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Investigation of the role of a β(1–4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*

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Gracilaria species are an important source of agar. The South African *Gracilaria* industry has experienced a number of setbacks over the last decade in the form of complete or partial die-offs of the agarophyte growing in Saldanha Bay, which may be attributed to bacterial infection. Since a positive correlation was observed between the presence of agarolytic epiphytes and bacterial pathogenicity, we investigated the role of an agarase in the virulence mechanism employed by a bacterium that elicits disease in *Gracilaria gracilis*. The recombinant plasmid pDA1, isolated from a *Pseudoalteromonas gracilis* B9 genomic library, was responsible for the agarolytic activity exhibited by *Escherichia coli* transformants when grown on solid medium. A BLAST search of the GenBank database showed that an 873 bp ORF (*aagA*) located on pDA1 had 85 % identity to the β-agarase (*dagA*) from *Pseudoalteromonas atlantica* ATCC 19262^T (or IAM 12927^T) at the amino acid level. AagA was purified from the extracellular medium of an *E. coli* transformant harbouring pDA1 by using a combination of gel filtration and ion-exchange chromatography. AagA has an M_r of 30 000 on SDS-PAGE. TLC of the digestion products of AagA showed that the enzyme cleaves the β-(1,4) linkages of agarose to yield predominately neoagarotetraose. Western hybridization confirmed that the cloned agarase was in fact the extracellular β-agarase of *P. gracilis* B9. The observed relationship between disease symptoms of *G. gracilis* and the agarolytic phenotype of *P. gracilis* B9 was confirmed. Transmission electron microscope examination of cross sections of both healthy *G. gracilis* and *G. gracilis* infected with *P. gracilis*, revealed a weakening of the cell structure in the latter plants. Immunogold-labelled antibodies localized the agarase *in situ* to the cell walls of bleached *G. gracilis*. Thus, the weakening observed in the cell structure of *G. gracilis* infected with *P. gracilis* can be attributed to degradation of the mucilaginous component of the cell wall of the bleached thalli.

INTRODUCTION

Gracilaria species are of great economic importance since they are the source of two important grades of agar, namely food grade and sugar reactive agar (Armisen, 1995; Murano, 1995). Since the 1950s, South Africa has benefited substantially from the export of *Gracilaria gracilis* that occurs naturally in Saldanha Bay (Anderson *et al.*, 1989). However, the South African *Gracilaria* industry, which depends solely on the natural *G. gracilis* resource in Saldanha Bay, has experienced a number of setbacks in the last decade or so. These setbacks were due to the major collapse experienced by the natural *G. gracilis* population in Saldanha Bay in 1989, a number of partial die-offs of raft-cultivated *G. gracilis* during the summer months in Saldanha Bay post-1989 and another substantial collapse of both natural and raft-cultivated *G. gracilis* during October–December 1996 (Anderson *et al.*, 1989, 1992, 1999).

To better understand the reasons for the die-offs of *G. gracilis* in Saldanha Bay, Jaffray & Coyne (1996) developed an *in situ* assay to identify putative bacterial pathogens of this macroalga. Of the epiphytic bacteria tested, a positive correlation between an agarolytic phenotype and bacterial pathogenicity was discovered (Jaffray & Coyne, 1996). Other than the aforementioned example, four other incidences of disease, namely 'ice–ice' white powdery disease in *Eucheuma* and *Kappaphycus* species, the disease

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Abbreviations: Ap, ampicillin; LA, LB agar; SSW, sterile seawater.

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in the red alga *Rhodella retriculata*, the 'rotten-thallus' syndrome in a *Gracilaria* species and the 'white-tip disease' of *Gracilaria conferta*, were all attributed to agarolytic bacteria (Friedlander & Gunkel, 1992; Largo *et al.*, 1995; Lavilla-Pitogo, 1992; Toncheva-Panova & Ivanova, 1997). However, the role of the agarases in the virulence mechanism of these bacterial pathogens was only hypothesized.

Agar-decomposing bacteria were first isolated by Gran in 1902 (Yaphe, 1957). Consequently, several agarolytic bacterial strains were isolated from marine and other environments. Some of the bacterial isolates have been assigned to the genera *Pseudoalteromonas* (Akagawa-Matsushita *et al.*, 1992; Belas *et al.*, 1988; Groleau & Yaphe, 1977; Leon *et al.*, 1992; Potin *et al.*, 1993; Vera *et al.*, 1998), *Pseudomonas* (Ha *et al.*, 1997; Hofsten & Malmqvist, 1975; Kong *et al.*, 1997; Lee *et al.*, 2000; Malmqvist, 1978; Nomura *et al.*, 1998), *Cytophaga* (Duckworth & Turvey, 1969; Van der Meulen & Harder, 1975), *Vibrio* (Aoki *et al.*, 1990; Araki *et al.*, 1998; Fukasawa *et al.*, 1987; Sugano *et al.*, 1993) and *Streptomyces* (Bibb *et al.*, 1987; Buttner *et al.*, 1987; Kendall & Cullum, 1984). Yaphe and co-workers were the first to describe an agar-degrading enzyme system from a marine bacterium (Day & Yaphe, 1975; Groleau & Yaphe, 1977). The agar-degrading enzyme system was that of a bacterial isolate classified as *Pseudoalteromonas atlantica* ATCC 19292^T (IAM 12927^T) (Gauthier *et al.*, 1995). The pathway of agar metabolism in this organism involves the initial cleavage of the agarose (alternating 3-*O*-linked *β*-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose) moiety of agar by an endo-acting enzyme, *β*-Agarase I, yielding neoagaro-oligosaccharides limited by the disaccharide, neoagarobiose unit [*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-*O*-*β*-D-galactose], but with predominance of the tetramer, neoagarotetraose [*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-*O*-*β*-D-galactopyranosyl-(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-galactose], as the major end product. The neoagarotetraose is in turn cleaved at its central *β*(1–4) linkages by a neoagarotetraose hydrolase yielding neoagarobiose. However, this enzyme was also shown to be able to degrade species of oligosaccharides larger than neoagarotetraose, and hence it was given the name *β*-Agarase II. It was shown to hydrolyse agar by an endomechanism to produce neoagaro-oligosaccharides, hexasaccharides, tetrasaccharides and neoagarobiose, with neoagarobiose being the limiting and predominant species. The third and final enzyme in the agar-degrading system of *P. atlantica* is a neoagarobiose hydrolase that cleaves the *α*(1–3) linkage in neoagarobiose to yield the monomeric sugars D-galactose and 3,6-anhydro-L-galactose.

In this study we report the cloning of one of the genes responsible for the agarolytic activity associated with an epiphytic bacterial pathogen of *G. gracilis* from Saldanha Bay. The enzyme was purified and used as a tool to elucidate its role in the virulence mechanism employed by the bacterium in eliciting disease in *G. gracilis*.

METHODS

Bacteria, plasmids, media and culture conditions. *Pseudoalteromonas gracilis* B9 was isolated from the surface of *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine & Farnham in Saldanha Bay, South Africa. The isolate was maintained at 22 °C in Marine broth and aerated on an orbital shaker at 100 r.p.m. (Jaffray *et al.*, 1997). Alternatively, the bacteria were cultured on Marine agar plates (Jaffray *et al.*, 1997). The *Escherichia coli* strains were cultured in either Luria–Bertani broth (LB) or on Luria–Bertani agar (LA) at 37 °C (Sambrook *et al.*, 1989). *Escherichia coli* transformants that harboured recombinant pEcoR251 and pBluescript KS plasmids were grown in LB or LA containing 100 μg ampicillin (Ap) ml^{-1}. The bacterial strains and plasmids used to clone and characterize the *β*-agarase gene from *P. gracilis* B9 are listed in Table 1.

Assay for bacterial pathogens of *G. gracilis*. Axenic *G. gracilis* (thalli were 5 cm in length) was produced and employed in a pathogenicity assay as described by Jaffray & Coyne (1996) to confirm whether *P. gracilis* B9 is a bacterial pathogen of *G. gracilis*. Thalli were injected with either 100 μl of a bacterial suspension containing 1×10^9 c.f.u. ml^{-1} or 3 μg pure AagA.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant features	Reference
E. coli HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Sambrook *et al.* (1989)
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F′(*traD36 proAB′ lacI*^{q} *lacZ*ΔM15)	Sambrook *et al.* (1989)
P. gracilis B9	Saldanha Bay, South Africa	This work
Isolate SS5g	Saldanha Bay, South Africa	Jaffray & Coyne (1996)
pBluescript KS	Ap^r, *β*-galactosidase	Short *et al.* (1988)
pEcoR251	Ap^r, *Eco*RI endonuclease	Zabeau & Stanley (1982)
pDA1	pEcoR251 containing ~7 kb *P. gracilis* B9 genomic DNA	This work
pDA10	Derivative of pDA1 containing ~6·5 kb *P. gracilis* B9 genomic DNA	This work
pDA11	Derivative of pDA1 containing ~5 kb *P. gracilis* B9 genomic DNA	This work
pDA12	Derivative of pDA1 containing ~2·6 kb *P. gracilis* B9 genomic DNA	This work
pDA15	Derivative of pDA1 containing ~3 kb *P. gracilis* B9 genomic DNA	This work
pDA16	Derivative of pDA1 containing ~1·3 kb *P. gracilis* B9 genomic DNA	This work
pDA012	2·75 kb *Hin*dIII–*Xho*I fragment from pDA11 cloned into pBluescript KS	This work

Molecular techniques. A genomic library was constructed in *E. coli* HB101, using the plasmid pEcoR251 (Table 1). Genomic DNA was extracted from *P. gracilis* B9 and partially digested as described by Ausubel *et al.* (1989) with *Sau*3A. The *Sau*3A DNA restriction fragments were size-fractionated on a 10–40 % (w/v) sucrose gradient (Sambrook *et al.*, 1989). DNA fragments of 10 kb in size were pooled and ligated with T4 ligase into the *Bgl*II site of plasmid pEcoR251 at 15 °C overnight (Ausubel *et al.*, 1989). The recombinant plasmids were recovered by transformation into *E. coli* HB101 (Dagert & Ehrlich, 1979). The genomic library was screened for agarolytic activity on LA (Ap) with Gran's Iodine reagent (Groleau & Yaphe, 1977). Plasmid DNA was isolated using a Nucleobond AX plasmid purification kit (Macherey–Nagel), according to the manufacturer's instructions, and digested with restriction enzymes obtained from Boehringer Mannheim and Amersham. Agarose gel electrophoresis was performed in Tris/acetate buffer (Ausubel *et al.*, 1989). Both the Southern hybridization and Heinikoff shortening procedures were followed as described by Ausubel *et al.* (1989). Sequencing was performed with the dideoxynucleotide chain-termination method using either the Sequenase sequencing kit (Amersham Pharmacia) and [α-^{35}S]dATP (Sanger *et al.*, 1977) or a Thermosequenase cycle-sequencing kit (Amersham) and an ALFexpress automated sequencer (AM version 3.01; Pharmacia Biotech). Sequence data were analysed using DNAMAN version 4.13 (Lynnon BioSoft) and DNASIS software version 2.1 (Hitachi Software Engineering). Homology searches with both DNA and protein sequences were carried out using the BLAST algorithm (Altschul *et al.*, 1990) provided by the Internet service of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/).

Purification of AagA. Five 1 l *E. coli* JM109(pDA16) cultures (Table 1) were grown in LB (Ap) for 24 h at 22 °C on an orbital shaker at 100 r.p.m. The cultures were centrifuged and the supernatants collected. The following procedures were performed at 4 °C. The supernatant was adjusted to a final ammonium sulphate saturation of 85 % (w/v) (Englard & Seifter, 1990). The precipitate was collected by centrifugation, resuspended in 20 mM Tris/HCl buffer (pH 7) and dialysed multiple times against the same buffer. The following procedures were carried out at 20 °C. A column (3 × 28 cm) of DEAE-Sephadex A-50 (Pharmacia) was activated with 5 M NaCl and then equilibrated with 20 mM Tris/HCl (pH 7) (Rossomando, 1990). The dialysed concentrate was applied to the column and the column was washed with 20 mM Tris/HCl (pH 7). The proteins were eluted from the column in a stepwise fashion with an increasing NaCl molarity (0·1–1 M NaCl). Fractions were collected with a Gilson FC 204 Fraction Collector. The resultant pooled total volume was reduced with an Amicon Centricon PM10 filter system. A column (6 × 1 m) of Sephadex G75 (Pharmacia) that had been equilibrated with 20 mM Tris/HCl (pH 7) was prepared. The active concentrate was applied to the column and fractions were collected. The active fractions were pooled and concentrated. Finally, the active concentrate was dialysed against 10 mM phosphate buffer (pH 7).

Enzymic assays. Protein concentrations were determined by the Bradford method (Ausubel *et al.*, 1989). Agarase activity was determined by the ferricyanide reducing sugar assay (Park & Johnson, 1949).

SDS-PAGE and zymograms. Samples were separated on a 12 % (w/v) SDS-PAGE gel in accordance with the Laemmli method (Ausubel *et al.*, 1989) and detected by silver staining (Sammons *et al.*, 1981). Zymogram detection of the extracellular agarase was performed as follows: a final concentration of 0·1 % (w/v) agarose was incorporated into the separating gel matrix of a 12 % (w/v) SDS-PAGE gel. After electrophoresis, the gel was soaked in 10 mM phosphate buffer (pH 7) at 22 °C. The buffer was replaced hourly for 3 h before incubation at 37 °C for 12 h. Zones of hydrolysis were visualized by staining the gel with Gran's Iodine.

TLC analysis of agarase hydrolysates. Purified enzyme (600 ng) was added to 100 µl freshly prepared 1 % (w/v) agarose substrate and 200 µl 20 mM PIPES solution (Yaphe, 1957). Similarly, 600 ng of purified enzyme was added to 50 µl of each of three oligosaccharides: neoagarobiose, neoagarotetraose and neoagarhexaose (Promega) (2·5 µg µl^{-1} final concentration) and 140 µl 20 mM PIPES solution. The reaction mixes were incubated at 37 °C for 1 h. TLC was performed on Silica gel 60 aluminium foil (Merck) and developed with the solvent *n*-butanol/acetic acid/water (2 : 1 : 1). The digests were visualized with naphthoresorcinol (Yaphe, 1957).

Antibody production against purified AagA. Polyclonal antibodies against purified AagA protein were obtained by immunizing a rabbit with 150 µg AagA, purified from the *E. coli* JM109(pDA16) transformant, together with Freund's incomplete adjuvant (Ausubel *et al.*, 1989).

Ultrastructure evaluation and colloidal gold immunolabelling. The procedure described by Dykstra (1993) was adapted for the preparation of pathogenicity assay samples. Thalli (6 mm in length) were washed in a base buffer [PBS, pH 7, 2·4 % (w/v) NaCl] to remove any excess material. The samples were fixed by overnight immersion in 5–10 × sample volume of 2·5 % (w/w) glutaraldehyde in base buffer at 4 °C. The tissues were rinsed twice (5 min each) in base buffer. The samples were post-fixed in 1 % (w/v) osmium tetroxide for 1 h at 22 °C. The tissues were rinsed twice in water (5 min each). Dehydration of the samples was carried out by passing them through the following alcohol dilution series: 30, 50, 70, 80, 90 and 95 % ethanol for 5 min respectively. The samples were then passed through 100 % ethanol for 10 min (twice) and finally through 100 % acetone for 10 min (twice). The samples were then infiltrated with Spurr resin (Spurr, 1969) as follows. The thalli were placed individually in moulds and covered with Spurr resin. The samples were polymerized in a 60 °C oven for 2 days, after which the polymerized wedges were stored at 22 °C. Ultrathin cross sections of the thallus were obtained with a Leica ultracuts ultramicrotone and mounted on carbon-coated nickel grids using a modification of the method described by Beesley (1989). The grids were first floated, section downwards, on PBS containing 1 % BSA (PBS-BSA) for 5 min. The grids were transferred to PBS containing glycine for 3 min and washed twice (1 min each) with PBS-BSA. Duplicate grids were floated on anti-AagA-containing serum obtained from either the first or the fifth bleed for 12 h and washed five times (1 min each) with PBS-BSA containing 0·1 % (w/w) Tween. The grids were washed thrice (1 min each) with PBS-BSA. The grids were then floated on a 1 : 50 dilution of 15 nm gold anti-rabbit probe in PBS-BSA for 2 h. The grids were rinsed five times (1 min each) with PBS-BSA containing 0·1 % (w/w) Tween, followed by three washes (1 min each) with PBS-BSA. The conjugant label complexes were fixed with 1 % (w/w) glutaraldehyde in PBS at 22 °C for 3 min. The grids were rinsed five times (1 min each) in ultrapure water. The grids were stained with 2 % (w/w) uranyl acetate for 10 min and washed five times (1 min each) with ultrapure water. The sections were then stained with a second stain, Reynolds lead citrate, for 5 min and the grids were washed in a stream of ultrapure water for 2 min. The samples were visualized with a JEM-200CX transmission electron microscope (JEOL).

RESULTS

Pathogenicity assay

The pathogenicity assay developed by Jaffray & Coyne (1996) was used to evaluate whether *P. gracilis* B9 is indeed

a pathogen of *Gracilaria gracilis*. The results obtained with this assay were consistent over the 5 day incubation period in three independent experiments. The control axenic thalli that were injected with sterile seawater (SSW), which was incubated in Marine broth at 22 °C, remained dark and healthy throughout the incubation period. No bacterial growth was observed from the thalli after the 5 day incubation. Similarly, healthy, dark thalli were observed following injection with isolate SS5g. After the 5 day incubation period with bacterium SS5g, only the SS5g isolate was recovered from the injected thalli. In comparison, thallus bleaching (lesion length: $1 \pm 0{\cdot}5$ mm) appeared after 4 days when thalli were injected with *P. gracilis* B9 (1×10^9 c.f.u. ml^{-1}). Only *P. gracilis* B9 was reisolated from thalli injected with this bacterium.

Restriction enzyme mapping of the recombinant plasmid pDA1

Three *E. coli* HB101 transformants capable of hydrolysing agar were isolated after screening the *P. gracilis* B9 genomic DNA library for agarase-encoding genes. A preliminary restriction enzyme digest performed on the three agarolytic recombinant plasmids isolated from the three *E. coli* HB101 transformants revealed that the plasmids were identical (data not shown). A representative was mapped further and the recombinant plasmid was designated pDA1 (Fig. 1a).

Southern hybridization studies

The 550 bp *Eco*RI–*Eco*RI fragment isolated from pDA1 (Fig. 1a) was used as a probe against wild-type *P. gracilis* B9 chromosomal DNA to verify that the inserted DNA fragment carried by the recombinant plasmid was of *P. gracilis* B9 origin. Indeed, the *Eco*RI (6400)–*Eco*RI (6950) fragment hybridized to two *Sac*I fragments of 32 and 22 kb in size (lane 1), an 8·5 kb *Pst*I fragment (lane 2), an 8·25 kb *Hin*dIII–*Pst*I fragment (lane 3) and a 9 kb *Hin*dIII fragment (lane 4) (Fig. 1b).

Deletion analysis of pDA1

Since the recombinant plasmid pDA1 harboured a large insert of *P. gracilis* B9 genomic DNA (pDA1 has an insert of ~7·0 kb) it was necessary to identify which region of the *P. gracilis* B9 DNA fragment was responsible for the agarolytic activity observed in the *E. coli* HB101 clones. This was achieved by deleting various fragments from pDA1 and visually scoring for agarolytic activity (Fig. 2). The 1·2 kb *Hin*dIII–*Eco*RI fragment of pDA1 was found to include the *P. gracilis* agarase gene(s).

DNA sequencing of the agarase gene cloned from *P. gracilis* B9

To characterize the agarolytic gene(s) encoded by pDA1, a 2·75 kb *Hin*dIII–*Xho*I restriction enzyme fragment from pDA11 was subcloned into the multiple cloning site of pBluescript KS to generate pDA012. The 2·75 kb *Hin*dIII–*Xho*I restriction fragment, as opposed to the smaller 1·15 kb *Hin*dIII–*Xho*I fragment, was chosen to increase the likelihood of obtaining the sequence of any regulatory regions possibly controlling the agarolytic gene(s) (compare agarase expression of pDA11 and pDA16, Fig. 2). pDA012 was sequentially deleted from both ends of the insert and the resultant deletion plasmids were sequenced. Upon translation of the nucleotide sequence in all six reading frames, a single ORF was identified which encodes a 290 aa protein with a hypothetical size of 31·87 kDa (GenBank accession no. U61972). The deduced protein sequence includes features commonly associated with signal peptides of secreted proteins. Thus, cleavage of a putative peptidase cleavage site, located between residues 21 and 22, would yield a mature protein of 269 aa with a molecular mass of 30·23 kDa.

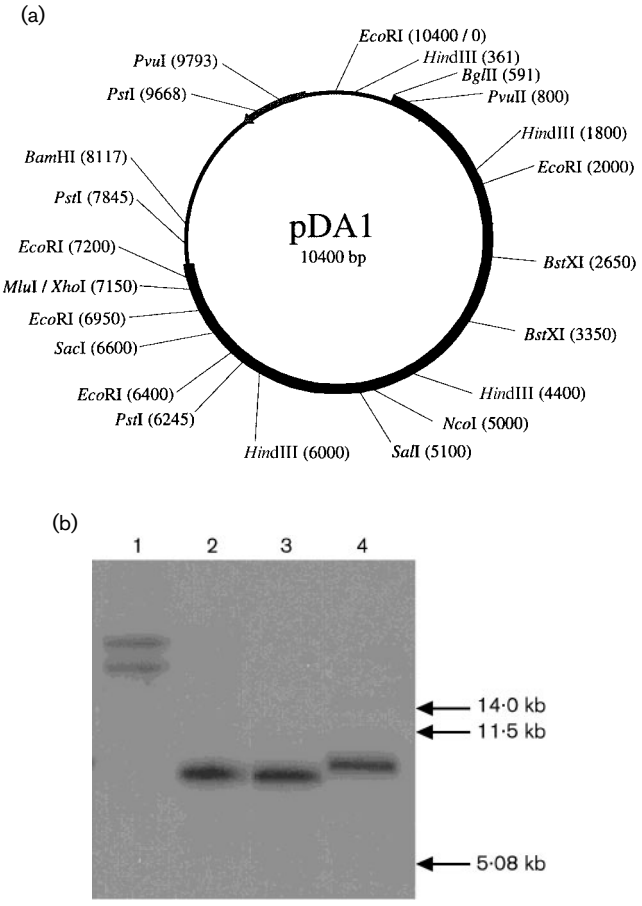

Fig. 1. (a) Restriction enzyme map of the recombinant plasmid pDA1. The thick line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. The numbers in parentheses indicate the positions in bp of the various restriction enzyme sites. The position of the β-lactamase gene (arrow) is shown. (b) Southern hybridization of the 550 bp *Eco*RI (6400)–*Eco*RI (6950) restriction fragment of pDA1 against *P. gracilis* B9 genomic DNA. Genomic DNA was digested with *Sac*I (lane 1), *Pst*I (lane 2), *Hin*dIII/*Pst*I (lane 3) and *Hin*dIII (lane 4). The arrows indicate the positions of the λ DNA markers.

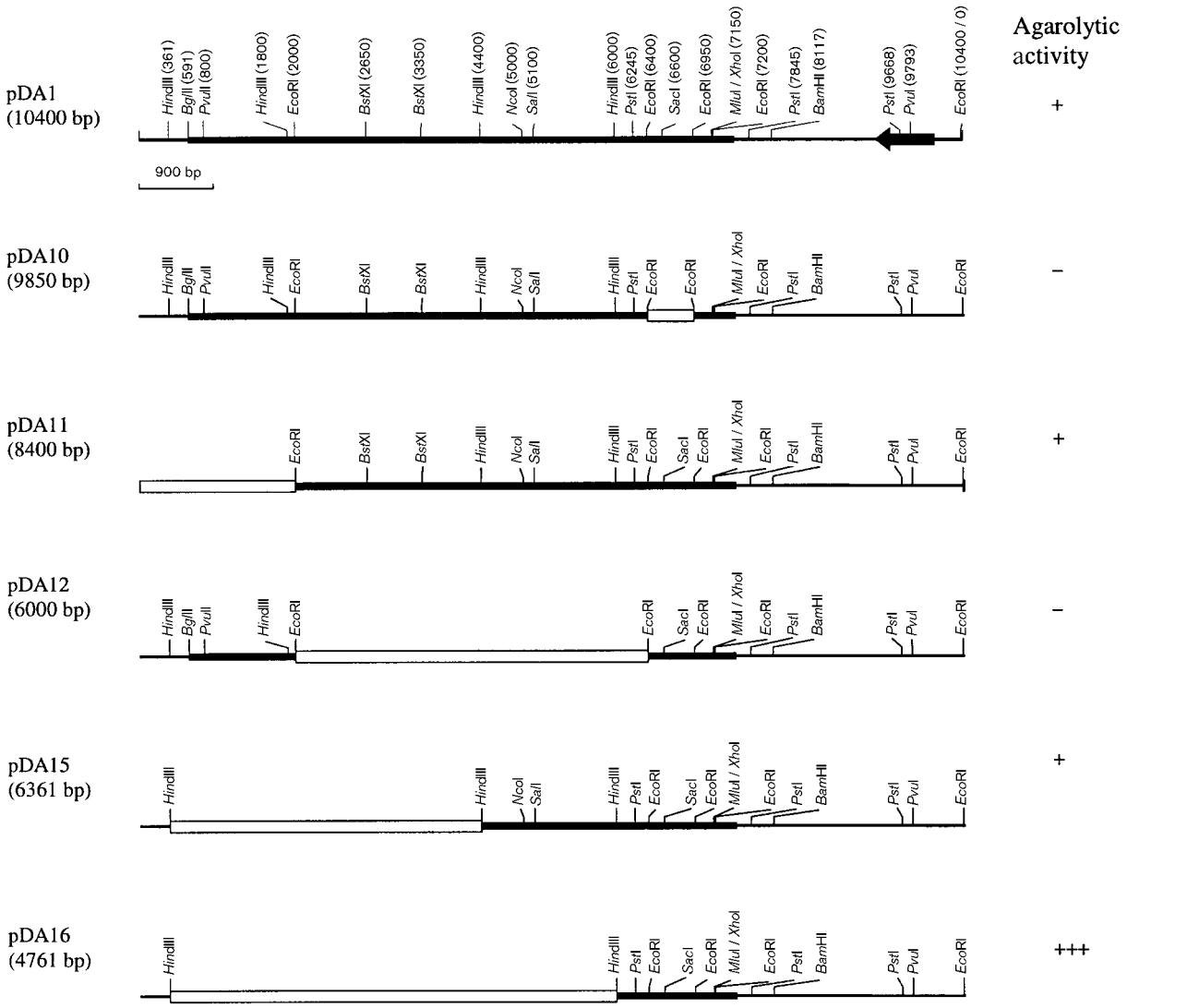

Fig. 2. Determination of the location of the agarase gene in pDA1 by deletion mapping. The open boxes represent the fragments deleted from pDA1 to create plasmids pDA10, pDA11, pDA12, pDA15 and pDA16. The thick solid line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. The position of the β-lactamase gene (arrow) is shown. The plus or minus signs represent the presence or absence of agarolytic activity, respectively. The sizes of the recombinant plasmids and the positions of the various restriction enzyme sites are indicated in bp.

Homology searches

Several databases were searched for homologous sequences to determine the putative identity of the protein encoded by the ORF on the 2·75 kb *Hin*dIII–*Xho*I fragment of pDA012. The ORF was found to have 76 and 85 % identity to the β-agarase (*dagA*, M73783) from *Pseudoalteromonas atlantica* ATCC 19262^T (IAM 12927^T) at the DNA and amino acid level, respectively. It also shared a lesser amino acid identity with the two β-agarases (AgaA, AF098954, and AgaB, AF098955) from *Cytophaga drobachiensis* and the β-agarase (DagA, P07883) from *Streptomyces coelicolor* A3(2); i.e. 51, 44 and 34 %, respectively. Therefore, it was concluded that the 873 bp ORF encoded a putative β-agarase and was designated *aagA*.

Purification and characterization of AagA from *E. coli* JM109(pDA16)

AagA was purified from the extracellular medium of *E. coli* JM109(pDA16) by using a combination of gel filtration and ion-exchange chromatography (data not shown). The overall yield of AagA was 21·4 % for a purification of 21·7-fold (Table 2). An aliquot of the final concentrate, subjected to SDS-PAGE, exhibited a single band at 30 kDa (Fig. 3a, lane 1). The zymogram confirmed that the purified

Table 2. Purification of AagA from *E. coli* JM109(pDA16)

Purification step	Total protein (mg)	Total activity (U*)	Specific activity (U* mg^{-1})	Recovery (%)	Purification (-fold)
Culture supernatant	135·9	4 331 310	31 881	100·0	1·0
Ammonium sulphate preparation	86·6	3 479 770	40 168	80·3	1·3
Sephadex A50	2·1	1 321 480	615 214	30·5	19·3
Sephadex G75	1·3	926 990	690 238	21·4	21·7

*U, μg galactose ml^{-1} min^{-1} at 37 °C.

protein was an agarolytic enzyme (Fig. 3b). TLC revealed that AagA hydrolyses β(1–4) linkages in agarose to predominantly yield neoagarotetraose as the major end product (Fig. 4, lane 1). In addition, AagA hydrolysed the neoagarohexaose to produce neoagarotetraose and neoagarobiose (Fig. 4, lane 2). AagA did not hydrolyse neoagarotetraose and neoagarobiose (Fig. 4, lanes 3 and 4).

In situ localization of the *P. gracilis* B9 agarase in infected *G. gracilis*

To determine the specificity of the polyclonal antibodies raised against AagA purified from *E. coli* JM109(pDA16), extracellular fractions of *P. gracilis* B9 and *E. coli* JM109(pDA16) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western hybridization showed that the anti-AagA antibodies cross-reacted solely and strongly to both AagA purified from the *E. coli* JM109(pDA16) transformant and the extracellular β-agarase purified from *P. gracilis* B9 (data not shown). The anti-AagA antibodies detected the presence of the 30 kDa AagA in the spent growth medium, but not in the cellular fraction, of *E. coli* JM109(pDA16) from where the protein was originally purified (data not shown). The antibodies also detected the extracellular β-agarase in the spent growth medium, but not in the cellular fraction, of the wild-type B9 strain (data not shown).

To evaluate the role of the *P. gracilis* B9 agarase in the infection process, *G. gracilis* thalli were injected with purified agarase and examined for disease symptoms over a 5 day period. Thalli injected with purified AagA exhibited the largest lesions (10 ± 1 mm). Bleaching in these thalli occurred 2 days post-injection. No bacteria were isolated from AagA-injected thalli after the 5 day incubation period.

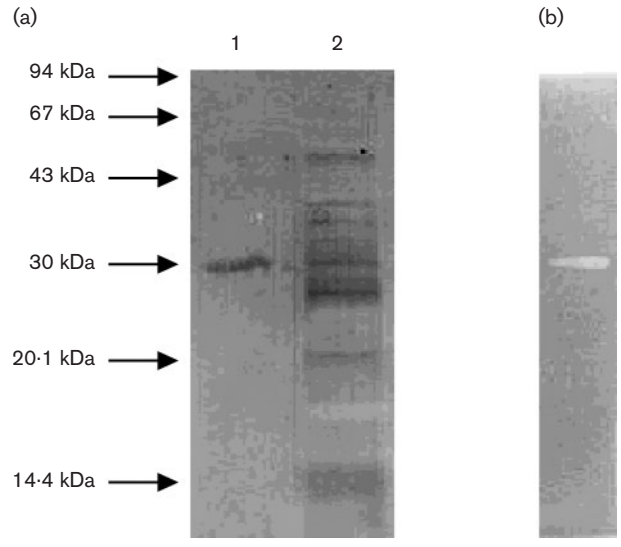

Fig. 3. (a) SDS-PAGE of samples obtained at different stages during purification of AagA from *E. coli* JM109(pDA16). Lanes: 1, final concentrate; 2, ammonium sulphate concentrate. The proteins were visualized by silver staining. The sizes and positions of the molecular mass markers are shown. (b) Zymogram of the final concentrate depicted in (a), lane 1. The enzymically active band was detected by staining the gel with Gran's Iodine.

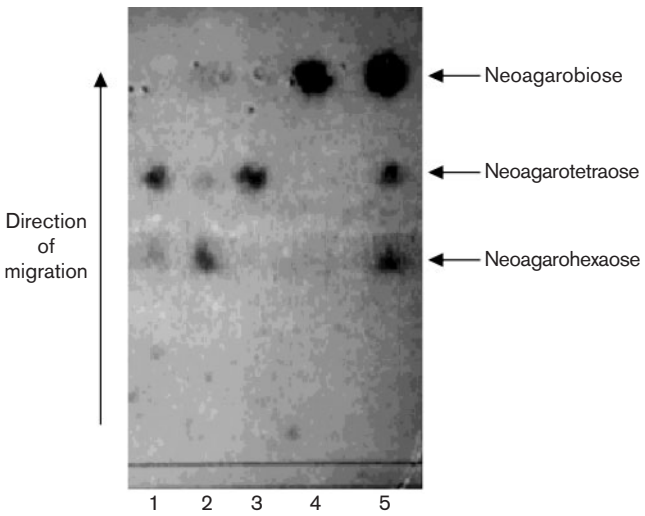

Fig. 4. TLC analysis of the oligosaccharides formed following AagA degradation of various saccharide substrates. Lanes: 1, purified agarase incubated with agarose; 2, purified agarase incubated with neoagarohexaose; 3, purified agarase incubated with neoagarotetraose; 4, purified agarase incubated with neoagarobiose; 5, molecular size markers.

The injected areas, corresponding to the bleached areas of thalli that had been injected with either *P. gracilis* B9 or AagA, were washed in a base buffer to remove any excess material and prepared for transmission electron microscopy. A comparison of the cross-sections of the thalli that had been injected with either SSW, *P. gracilis* B9 or bacterium SS5g revealed no apparent differences in the cell structure. However, a comparison between any of these three cross-sections and that of AagA-injected thalli revealed a clear disruption of the algal cell structure, i.e. the cell walls generally appeared more swollen in comparison to thalli injected with bacterium SS5g (Fig. 5).

Immunogold-labelled antibodies revealed that the β-agarase was associated with the cell walls of bleached *G. gracilis*. The immunogold was localized to the intercellular matrix of the cell wall of the thalli that had been injected with either AagA or *P. gracilis* B9 (Fig. 6b and c). Immunogold was not detected in the cell walls of the thalli that had been injected with either SSW or bacterium SS5g (Fig. 6a). The fibrillar component of the intercellular matrix between the cortical and medullary cells was disrupted in the severely bleached thallus, i.e. the fibrillar appearance of the cell walls had been lost in the thalli that had been injected with AagA (Fig. 6c). Even though the immunogold was localized to the intercellular matrix of the cell wall of thalli injected with *P. gracilis* B9, the fibrillar appearance of the intercellular matrix remained evident (Fig. 6b). Similarly, the fibrillar nature of the intercellular matrix was apparent in thalli injected with either SSW or bacterium SS5g (Fig. 6a).

DISCUSSION

Jaffray & Coyne (1996) developed a reliable assay for detection of bacterial pathogens of *G. gracilis*. This assay was used to test whether *P. gracilis* B9 was pathogenic towards *G. gracilis*. *Pseudoalteromonas gracilis* B9 fulfilled Koch's postulates, indicating that thallus bleaching could only be due to the bacterium since bacteria-free controls remained unbleached. Thus, from the pathogenicity assay it could be concluded that *P. gracilis* B9 is indeed a potential pathogen of *G. gracilis*.

A *P. gracilis* B9 genomic library was screened for agarolytic activity by visual inspection of the agar for zones of pitting around *E. coli* transformants growing on solid media. Agar-digesting *E. coli* clones were found to harbour a recombinant plasmid designated pDA1. Deletion analysis of pDA1 revealed the location of the gene responsible for agarolytic activity. A BLAST search of the GenBank database showed that the ORF situated in the *P. gracilis* B9 DNA of pDA012 had sequence identity to a number of β-agarases. The β-agarase DagA (M73783) of *P. atlantica* ATCC 19262^T had the greatest similarity to the *P. gracilis* B9 gene. Therefore, it was concluded that the 873 bp ORF encoded a putative β-agarase and was designated *aagA*.

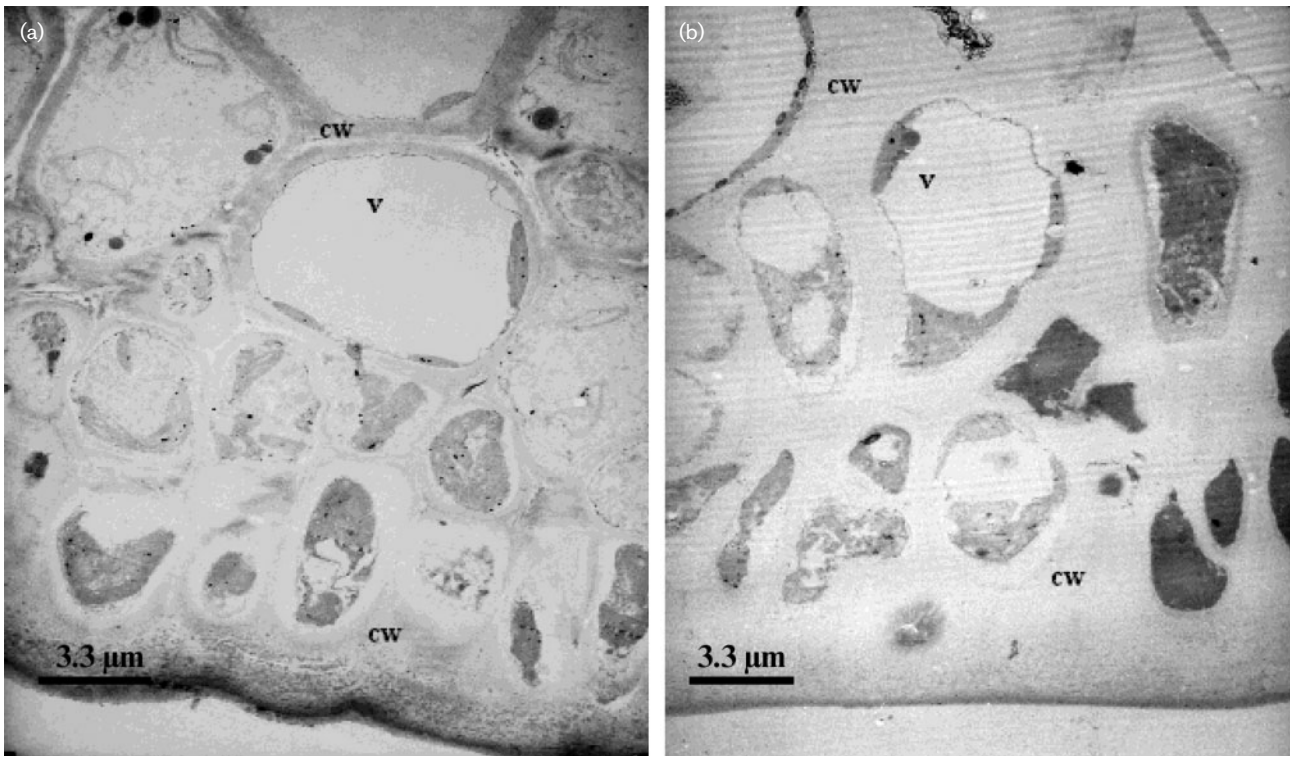

Fig. 5. Transmission electron micrographs of (a) a cross-section through a mature thallus injected with the bacterium SS5g and (b) a cross-section through a mature thallus injected with AagA. v, Vacuoles; cw, cell wall.

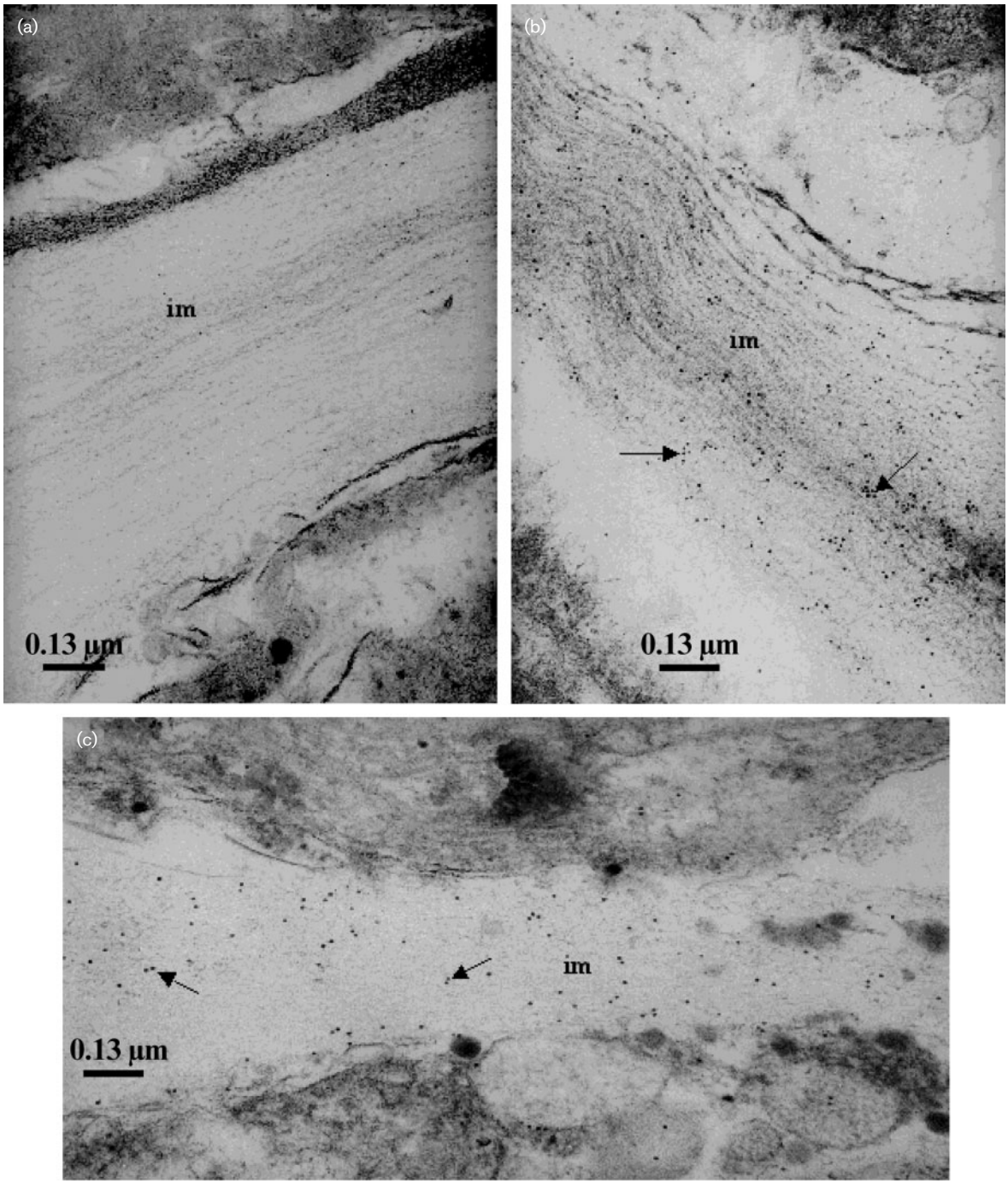

Fig. 6. Transmission electron micrographs of (a) a cross-section through a mature thallus injected with SSW, (b) a cross-section through a mature thallus injected with *P. gracilis* B9 and (c) a cross-section through a mature thallus injected with AagA. im, Intercellular matrix; arrows, 15 nm gold particles.

A 30 kDa extracellular β-agarase was purified from the spent growth medium of the transformant *E. coli* JM109(pDA16). The size of the purified β-agarase was consistent with the theoretical size predicted for the mature β-agarase. In addition, zymogram analysis confirmed that the purified protein was agarolytic. TLC data confirmed that the purified extracellular agarase was indeed a β(1–4) agarase; i.e. the agarase hydrolysed the β(1–4) linkages of agarose to predominately produce neoagarotetraose. However, the β(1–4) agarase only hydrolysed saccharides larger than neoagarotetraose. This is consistent with the extracellular β-agarase type I enzyme

from *P. atlantica* ATCC 19262^T (Day & Yaphe, 1975; Morrice *et al.*, 1983).

The polyclonal antibodies raised against the β-agarase purified from the *E. coli* JM109(pDA16) transformant specifically cross-reacted with AagA and the extracellular β-agarase purified from *P. gracilis* B9. Since a 30 kDa band was the only protein detected in the extracellular extract of *P. gracilis* B9, it is highly likely that AagA is the only β-agarase secreted into the medium under the growth conditions tested. However, we cannot rule out the possibility that *P. gracilis* B9 may produce additional extracellular agarases under different growth conditions. For example, when the culture conditions used to grow *Vibrio* sp. JT0107 were changed, another agarase, designated agarase 0072, was produced by the bacterium (Sugano *et al.*, 1995).

The relationship observed between disease symptoms exhibited by infected *G. gracilis* and the agarolytic phenotype of *P. gracilis* B9 was confirmed. Microscopic examination of cross sections prepared from severely bleached thalli that had been injected with purified AagA revealed disruption of the cell structure. The cell wall appeared to have weakened, i.e. the ultrastructure of the cell wall was lost upon treatment with gluteraldehyde during sample preparation, resulting in a swollen appearance in comparison to cross sections of thalli that had been injected with either SSW, *P. gracilis* B9 or bacterium SS5g. Immunogold-labelled antibodies localized the β-agarase, *in situ*, to the intercellular matrix of the cell walls of thalli injected with either AagA or *P. gracilis* B9. In addition, no immunogold-labelled antibodies were detected in the SSW and SS5g sections. There seems to be a direct relationship between the severity of thallus bleaching and the degree of disruption of the fibrillar component of the cell walls. The cellulosic fibrillar cell wall component functions in concert with the mucilaginous agar component of the cell wall to strengthen the thallus (Christiaen *et al.*, 1987). We hypothesize that β-agarase degradation of the mucilaginous component weakened the overall structure of the cell wall and consequently, resulted in the collapse of the fibrillar component in the AagA-injected thallus. The swollen appearance of the cell wall of AagA-injected thalli could be attributed to an overall loss in cell wall strength of the bleached thalli.

Weinberger *et al.* (1999) showed that *Gracilaria conferta* responded with an oxidative burst, a rapid increase in respiration and halogenation when it detected the breakdown products of agar, namely neoagarohexaose and neoagarotetraose. Neoagarohexaose elicited a release of hydrogen peroxide that resulted in an immediate increase in algal brominating activity. Bleached thallus tips appeared a few hours after the addition of neoagarohexaose. These observations are consistent with our results. The end products released as a result of AagA activity on the mucilaginous component of the cell wall could elicit a similar response. Thus, the extensive bleaching that followed injection of pure enzyme (AagA) into the *G. gracilis* thallus, and the thallus bleaching that occurred as a consequence of secretion of *P. gracilis* B9 agarase into the thallus of infected *G. gracilis*, could be due to the macroalga responding to the end products (neoagarohexaose and neoagarotetraose) produced by the extracellular β-agarase of *P. gracilis* B9 as a consequence of agar degradation.

Even though the central medullary cells were employed as the site of injection in the pathogenicity assay, β-agarase was detected in both the medullary and cortical cell walls of thalli that had been injected with either AagA or *P. gracilis* B9. Since bacteria were only observed at the site of injection, our data suggest that the enzyme spread through the cell wall from the site of injection to other areas of the thallus. Thus, it could be hypothesized that the β-agarase is secreted into the thalli by the bacterial pathogen and becomes associated with the mucilaginous component (agar) of the cell wall. The breakdown products of the hydrolysed agar, namely neoagarohexaose and neoagarotetraose, are then released from the polymer into the surrounding medium, possibly by diffusion stimulated by wave action. Of the agarolytic pathways characterized to date, more than one enzyme has been shown to be required for complete agar hydrolysis, and furthermore, each pathway included both an extra- and intracellular enzyme (Belas *et al.*, 1988; Potin *et al.*, 1993). Hence, it is most likely that *P. gracilis* B9 hydrolyses the oligosaccharides further by means of a cell-bound enzyme(s) that has not yet been identified.

The extracellular β-agarase was localized to the *G. gracilis* cell wall following injection with *P. gracilis* B9. However, the ultimate effect of the enzyme on the thallus had not yet occurred when the cross sections were prepared for microscopic examination, i.e. complete disruption of the cellulosic fibrillar appearance of the cell wall as a consequence of the degradation of the mucilaginous agar component and severe thallus bleaching. However, when *P. gracilis* B9-injected thalli were maintained at an incubation temperature of 30 °C, as opposed to 22 °C, the characteristic bleached phenotype was observed (data not shown). The higher incubation temperature results in an increased enzyme activity, which was observed in *in vitro* enzyme assays (data not shown). Thus, the higher incubation temperature sped up the effect of the enzyme on the thallus. Consequently, we postulate that the bacterial epiphyte *P. gracilis* B9 becomes pathogenic towards *G. gracilis* in response to specific environmental conditions.

The immunogold detection technology described in this study could be used for a thorough and detailed investigation of the causes of the disease experienced by *G. gracilis* at Saldanha Bay. It is difficult to pinpoint the exact factors that cause disease in *G. gracilis* at Saldanha Bay since the early stages of infection cannot be detected with the naked eye. The symptoms only become visible once the disease has progressed to the point where thallus bleaching and breakage occurs. However, by this time the agar component of the cell wall is degraded and the crop is rendered useless.

In situ monitoring of bacterial extracellular β-agarases at various times during the *G. gracilis* growth season, either in natural or raft-cultivated populations at Saldanha Bay, would allow elucidation of environmental cues that result in bacterial infection and the subsequent collapse of *G. gracilis* populations. Advances in immunocytochemistry have led to the development of non-microscopic techniques such as pregnancy diagnostic kits (Beesley, 1989). Similarly, an *in situ* agarase detection kit could be developed to assist *Gracilaria* farmers in early detection of the onset of disease and thus allow for early harvesting, which would in turn circumvent the complete collapse of the entire *Gracilaria* crop, thus avoiding financial ruin.

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