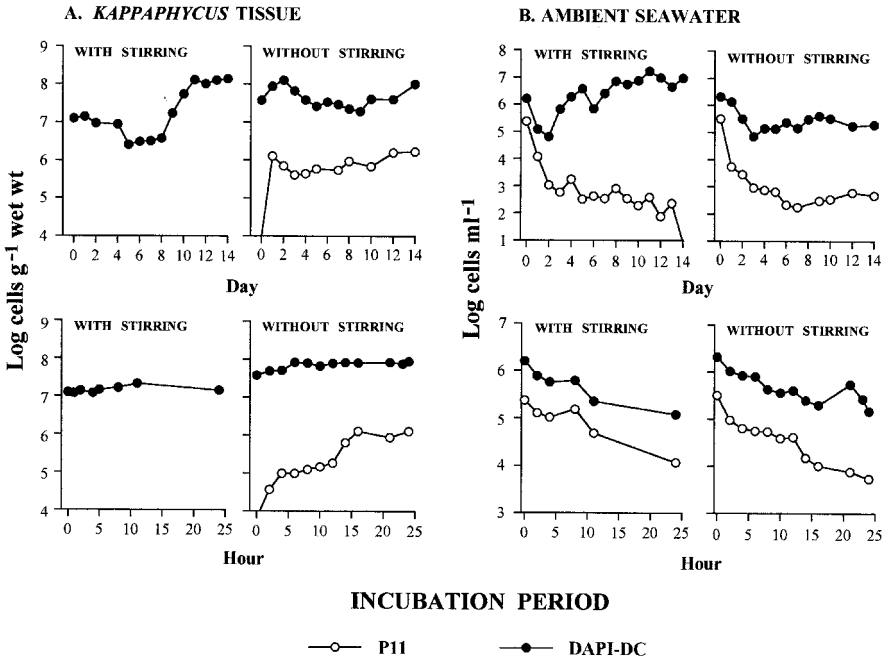

Figure 4. Behaviour of *Vibrio* sp. P11 in non-stressed *K. alvarezii* in a continuous culture system with an exchange rate of 1 volume d^{-1} with and without stirring. A: counts of bacteria from seaweed. B: counts of bacteria from ambient seawater medium. Note the absence of P11 in seaweed tissue from the stirred system. Each count represents the average of duplicate subsamples. Legends apply to all panels.

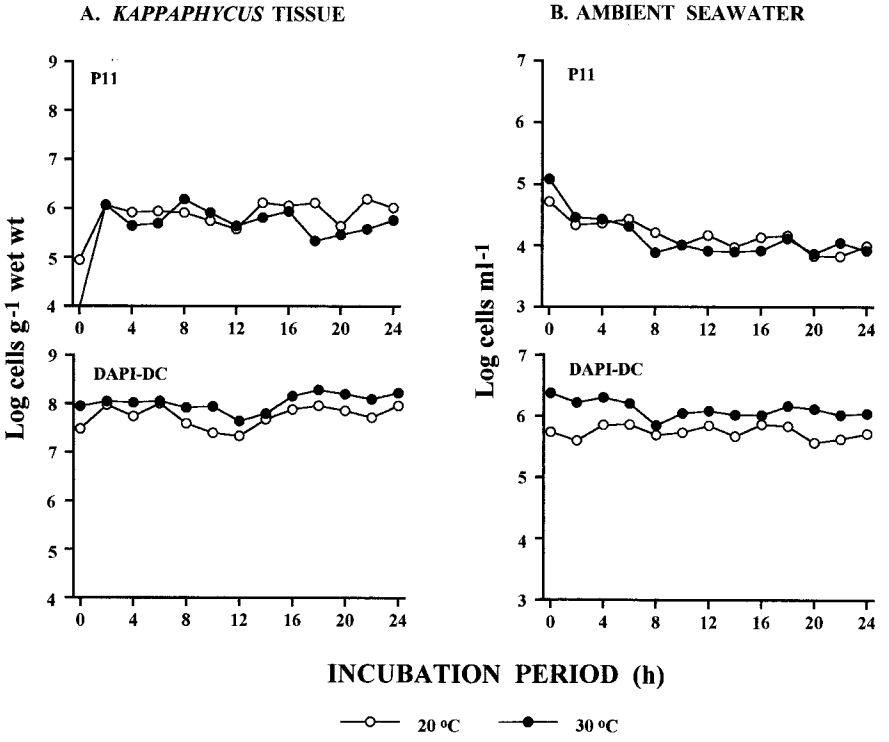

Figure 5. Effect of lower (20 °C) and upper (30 °C) range of temperature requirement of *K. alvarezii* on the behaviour of P11 on non-stressed thalli incubated using a continuous culture system without stirring. A) counts of bacteria from seaweed tissue. B) counts of bacteria from ambient seawater medium. Each point represents the average count of duplicate subsamples. Legends apply to all panels.

Discussion

The development of ice-ice disease in *Kappaphycus alvarezii* depends on several factors to which the seaweed is exposed. The combined effect of stress and biotic agents, such as opportunistic pathogenic bacteria, are primary factors of the ice-ice disease. The infection of the seaweed by these pathogens may depend, initially, on the bacteria's ability to establish themselves on the seaweed surface. This ability, however, could be influenced by the members of the bacterial community or, possibly, with other co-existing micro-organisms. Initial experiments using the agar plate method showed that *Vibrio* sp. P11 could be recovered only from ice-ice diseased thalli parts but its competitive ability against other bacteria was weak. In the absence of other bacteria causing ice-ice disease, non-stressed thalli created an environment in which P11 promoted the ice-ice disease. Our previous study showed that P11 induces the ice-ice disease within 7 days after infection (Largo et al., 1995a). On the other hand, the high cell density of *Cytophaga* sp. P25 recovered from both healthy and ice-ice diseased thalli parts suggests that this bacterium has more competitive ability against other bacteria and that it is part of the natural flora. Unfortunately, we were unable to produce an immunofluorescent probe for *Cytophaga* sp. P25 and thus, we could not study in detail the behaviour of this strain on the seaweed.

The attachment of *Vibrio* sp. P11 to the seaweed thalli is required for the bacterium to promote the ice-ice disease and it is enhanced by the flagellum of the micro-organism (Largo et al., unpublished data). Previous studies on the behaviour of motile and non-motile mutants of *Pseudomonas fluorescens* (Korber et al., 1994) and *Ps. syringae* (Haefele & Lindow, 1987) showed that motile strains colonised the substrate more successfully than non-motile ones, due to their active flagella. In the present study, the quick build-up of P11 cells on the thalli, reaching concentrations above 10^6 cells g^{-1}, is necessary in the ice-ice disease development and is one of the advantageous properties of P11 as an ice-ice causative bacterium. The development of ice-ice symptoms after a rapid increase in numbers of bacterial cells is typical of that found in higher plants. Alfano and Collmer (1996) described this phenomenon as a compatible response (disease-causing) of the plant host against an increased number of pathogenic bacteria. However, in non-stressed thalli of *K. alvarezii*, the observed decline in bacterial density, delaying for several days the development of the ice-ice symptoms, could result from a form of algal defence mechanism against invasive bacterial pathogen. The reversible attachment of P11 could be due to the presence of an inhibitory factor. *Kappaphycus alvarezii* was found to possess an antibacterial agent which selectively inhibits several strains of bacteria, depending on the season of seaweed collection and the thalli part where the antibacterial extract was obtained (Largo et al., unpublished results).

Another factor shown to affect the general attachment of P11 was water movement; continuous stirring clearly diminishes bacterial fixation. In the continuous culture system, when stirring was not used, some of the inoculated P11 cells were able to colonise the alga, but most of them were easily lost during the course of incubation. This has also been observed by Korber et al. (1994) for the motile strain of *Pseudomonas fluorescens* in a continuous culture system and may be due to physical factors such as hydrodynamic conditions and shear stress continually affecting the attaching cells. Even among already attached cells, after they reached an equilibrium, they remain dynamic as they are continually adhering and desorbing from the surface (Lawrence et al., 1995). Moreover, the number of P11 on the seaweed tissue, especially after a period of incubation, can also be affected by the increase in number of the pre-existing bacteria. Since these bacteria already occupied most of the seaweed surface prior to inoculation, despite the antibiotic treatment, this gave them a more competitive edge over that of the inoculated P11.

The attachment of P11 did not vary much in response to water temperatures within the lower or upper optimal range required for *K. alvarezii*. Both temperature regimes allowed attachment of P11 into the seaweed within the first 24 h although they were in a very unstable condition, suggesting reversibility in its attachment, at least during the early period.

Based on the findings presented in this study, it can be concluded that *Vibrio* sp. P11, by virtue of its motility, has the ability to attach quickly and colonise seaweed tissue as a first step of infection, provided that the prevailing conditions, both on the seaweed surface and in the ambient seawater, allow successful attachment of the bacterium. The subsequent build-up of the bacterial cells is a prerequisite to promoting the ice-ice disease. The second step of infection by *Vibrio* sp. P11 is based on its ability to utilise carrageenan in place of agar as a culture medium (Largo et al., 1995a), and therefore could be using this cellular polysaccharide as a carbon source which is abundant in

K. alvarezii cell wall matrix. The bacterium has been microscopically observed to penetrate the medullary layer of an P11-infected thalli (Largo et al., 1998), suggesting the possible involvement of hydrolytic enzymes such as carrageenase. Zablackis et al. (1993) showed that cellulase and carrageenase, which were originally isolated from bacteria, release epidermal as well as medullary cell protoplasts in *K. alvarezii*. This hydrolytic activity could be the reason for thalli whitening, symptomatic of ice-ice disease. Once P11 bacterial cells have colonised the thalli at high concentrations and start to utilise carrageenan (1–2 days), penetration into the medulla could cause the further weakening and eventual collapse of the thalli in the affected part. Water current in the cultivation site then causes the affected thalli to fragment prematurely.

From the standpoint of seaweed crop management, the disadvantageous factors affecting P11 attachment into *K. alvarezii* are also advantages when considering preventive measures of ice-ice disease. Areas with strong water exchange are normally ideal sites for seaweed farming. Thus, healthy thalli of *K. alvarezii*, as a result of efficient uptake of nutrients in well-flushed and growth-conducive areas, can themselves ward-off potential pathogens possibly by the production of an antimicrobial compound. This is an important consideration when selecting suitable sites for *Kappaphycus/Eucheuma* farming.

Acknowledgement

D.B. Largo is grateful to the Ministry of Education, Science and Culture, Government of Japan, for the fellowship grant under which this study was conducted as part of his PhD dissertation.

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