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NOTE

Cross-Host Protection of Marine Bacteria Against Macroalgal Disease

Jiasui Li[1] · Florian Weinberger[2] · Mahasweta Saha[2,3] · Marwan E. Majzoub[1] · Suhelen Egan[1]

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Abstract

Despite an increasing awareness of disease impacts on both cultivated and native seaweed populations, the development of marine probiotics has been limited and predominately focused on farmed animals. Bleaching (loss of thallus pigmentation) is one of the most prevalent diseases observed in marine macroalgae. Endemic probiotic bacteria have been characterized to prevent bleaching disease in red macroalgae *Agarophyton vermiculophyllum* and *Delisea pulchra*; however, the extent to which probiotic strains provide cross-protection to non-endemic hosts and the influence of native microbiota remain unknown. Using *A. vermiculophyllum* as a model, we demonstrate that co-inoculation with the pathogen *Pseudoalteromonas arctica* G-MAN6 and *D. pulchra* probiotic strain *Phaeobacter* sp. BS52 or *Pseudoalteromonas* sp. PB2-1 reduced the disease risks compared to the pathogen only treatment. Moreover, non-endemic probiotics outperformed the endemic probiotic strain *Ralstonia* sp. G-NY6 in the presence of the host natural microbiota. This study highlights how the native microbiota can impact the effectiveness of marine probiotics and illustrates the potential of harnessing probiotics that can function across different hosts to mitigate the impact of emerging marine diseases.

Keywords *Agarophyton vermiculophyllum* · Bleaching mitigation · *Gracilaria vermiculophylla* · Probiotics · Seaweed disease · *Delisea pulchra*

Infectious diseases have been reported as one of the main factors threatening marine macroalgae (seaweeds) in wild populations [1–3] and farmed species [4, 5]. However, current disease management strategies, involving specific cultivation practices or chemical treatments [6, 7], are often highly labor-intensive and impractical for the application to wild seaweed populations. The use of probiotics for disease control in industrial and natural systems is gaining interest [8–12], yet in the marine environment disease protective bacteria have predominately been characterized for aquaculture animals [13, 14] with few attempts made to discover macroalgal probiotics [15–17]. To date, research has focused either on the isolation of endemic probiotic bacteria from the target hosts and their environment or on the use of well-characterized human/animal dietary probiotics such as *Lactobacillus* spp. or *Bacillus* spp. [14]. While such approaches contribute to the development of new probiotics, animal probiotics may not be well adapted to the marine macroalgal environment. Moreover, little is understood about the extent to which the function of a marine probiotic strain is host specific (but see [18, 19]). Addressing these knowledge gaps serves as the cornerstone of developing common probiotics as a generalized solution to emerging aquatic diseases.

Bleaching is one of the most common diseases observed in macroalgae [2, 20–22] including *Agarophyton vermiculophyllum* in the Baltic Sea [16] and wild *Delisea pulchra* populations in Australia [23]. A common feature of bleaching is that disease appears to be a result of opportunistic pathogens exploiting the stressed hosts. We have recently identified a range of bacteria that can antagonize pathogens that cause *D. pulchra* bleaching disease, including two strains (*Pseudoalteromonas* sp. PB2-1 and *Phaeobacter* sp. BS52) that can reduce the pathogen-induced bleaching in vivo [15]. Similarly, endemic bacterial strains have been shown to reduce the risk of disease in *A. vermiculophyllum*

✉ Suhelen Egan
s.egan@unsw.edu.au

[1] Centre for Marine Science and Innovation, School of Biological, Earth and Environmental Sciences, Faculty of Science, The University of New South Wales, Kensington, NSW 2052, Australia

[2] Marine Ecology Division, GEOMAR Helmholtz Centre for Ocean Research Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany

[3] Marine Ecology and Biodiversity, Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, UK

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in the presence of pathogens [16]. However, previous studies were conducted on *A. vermiculophyllum* pre-treated with antibiotics; thus, the extent to which probiotics are effective in reducing the risk of disease in the presence of the macroalga's undisturbed natural microbiota remains unknown. Here we test the hypothesis that protective bacteria of *D. pulchra* are also effective in controlling pathogen-induced disease in *A. vermiculophyllum* and that disease protection is influenced by the host's surface microbiota.

Healthy *A. vermiculophyllum* [24] (synonym: *Gracilaria vermiculophylla* (Ohmi) Papenfuss) samples without any apparent thallus discoloration were collected from the sandy shores at Nordstrand (53°29′10.25″N, 8°38′35.33″E), from December 2018 to March 2019, and transported to the GEOMAR Helmholtz Centre for Ocean Research labs (Kiel, Germany) within 2 h. Macroalgal maintenance and in vivo bleaching disease assays were conducted as previously described [16] (see also Supplementary Information).

We first determined if the non-endemic candidate probiotics (*Pseudoalteromonas* sp. PB2-1; *Phaeobacter* sp. BS23, BS34, and BS52; *Photobacterium* sp. BS55; and *Vibrio* sp. BL95) had a detrimental impact on *A. vermiculophyllum* health and compared these to both the representative pathogen *Pseudoalteromonas arctica* G-MAN6 and the endemic protective strain *Ralstonia* sp. G-NY6 [16] (Table 1). Each strain was inoculated separately in bleaching assays of *A. vermiculophyllum* with a disturbed (pre-treated with antibiotics vancomycin and cefotaxime each at a final concentration of 0.1 mg·ml^{-1} for 2 days following the procedures outlined in [16]) or natural microbiota (i.e., no antibiotic pre-treatment). The number of healthy and bleached tips from each fragment was counted after 5 days using a binocular microscope (Fig. S1). The relative risk (RR) for each treatment compared to the seawater control (SW) was calculated using the formula below [25] and the effect of each treatment tested using a Generalized Linear Mixed-effect Model with a p value of < 0.05 considered significant (see also Supplementary Information).

$$\text{Absolute risk of tip bleaching in Treatment } X\ (\%) = \frac{\text{Number of bleached tips in Treatment } X}{\text{Number of tested tips in Treatment } X} \times 100 \quad (1)$$

$$\text{Absolute risk of tip bleaching in Control } (\%) = \frac{\text{Number of bleached tips in Control}}{\text{Number of tested tips in Control}} \times 100 \quad (2)$$

A RR = 1 means the risk of tip bleaching is identical in the treatment and control. An RR < 1 or > 1 means the treatment reduces or increases the risk compared to the control.

The addition of non-endemic bacteria did not alter the disease risk of *A. vermiculophyllum* compared to the control samples (Fig. 1; Table S1). In contrast, adding *P. arctica* G-MAN6 to *A. vermiculophyllum* resulted in a significant increase in bleaching compared to the control, in samples with both a disturbed and natural microbiota ($RR_{\text{disturbed microbiota}} = 2.36$, $RR_{\text{natural microbiota}} = 2.25$; Fig. 1, Table S1), thereby confirming its role as a pathogen. Interestingly, inoculation of the endemic probiotic strain *Ralstonia* sp. G-NY6 lowered the disease risk to approximately one-third compared to the SW control in *A. vermiculophyllum* with disturbed microbiota, but doubled the disease risk in the presence of the natural microbiota ($RR_{\text{disturbed microbiota}} = 0.36$, $RR_{\text{natural microbiota}} = 2.11$; Fig. 1, Table S1). These results suggest that *Ralstonia* sp. G-NY6 could compromise host health through interacting with other microbiota members and highlight the importance of testing probiotics in the presence of a natural microbiota.

Table 1 Bacteria tested in *Agarophyton vermiculophyllum* bleaching disease assays

Bacterial ID	Taxonomy	Original habitat	Ecological role	Reference/Provider
G-MAN6	*Pseudoalteromonas arctica*	Surface of *A. vermiculophyllum*	*A. vermiculophyllum* pathogen	[16]
G-NY6	*Ralstonia* sp.	Surface of *A. vermiculophyllum*	*A. vermiculophyllum* bleaching protective strain	[16]
PB2-1	*Pseudoalteromonas* sp.	Surface of *D. pulchra*	*D. pulchra* bleaching protective strain	[15, 26]
BS23	*Phaeobacter* sp.	Surface of *D. pulchra*	*D. pulchra* pathogen-antagonistic strain	Sharon Longford, UNSW Sydney; [15]
BS34	*Phaeobacter* sp.	Surface of *Ulva australis*	*D. pulchra* pathogen-antagonistic strain	[15, 27]
BS52	*Phaeobacter* sp.	Surface of *D. pulchra*	*D. pulchra* bleaching protective strain	[15, 27]
BS55	*Photobacterium* sp.	Surface of *D. pulchra*	*D. pulchra* pathogen-antagonistic strain	[15, 27]
BL95	*Vibrio* sp.	Surface of *D. pulchra*	*D. pulchra* pathogen-antagonistic strain	Vipra Kumar, UNSW Sydney; [15]

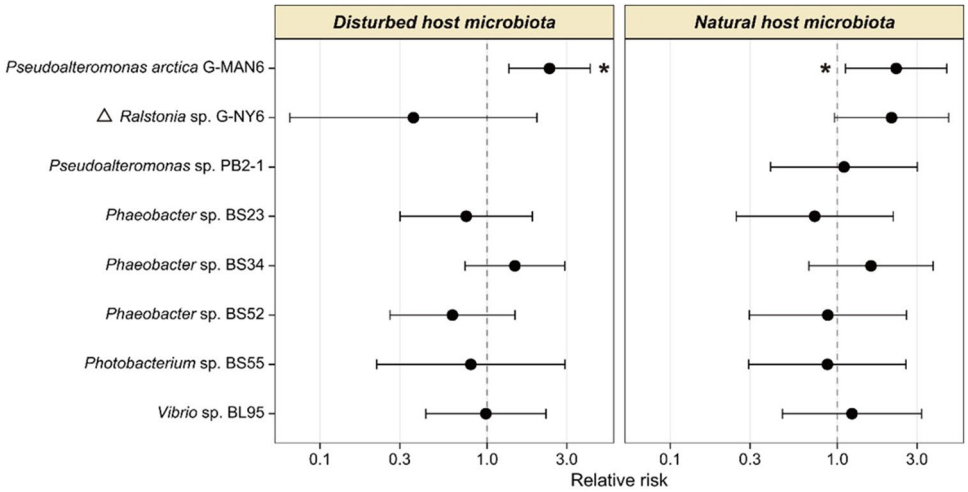

Fig. 1 Effect of single bacterial strain inoculums on the risk of bleaching disease in *Agarophyton vermiculophyllum* with a disturbed or a natural microbiota. The *x*-axis shows the relative risk (RR, calculated for three independent experiments with six biological replicates each) and 95% confidence intervals (CIs). Note that no diseased tips were observed in *Pseudoalteromonas* sp. PB2-1 treatment in *A. vermiculophyllum* with disturbed microbiota; thus, the CIs could not be estimated. The vertical dashed line indicates RR = 1.0 (i.e., the risk of test strain treatment equals to the corresponding control). The *y*-axis shows the taxonomic affiliation and identification number (ID) of test bacteria. Multiple comparisons (*emmeans* function in R emmeans package) on a two-way Poisson GLMM ($Df = 83$) were performed to examine the differences between treatments and SW control (* indicates a significant difference), and between host microbiota status (△ indicates a significant difference). The confidence levels and *p* values were adjusted using a mvt method. An adjusted *p* value of < 0.05 was considered significant

After showing that the non-endemic candidate probiotics did not themselves result in an increased risk of tip bleaching (Fig. 1; Table S1), we further tested whether any of them could reduce pathogen-induced bleaching in *A. vermiculophyllum*. Each of the candidate probiotics was co-inoculated with the pathogen *P. arctica* G-MAN6 in *A. vermiculophyllum* with a disturbed or natural microbiota. In *A. vermiculophyllum* with a disturbed microbiota, co-inoculations with *Phaeobacter* sp. BS23, BS34, and BS52 or *Ralstonia* sp. G-NY6 resulted in a significant decrease in the disease risks compared to the pathogen only treatment ($RR_{BS23\ +\ G\text{-}MAN6} = 0.20$, $RR_{BS34\ +\ G\text{-}MAN6} = 0.15$, $RR_{BS52\ +\ G\text{-}MAN6} = 0.18$, $RR_{G\text{-}NY6\ +\ G\text{-}MAN6} = 0.32$), while there was no statistical support for a change in these disease risks compared to the SW controls (Fig. 2; Table S2). However, when the microbiota was present, co-inoculation of *P. arctica* G-MAN6 and either *Phaeobacter* sp. BS23, *Phaeobacter* sp. BS52, or *Pseudoalteromonas* sp. PB2-1 resulted in a significant decrease in disease risks compared to the pathogen alone ($RR_{BS23\ +\ G\text{-}MAN6} = 0.29$, $RR_{BS52\ +\ G\text{-}MAN6} = 0.19$, $RR_{PB2\text{-}1\ +\ G\text{-}MAN6} = 0.24$; post hoc tests on Poisson GLMM: $p_{adjusted} < 0.05$). Moreover, *Phaeobacter* sp. BS23 and BS52 strains outperformed *Ralstonia* sp. G-NY6 in disease protective effects in *A. vermiculophyllum* (Fig. 2).

Collectively these results show that *D. pulchra* bleaching-protective strains *Phaeobacter* sp. BS52 and *Pseudoalteromonas* sp. PB2-1 [15] could also alleviate the pathogen-induced bleaching in *A. vermiculophyllum*. Bacteria belonging to the genera *Phaeobacter* and *Pseudoalteromonas* have also been reported as probiotics in a variety of marine systems, including corals [28], shellfish [11, 29, 30], and fish larvae [31–33]. Together with these studies, our current work supports the idea that *Phaeobacter* and *Pseudoalteromonas* strains show promise as general marine probiotics.

Factors that contribute to the success of *Pseudoalteromonas* spp. as a probiotic could be due to direct or indirect inhibition of pathogens, e.g., the production of antibiotics [34, 35] or bacterial quorum sensing inhibitors [36]. It should be noted, however, that *Pseudoalteromonas* sp. PB2-1 provided significant protection to *A. vermiculophyllum* in the presence of the host natural microbiota, but only displayed moderate protection on *A. vermiculophyllum* with a disturbed microbiota. These observations suggest that the direct antibiotic activities of *Pseudoalteromonas* sp. PB2-1 may not be sufficient to protect *A. vermiculophyllum* against disease. Rather PB2-1 may have an indirect mode of action for disease protection, such as mitigating pathogen-induced dysbiosis (e.g., see [15, 37, 38]), or acting in synergy with other members of the microbiota to afford protection to the host.

Host microbiota have previously been hypothesized to play a key role in health maintenance through inhibiting detrimental colonizers including pathogens [39]. However, our results suggest that different probiotic strains can perform differently depending on the host microbiota. For example,

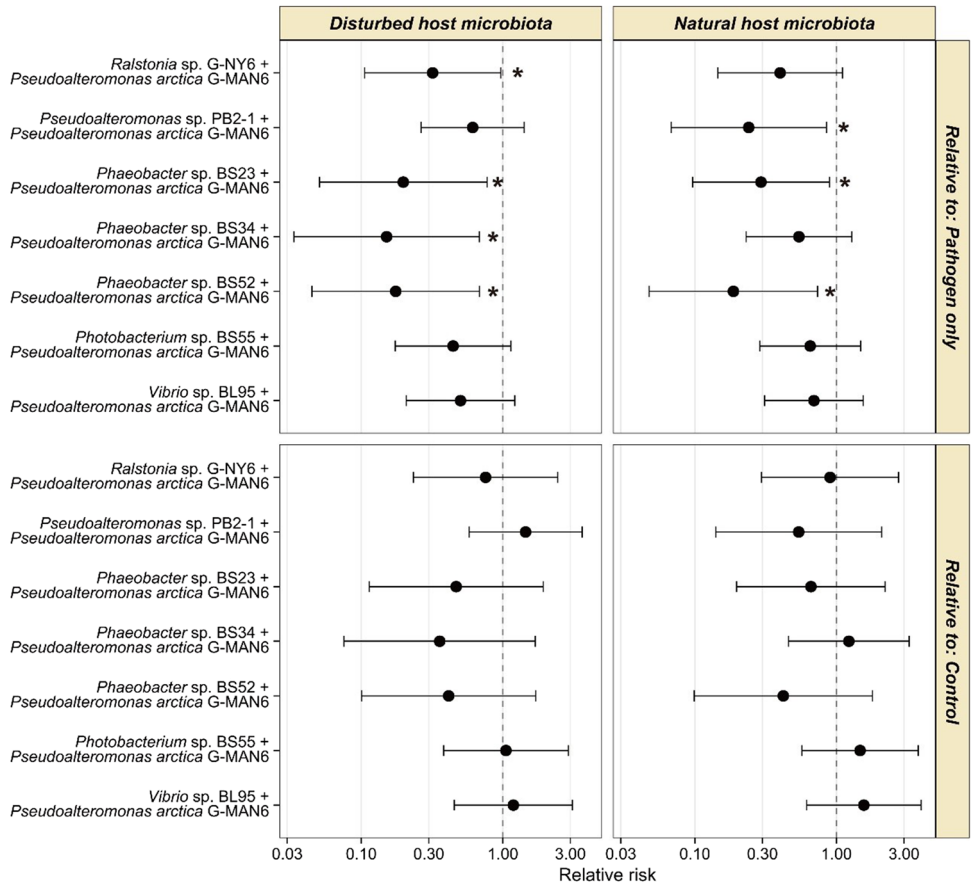

Fig. 2 Effect of co-inoculation of candidate protective bacteria and the pathogen *Pseudoalteromonas arctica* G-MAN6 on the risk of bleaching disease in *Agarophyton vermiculophyllum* with a disturbed or a natural microbiota. The pathogen only treatment and SW control are used as references to calculate the relative risk (RR) of different co-inoculation treatments (results are shown in top and bottom panels, respectively). The *x*-axis shows the RR (calculated for three independent experiments with six biological replicates each) and 95% confidence intervals (CIs). The vertical dashed line indicates RR = 1.0 (i.e., the risk of co-inoculation treatment equals to the corresponding pathogen only treatment or SW control). The *y*-axis shows the taxonomic affiliation and identification number (ID) of inoculated bacteria. Multiple comparisons (*emmeans* function in R emmeans package) on a two-way Poisson GLMM ($Df = 83$) were performed to examine the differences between treatments and pathogen only or SW control (* indicates a significant difference), and between host microbiota status (△ indicates a significant difference). The confidence levels and *p* values were adjusted using a mvt method. An adjusted *p* value of < 0.05 was considered significant

while *Pseudoalteromonas* sp. PB2-1 was more effective in the presence of the host microbiota (see above), the presence of a natural surface microbiota did not change the effectiveness of *Phaeobacter* sp. strains BS52 or BS23. In contrast *Ralstonia* sp. G-NY6 and *Phaeobacter* sp. BS34 were protective only when the *A. vermiculophyllum* surface microbiota was disturbed. One possible explanation for this reduced probiotic effect is that the host microbiota offer some protection to the pathogen. Similar microbiota influences on interactions between probiotics and the target pathogen have been reported for the aquaculture probiotic strain *Phaeobacter inhibens* DSM 17,395. For example, introduction of *P. inhibens* DSM 17395 reduces the abundance of the fish pathogen *Vibrio anguillarum* in axenic artemia cultures, but only limits pathogen growth (i.e., by keeping the pathogen counts similar to the inoculum levels) in non-axenic cultures [40]. The mechanisms through which tripartite interactions among the host microbiota, pathogens, and probiotics affect the final disease outcomes on the host will be an important area for future research and our findings highlight the necessity of testing probiotics in near natural systems.

In summary, we have shown that *Phaeobacter* sp. BS52 and *Pseudoalteromonas* sp. PB2-1 can protect the two phylogenetically and geographically distinctive red seaweeds *D. pulchra* and *A. vermiculophyllum* from their pathogen-induced bleaching disease, even in the presence of a native microbiome. Understanding these ecological interactions will help pave the way for the use of common probiotics to facilitate marine host conservation and sustainable aquaculture.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01909-2.

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Author Contribution JL, FW, MS, and SE contributed to the study conception and design. FW and JL prepared the experimental materials. JL and MS performed the experiments. JL analyzed the data. JL and SE wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Raw data of alga infection assays are deposited at https://doi.org/10.6084/m9.figshare.16576244.

Code Availability Code for analyses is available at: https://doi.org/10.6084/m9.figshare.16576244.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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