Journal of Applied Phycology **8**: 409–414, 1996.

Development of an *in situ* assay to detect bacterial pathogens of the red alga *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham

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Received 1 June 1996; revised 15 August 1996; accepted 16 August 1996

Key words: Gracilaria gracilis, bacterial pathogens, agarolytic, Saldanha Bay, South Africa

Abstract

Since current protocols were found to be inadequate for the identification of bacteria pathogenic to *Gracilaria gracilis*, an assay was developed which made use of 'axenic' algae in order to attribute disease symptoms directly to the presence of a specific bacterial isolate. However, this assay proved to be as unreliable as existing procedures and failed to satisfy Koch's postulates. A second pathogenicity assay was developed which proved more reliable in that each bacterial strain consistently induced a particular symptom in the infected algal thallus. The bacterial strain LS2i was identified as a possible pathogen of *G. gracilis* using this assay. However, this assay did not satisfy Koch's criteria for establishing an unequivocal link between the observed disease symptoms and strain LS2i, since significant bacterial contamination of the test alga occurred. A third protocol generated consistent results and satisfied Koch's postulates, enabling the identification of six pathogens of *G. gracilis*. All the pathogenic bacterial strains were agarolytic.

Introduction

The red algal genus *Gracilaria* is a commercially important source of industrial food agar (McHugh, 1991; Armisen, 1995). A large natural population of *G. gracilis* exists in Saldanha Bay, South Africa. In addition, raft culture of *G. gracilis* is being investigated experimentally at Saldanha Bay by the Sea Fisheries Research Institute (Anderson et al., 1989), and has recently been initiated on a commercial scale by Sea Harvest (R. J. Anderson, pers. comm.), in order to increase the yield of Saldanha Bay *G. gracilis* for commercial exploitation.

In 1989, the natural *G. gracilis* population at Saldanha Bay collapsed and no commercial collections were obtained for the following three years. The initial cause of the collapse remains unclear, but once underway, the seaweed beds were further decimated by invertebrate grazers (Anderson et al., 1993). The collapse of the Saldanha Bay population in 1989 may have been the result of disease, since plants examined during subsequent partial die-offs of the Saldanha Bay beds (e.g. in January 1993) exhibited symptoms such as lesions and thallus bleaching (R. J. Anderson, pers. comm.).

Very little is known concerning bacterial pathogens of algae (Andrews, 1976; Correa & Craigie, 1991), and *Gracilaria* species in particular. 'Ice-ice', a white powdery growth observed on a *Eucheuma* species growing in the Phillipines, was thought to be caused by bacteria (Uyenco et al., 1981). The same disease, identified in *E. denticulatum* and *Kappaphycus alvarezii* from the Phillipines, was shown to be triggered by bacteria in laboratory experiments (Largo et al., 1995a). Agar-degrading bacteria are responsible for a white tip disease of *G. conferta* growing in seawater ponds (Friedlander & Gunkel, 1992; Weinberger et al., 1994; Friedlander & Levy, 1995). Lavilla-Pitogo (1992) reported that agar-digesting bacteria were associated with 'rotten thallus syndrome' in another *Gracilaria* species.

G. gracilis from Saldanha Bay supports a diverse community of bacterial epiphytes, many of which are agarolytic (A. E. Jaffray et al., unpublished results). Since the cell wall of *G. gracilis* is composed primari-

ly of agar (McHugh, 1991), it is possible that agarases secreted by epiphytic bacteria may be responsible for disease symptoms such as thallus bleaching and lesion formation in this algal species. Indeed, this has been confirmed with respect to bacterial pathogens of other *Gracilaria* species (Friedlander & Gunkel, 1992; Weinberger et al., 1994; Friedlander & Levy, 1995; Lavilla-Pitogo, 1992). Furthermore, Uyenco et al. (1981) postulated that low levels of nutrients such as phosphates were responsible for making a particular *Eucheuma* species more susceptible to bacterial disease, while Largo et al. (1995b) showed that 'ice-ice' can be triggered artificially by subjecting *E. denticulatum* and *K. alvarezii* to sub-optimal levels of light intensity, temperature and salinity during laboratory culture. It is possible that unfavourable environmental factors such as increased water temperature and nutrient deprivation, both of which are known to occur in Saldanha Bay during the summer months (Anderson et al., 1996), may induce the onset of bacterial disease in *G. gracilis*.

In order to identify the causal agent of a disease, it is necessary to fulfil Koch's postulates (Black, 1996). Thus, specific symptoms must be induced by a particular bacterium when introduced into a susceptible host, and subsequently, the identical bacterium should be re-isolated from the diseased host.

Largo et al. (1995a) have shown that epiphytic bacteria isolated from *K. alvarezii* are pathogenic to this alga by inoculating a non-axenic algal culture with a specific bacterial isolate. Similar methods were employed by Weinberger et al. (1994) to demonstrate the pathogenicity of *G. conferta* bacterial epiphytes. However, the above methodology proved inadequate for the identification of bacterial pathogens of *G. gracilis* from Saldanha Bay, in that assay results were not reproducible, control plants developed disease symptoms, the bacterial strain inoculated into the algal culture could not always be re-isolated following the onset of disease, and lastly, the use of non-axenic *G. gracilis* resulted in bacterial contamination which prevented identification of a particular bacterial strain as the causative agent of the disease symptoms manifested by the inoculated alga.

In this study, we describe the development of a method for generating axenic *G. gracilis* and an assay suitable for screening bacterial epiphytes of this alga for pathogenicity according to Koch's postulates.

Materials and methods

Generation of axenic G. gracilis

Five-gram quantities (wet wt) of healthy *G. gracilis* (dark red and free of algal epiphytes) were immersed in 500 ml 0.2 μm-filtered distilled water for 4 h. The alga was then re-immersed in 100 ml sterile seawater (SSW) and sonicated in a sonicating bath (Elma) at 50–60 Hz for 4 min. The alga was subsequently immersed in 100-ml Povidone-iodine for 60 s and finally incubated for 24 h in 100 ml SSW containing a 'cocktail' of antibiotics. This was composed of (per litre SSW) streptomycin (500 mg), penicillin (500 mg), kanamycin sulphate (500 mg), cefotaxime (300 mg) and nalidixic acid (0.6 mg). The alga was rinsed thoroughly in SSW and tested for axenicity by rubbing 5–6 cm thallus pieces across the surface of synthetic marine agar: (per litre distilled water) NaCl (30.0 g), $MgCl_2$ ((2.3 g), KCl (0.3 g), glucose (2.0 g), casamino acids (5.0 g), yeast extract (1.0 g) and agar (20.0 g), adjusted to pH 7.0 with 1 M NaOH. The cultures were incubated for up to 14 days at 22 °C, during which time they were monitored for growth of bacterial colonies.

Pathogenicity assay: method 1

Bacterial epiphytes were previously isolated from the surface of *G. gracilis* (manuscript in preparation). Six-centimeter lengths of axenic thalli were rinsed thoroughly in SSW and each inoculated along its length with a single bacterial strain using an inoculating loop. Each inoculated thallus was placed in an Erlenmeyer flask containing 100 ml SSW and incubated at 22 °C. A 6-cm length of axenic thallus was placed in an Erlenmeyer flask containing 100 ml SSW as a control.

The thalli were scored for the appearance or absence of symptoms after 12 days. In order to satisfy Koch's postulates, 1-cm sections were cut off the inoculated thallus and inoculated onto synthetic marine agar. The cultures were incubated at 22 °C for 5 days and scored for the presence of bacterial colonies identical to the original inoculant.

Pathogenicity assay: method 2

Two previously-isolated epiphytic bacterial strains, designated LS2i and LS1a, were each cultured at 22 °C for 16 h in 10 ml marine broth (MB) on an orbital shaker (Gallenkamp) at 100 rpm. One hundred microlitres of each culture was subsequently transferred to 100 ml

MB in an Erlenmeyer flask and incubated under the same conditions until the culture reached an optical density (A_{600}) of between 1.20 and 1.50.

5-cm lengths of axenic *G. gracilis* thalli were injected at each end with 200 μl bacterial suspension to a depth of approximately 1 cm from the end of the thallus. Each injected thallus segment was placed onto synthetic marine agar and incubated at 30 °C for up to 7 days. A 5 cm length of axenic thallus injected with SSW, and an uninjected axenic thallus segment, served as experimental controls. The thalli were monitored daily for the appearance of symptoms and concurrently examined for the growth of bacterial colonies identical to the injected strains.

Pathogenicity assay: method 3

Ten previously-isolated epiphytic bacterial strains were cultured in MB as described in method 2. Five-centimeter lengths of axenic *G. gracilis* thalli were injected with the bacterial strains as described previously in method 2. Experimental controls were as above (method 2). Each injected thallus was placed in an Erlenmeyer flask containing 50 ml MB and incubated at 30 °C for 5 days.

Thalli were scored for the appearance of symptoms after 5 days. In order to satisfy Koch's postulates, bacteria from the culture media were inoculated on synthetic marine agar and incubated at 22 °C for 5 days. The cultures were scored for the growth of bacterial colonies identical to the strain originally injected into the thallus.

Results

Bacterial growth was not observed on synthetic marine agar over a 10-day period when axenic *Gracilaria* was tested for sterility. Thereafter, a variety of bacterial colonies were observed growing on the medium.

Ten bacterial epiphytes were individually tested for pathogenicity on axenic *G. gracilis* using method 1 (Table 1). The initial experiment identified 7 bacterial strains which elicited disease symptoms in inoculated thalli after 12 days incubation, while 3 bacterial isolates failed to affect the appearance of the host alga over the same time period. The symptoms observed varied from the entire thallus turning pale green or white, the appearance of pale pink and green bands encircling the thallus, to the appearance of mottled patches on some thalli and growth tips. The experimental control remained symptomless for at least 20 days incubation.

Table 1. Results of pathogenicity assay using method 1. Disease symptoms were scored after 12 days incubation.

Strain	Experiment number					
	1	2	3	1	2	3
	Disease symptoms			Re-isolation of inoculant		
LS1a	+	+	+	i	i	i
LS1q	+	+	+	i	+c	i
LS1ah	−	+	−	i	i	i
LS2i	−	+	−	i	i	i
LS2q	+	+	+	i	i	i
LS3f	+	+	+	i	i	i
LS3g	+	+	−	c	c	c
LS4d	+	+	+	i	i	i
SS4g	+	−	−	i	+c	i
SS5g	−	−	+	i	i	i
Control	−	+	+	c	c	c

+, symptoms present; −, symptoms absent; i, inoculant re-isolated; c, contaminant(s) re-isolated; +c, inoculant and contaminant(s) re-isolated

In a repeat experiment (Table 1; experiment 2), bacterial strains LS1ah and LS2i induced symptoms in the host plant, while strain SS4g no longer caused symptoms in axenic *G. gracilis*. In addition, disease symptoms were apparent in the control plant. A similar lack of consistency was observed when the experiment was performed a third time (Table 1; experiment 3). Furthermore, symptoms caused by particular bacterial isolates were inconsistent between experiments.

Nine of the ten bacterial strains tested for pathogenicity were re-isolated from infected algal thalli in all three experiments using assay method 1 (Table 1). Although colonies of both LS1q and SS4g were identifiable from thallus samples inoculated on synthetic marine agar, the thallus segments were also contaminated with other bacterial strains. Strain LS3g was not re-isolated from inoculated thallus, whereas contaminating bacteria were observed.

The second assay procedure was used to test the pathogenicity of bacterial strains LS1a and LS2i. Strain LS2i, a highly agarolytic bacterium (results not shown), caused bleaching along the injected portion of the algal thallus (Table 2). Strain LS1a, a non-agarolytic isolate, did not cause disease symptoms in *G. gracilis* thallus. Potential physical damage to the thallus as a consequence of injection was tested by injecting SSW into thallus segments. In all 3 replicates

Table 2. Pathogenicity assay using method 2. Disease symptoms were scored after 4 days incubation.

Strain	Experiment number					
	1	2	3	1	2	3
	Disease symptoms			Re-isolation of inoculant		
LS1a	+	+	+	+c	+c	+c
LS2i	–	–	–	i	i	i
Control	–	+	+	n	n	n

+, symptoms present; –, symptoms absent; i, inoculant re-isolated; +c, inoculant and contaminant(s) reisolated; n, no bacterial growth

Table 3. Pathogenicity assay using method 3. Disease symptoms were scored after 5 days incubation.

Strain	Experiment number					
	1	2	3	1	2	3
	Disease symptoms			Re-isolation of inoculant		
LS1a	–	–	–	c	i	i
LS1q	–	–	–	i	i	i
LS1ah	+	+	+	i	i	i
LS2i	+	+	+	i	i	i
LS2q	+	+	+	i	i	i
LS3f	+	+	+	i	i	i
LS3g	–	–	–	i	i	i
LS4d	+	+	+	i	i	i
SS4g	+	+	+	i	i	i
SS5g	–	–	–	i	i	i
Control	–	–	–	n	n	n

+, symptoms present; –, symptoms absent; i, inoculant re-isolated; c, contaminant(s) re-isolated; n, no bacterial growth

of the experiment, the thallus pieces remained in the same condition as that of the uninjected control. However, when the thalli injected with strain LS2i began exhibiting symptoms after 4 days incubation at 30 °C, a variety of different bacteria, in addition to LS2i, was observed growing around the thallus segment on the agar surface.

The ten bacterial strains tested in the first protocol were re-examined for pathogenicity to *G. gracilis* by means of method 3. Six of the ten strains elicited disease symptoms in healthy, axenic *G. gracilis* (Table 3). The results obtained using assay procedure 3 were consistent over the 3 to 5 day incubation period in three independent experiments. Symptoms were exhibited as bleached areas along the injected area of the thallus. Although some pathogens took a slightly longer time to manifest themselves than others, no new symptoms appeared after 5 days incubation. Interestingly, all the agarolytic strains tested caused symptoms, while the non-agarolytic strains did not affect the injected thallus. In all cases but one, the original bacterial strain was re-isolated from the injected thallus, thus satisfying Koch's postulates. Although LS1a was not re-isolated in the first experiment, bacterial growth was absent in the flask, indicating possible fault with the inoculating culture.

Discussion

Since bacterial growth was observed on synthetic marine agar 10 days after testing 'axenic' *G. gracilis* for sterility, it was hoped that disease symptoms would be induced by the test bacterium prior to excessive growth of any contaminating bacterial strains that may have been temporarily attenuated in the sterilisation process. In addition, the nutritionally-poor nature of SSW would not support optimal growth of any contaminants.

Disease symptoms were observed 12 days postinoculation when method 1 was employed to identify bacterial pathogens of *G. gracilis*. Although 9 of the 10 test strains could be re-isolated from infected thallus, the data were inconsistent in repeated experiments. It is possible that the 12-day incubation period was too long as it may have allowed contaminating bacterial strains present on the 'axenic' plant to grow rapidly, and thus, outgrow the inoculant. This explanation is supported by the observation that the control alga exhibited disease symptoms on two occasions and was found to be colonized by a variety of contaminating bacteria. It was therefore impossible for us to conclude that symptoms observed on the algal thalli were caused by the inoculated bacterial strains. Furthermore, it was expected that symptoms caused by a particular bacterium would be consistent. However, symptom characteristics varied between experiments even though thalli had been inoculated with the identical bacterial strain. This variation may also be attributed to bacterial contamination. The inconclusive results obtained using procedure 1 suggested that it may be better to develop a technique which facilitated optimum growth of the test bacterial strain.

Since many marine bacteria are agarolytic (Austin, 1988), it is possible that the symptoms caused by bacterial pathogens of *G. gracilis* could be due to bacterial secretion of agarases. It was decided to refine the pathogenicity assay using a minimum number of strains. A

highly agarolytic strain (LS2i) was selected, as it was hoped that this may rapidly induce the onset of marked symptoms on the algal thallus. A non-agarolytic strain (LS1a) was used as a comparison. The assay was performed using a nutrient medium that would encourage bacterial growth (synthetic marine agar). The bacterial strains were grown to a culture density at which maximum agarase activity occurred (results not shown). The bacteria were cultured at 30 °C, a temperature which has been shown to optimally induce agarase activity in strain LS2i (results not shown).

Results obtained using the second assay protocol were consistent in that thalli segments injected with LS2i developed disease symptoms, while those injected with LSla remained healthy. However, Koch's postulates could not be satisfied as a number of different bacterial colony-types, besides the injected strains, had grown around the thallus by the time disease symptoms appeared. The presence of contaminating bacteria in the injected thallus is difficult to reconcile with the fact that the control plants showed no signs of bacterial growth over a period of at least 7 days. One possibility is that the nutrient broth in which the test bacterial strain was suspended may have provided a rich nutrient source for injured marine bacteria remaining on the surface of the 'axenic' *G. gracilis*, allowing them to recover, and consequently, form visible colonies within 4 days. It was thus inappropriate to conclude that the symptoms had been caused by the injected strain LS2i.

The third protocol was designed to provide a nutrient medium which would enable rapid growth of the injected bacterium in order to prevent competition by any contaminating bacteria present on the treated *G. gracilis*. This method proved to be extremely reliable as a pathogenicity assay. Disease symptoms caused by each bacterial strain remained consistent in 3 separate experiments. Significant growth of each test bacterium was encouraged under the conditions of incubation, indicating an actively growing bacterial population. Symptoms appeared 3 to 5 days after injection of the thallus segment with a bacterial strain. The control thalli remained dark and healthy throughout the incubation period. Those thalli injected with bacterial strains which did not elicit disease remained in the same condition as the control plants. In all cases, the injected strain was re-isolated from the culture, satisfying Koch's postulates. It can thus be concluded that bacterial strains LS1ah, LS2i, LS2q, LS3f, LS4d and SS5g, are pathogens of *G. gracilis*.

This technique has a relatively short incubation period and requires little expense in terms of media and apparatus. It produces very consistent results and satisfies the criteria of Koch's postulates. It is a very simple assay which has identified several pathogens of *G. gracilis* and could be a useful tool for the detection of bacterial pathogens of other commercially important seaweeds. In particular, the pathogens responsible for causing 'ice-ice' in *Eucheuma* species in the Phillipines and 'rotten thallus syndrome' in another *Gracilaria* species could be unequivocally identified by means of this assay.

Acknowledgments

This work was supported by a Quest International research contract and a Foundation for Research and Development (FRD) research grant awarded to VEC, and a FRD Postgraduate Bursary awarded to AEJ. The authors are grateful to Dr Robert Anderson, Derek Kemp and Albertus Smit for their supply of *G. gracilis* and helpful advice.

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