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Algicidal bacteria associated with blooms of a toxic dinoflagellate in a temperate Australian estuary

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ABSTRACT: *Gymnodinium catenatum* is an introduced toxic dinoflagellate that blooms intermittently and causes shellfish farm closure in the Huon Estuary, Tasmania, Australia. Seventy-five bacteria isolated from the estuary were tested for algicidal activity against this and other toxic and non-toxic algal species. Five isolates produced algicidal extracellular exudates. These algicidal species were a *Pseudoalteromonas* sp. (ACEM 4), a novel *Zobellia* sp. (ACEM 20), a strain of *Cellulophaga lytica* (ACEM 21) and 2 Firmicutes: a novel *Planomicrobium* sp. (ACEM 22) and a strain of *Bacillus cereus* (ACEM 32). This study is the first time Gram-positive bacteria have been associated with algicidal activities. Further data are presented on an algicidal *Pseudoalteromonas* species previously isolated from the Huon Estuary (Strain y). Supernatant produced by all 5 strains caused cell lysis and death in *G. catenatum* vegetative cells. No change or reversible ecdysis was noted for 2 other endemic dinoflagellate species. Algicidal or inhibitory activity was not activated via homoserine lactones, but bacterial quorum sensing for the isolates was shown by means of the AI-2 mechanism. Algicidal activity from field isolates was also influenced by strain or environmental variation. Bacteria were capable of losing or switching off their algicidal ability indicating that the presence of an algicidal species in the environment may not necessarily signify that they are currently algicidal. Concentrations of algicidal compounds required for algal lysis in laboratory experiments indicate that the 5 bacterial species can be effective against *G. catenatum* vegetative cells if they dominate the bacterial population in the estuary, particularly when attached to particles.

KEY WORDS: Algicidal · *Gymnodinium* · *Pseudoalteromonas* · *Cellulophaga* · Flavobacteria · Harmful algal blooms

INTRODUCTION

The Huon Estuary in Tasmania is an unpolluted waterway with low nutrient levels. Despite this, the estuary experiences periodic outbreaks of the introduced toxic dinoflagellate *Gymnodinium catenatum* that are detrimental to the local shellfish industry. An extensive 2 yr scientific study of the biology, chemistry and physics of the estuary confirmed the importance of physical forcing; however, no role for macronutrients was obvious for this vertically migrating dinoflagellate (CSIRO Huon Estuary Study Team 2000).

The relationship between toxic algal blooms and algicidal bacteria has been the subject of extensive research (Stewart & Brown 1969, Imai et al. 1993, Doucette et al. 1998, Lovejoy et al. 1998, Nagasaki et al. 2000). Particular attention has been paid to the potential use of algicidal bacteria in bloom control (Kim et al. 1998, Yoshinaga et al. 1999, Nagasaki et al. 2000). However, to date, no effective, practical use of these bacteria in the marine environment has been achieved.

Algicidal bacteria include various species in the genus *Pseudoalteromonas* as well as species of the

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Class Flavobacteria, predominantly the genus *Cellulophaga* (Doucette et al. 1998, Nagai & Imai 1998). A *Pseudoalteromonas* strain previously isolated in the Huon Estuary demonstrated algicidal activity against *Gymnodinium catenatum* vegetative cells as well as other dinoflagellates and flagellates (Lovejoy et al. 1998). In the same study, another *Pseudoalteromonas* strain caused a detrimental but reversible effect on *G. catenatum* vegetative cells.

The mechanisms bacteria activate to produce algicidal components or to commence predatory attack are largely unknown. These mechanisms may include bacteria reaching a certain density before algicidal expression occurs (quorum sensing). This mechanism is typical of many bacteria that form biofilms but is also present in free-living bacteria (Schauder et al. 2001). Chemicals produced by bacteria such as acetylated homoserinelactones (AHL) can be used to communicate between bacteria in order to regulate expression (Bassler et al. 1993, Eberl 1999). Based on the concentration of AHL, bacteria activate or inactivate different functions that can regulate antibiotic production, conjugation, swarming, toxin production, biofilm formation, luminescence and exoprotease production (Bassler 1999, Eberl 1999). At certain AHL concentrations during mid- to late log phase, the receptor protein produces a phenotypic response and this is termed the threshold concentration. Many different bacterial species produce a signal molecule that is not an AHL but is termed an AI-2 inducer (Schauder et al. 2001) and thought to be significant for bacteria that exist in areas such as the marine environment where bacterial numbers are lower and species are diverse. Although AHL and AI-2 are different molecules they both use quorum sensing as the mode of control. Chemicals are used by bacteria to monitor their population density and could constitute possible regulatory mechanisms for the control of the expression of algicidal activity in the isolates. Algicidal isolates in this study were therefore tested for the 2 different types of signalling mechanisms, AHL and AI-2, that trigger luminescence in *Vibrio harveyi*, as well as the induction of many different genes (often involved in virulence) in a wide range of bacterial species.

The objectives of the present work were to screen a collection of cultured bacteria from the estuary and to identify the prevalence of algicidal activity against the toxic algal bloom species present in the estuary, *Gymnodinium catenatum*. The 5 positive bacterial isolates identified by this assay and their mode of action and activity against a wider range of organisms such as other dinoflagellates and diatoms was then examined. Implications of the presence of algicidal bacteria and evidence of whether complex control of expression and cell density influenced their algicidal expression was also investigated.

METHODS

Bacterial and algal strains studied. Over a 2 yr study of the Huon Estuary (August 1996 to September 1998), 5 biological stations were surveyed (CSIRO Huon Estuary Study Team 2000). The biological stations were part of a number of larger scale spatial surveys that included 40 sites throughout the estuary during different seasons. The biological stations were sampled frequently to resolve short-term changes and algal bloom behaviour. Bacterial isolates were collected from water samples from the Huon Estuary during this survey. Seventy-five bacterial isolates were catalogued as strains in the Australian Collection of Estuarine Microorganisms (ACEM). The algicidal activities of the 75 isolates were tested against the introduced toxic alga *Gymnodinium catenatum* using the procedure of Lovejoy et al. (1998). Genomic DNA was extracted from the cells of the algicidal isolates and purified using the procedure of Marmur & Doty (1962). The 16S rRNA genes from these strains were amplified by PCR using the primers 10f (AGT TTG ATC CTG GCT CA) and 1492r (TAC GGY TAC CTT GTT ACG ACT T). Conditions used for PCR are described in Bowman et al. (1996). The 5 algicidal strains then underwent additional testing against other algal species (Table 1). Further data was also obtained on Strain γ (referred to as ACEM 1), an algicidal *Pseudoalteromonas* species previously isolated from the Huon Estuary (Lovejoy et al. 1998).

Several other *Pseudoalteromonas* species, related to the 2 Huon Estuary *Pseudoalteromonas* strains (ACEM 1 and ACEM 4), were also tested for algicidal activity against *Gymnodinium catenatum* and *Chattonella marina*. The strains were *P. espejiana* (NCIMB 2127), *P. rubra* (NCIMB 1890), *P. luteoviolacea* (NCIMB 1893), *P. citrea* (NCIMB 1889) and *P. aurantia* (NCIMB 2033), 3 strains of *P. piscicida* (NCIMB 1938, 1142 and 645), *P. tunicata* (CCUG 26757) and *P. ulvae* (NCIMB 13762).

For maintenance of bacterial cultures, isolates were grown on marine agar (MA) using 1 l Huon Estuary water adjusted to a salinity of 28 with artificial sea salts (MA:14 g agar, 5 g bacteriological peptone, 1 g yeast extract). For biocidal assays, MA using 28 g artificial sea salts and 1 l distilled water was used in conjunction with an additional weaker nutrient media 1/10MA (salinity 28) in order that the nutrient levels were similar to those in the algal media.

Biocidal tests were carried out on the algal and microbial species listed in Table 1. Algal cultures were maintained at 17°C under cool white fluorescent light (100 photons m^{-2} s^{-1}) and a 12:12 h light:dark cycle. Algal media used either 0.2 µm filtered seawater or autoclaved 0.7 µm filtered Huon Estuary water. Salinity was adjusted to 28 using Milli-Q deionised filtered water or the addition of artificial sea salts (Sigma). Dinoflagellate spe-

Table 1. Taxon media strain and source of algal and bacterial species used in biocidal assays. Mix of Antarctic diatoms: concentrated mix of unidentified diatoms from Antarctic coastal seawater

Taxon	Class	Medium[a]	Strain	Strain source
Mix of Antarctic diatoms	Bacillariophyceae	f/2	–	R. van Denenden; Eastern Antarctica
Pseudonitzschia pseudodelitcassima	Bacillariophyceae	f/2	PPH03	J. Skerratt; Huon, Tasmania, Australia
Skeletonema costatum (Greville) Cleve	Bacillariophyceae	f/2	SkeHOA	C. Lovejoy; Huon, Tasmania, Australia
Alexandrium minutum Halim	Dinophyceae	GSe	AMAD 06	J. Cannon, S. Blackburn; Port River, South Australia
Gymnodinium catenatum Graham	Dinophyceae	GSe	GCDEO6; GCJP01	S. Blackburn; Derwent, Tasmania, Japan
Gymnoid Strains 1 and 2 and Dinophyta	Dinophyceae	GSe	GX1 GX2 GTR1	M. de Salas; Triabunna, Tasmania, Australia
Gyrodinium sp.	Dinophyceae	GSe	GYPA06	C. Bolch; Port Arthur, Tasmania, Australia
Polarella glaciacola Montresor et al.	Dinophyceae	f/2	FL2B	P. Thomson; Eastern Antarctica
Protoceratium reticulatum (Claparede & Lachmann) *Butschli*	Dinophyceae	GSe	DPR0	N. Parker; Derwent, Tasmania, Australia
Chattonella marina (Subrahmanyan) Hara & Chihara	Raphidophyceae	GSe	CMPL02	J. Marshall; Port Lincoln, South Australia, Australia
Brachionus plicatilis	Aschelminthes	–	–	DPIWE Marine Research Lab; Tasmania, Australia
Thraustochytridae Cavalier-Smith et al.	Chromista	Unpublished	ACEM A	T Lewis; South-Eastern Tasmania, Australia
Bacillus cereus	Firmicute	MA	ACEM 32	J. Skerratt; Huon, Tasmania, Australia
Planomicrobium sp.	Firmicute	MA	ACEM 22	J. Skerratt; Huon, Tasmania, Australia
Cellulophaga lytica	Flavobacteria	MA	ACEM 21	J. Skerratt; Huon, Tasmania, Australia
Zobellia sp.	Flavobacteria	MA	ACEM 20	J. Skerratt; Huon, Tasmania, Australia
Pseudoalteromonas sp.	γ-proteobacteria	MA	ACEM 1 (ibid Strain y)	C. Lovejoy; Huon, Tasmania, Australia
Pseudoalteromonas sp.	γ-proteobacteria	MA	ACEM 4	J. Skerratt; Huon, Tasmania, Australia

[a]GSe Media (Blackburn et al. 1989); f/2 (Guillard & Ruther 1962); MA (this paper)

cies were grown in GSe medium (Blackburn et al. 1989). Other cultures were grown in media listed in Table 1 (Guillard & Ryther 1962). Bacterial loads of the algal cultures were tested using both plate counts (CFU) and the fluorescent stain 4'6'-diamidino-2-phenylindole (DAPI, Sigma). All algal cultures tested were non-axenic, unialgal cultures with moderate to low bacterial loads (10^4 to 10^8 cells l^{-1} CFU, 10^4 to 10^9 cells l^{-1} DAPI).

Algal assays. The protocol described by Lovejoy et al. (1998) was used for algicidal assays. To test bacterial effects on different algal species, triplicate 1 ml samples of algal culture were added to a 24 well microplate (Iwaki, Japan). To this, 100 µl of a bacterial culture or filtrate or media control was added. Liquid media used for bacteria were MA and 1/10MA. Duplicate media controls were run in tandem with the experiments. The plates were sealed with parafilm and monitored at time intervals of 0, 5, 10, 15, 30, 60, 90, 120 and 180 min and then every hour for up to 6 h. Cultures were then monitored daily. Most algal cultures lysed within 3 h once the algicidal bacteria or exudate was added. A positive algicidal effect was considered to occur when 80% or more algal cells were lysed. A dilution series was completed on the algicidal bacterial media added to the algal cells to determine bacterial concentrations required to produce algicidal lysis (Fig. 1).

Characterisation of algicidal bacteria and compounds. Specific growth rates of the bacterial isolates were determined to identify differences between algicidal activity in logarithmic and stationary phase cultures. Late log phase cultures (1 ml) were inoculated into side arm flasks containing 1/10MA. Flasks were placed on a shaker table (22°C, 120 rpm) and growth curves were constructed by optical density measurements (OD: Absorbance 660 nm). Sub-samples were taken every hour until the stationary phase commenced. To determine the specific growth rate, the natural logs (ln) of the optical density readings were plotted versus time for each culture. From the plot, the data points that best represented the logarithmic phase of growth were used to make a linear least squares fit (number of points on line n = 7 to 10). From this equation, the slope was the calculated specific growth rate (Pirt 1975). Tests were carried out on the stability of the algicidal compounds with respect to temperature and the growth phase of the algicidal bacterial isolates. The algicidal abilities of the bacteria were tested in latent phase, early, mid- and late logarithmic phase, and early and late stationary phase. The heat stability of algicidal components was tested by incubating the bacterial supernatant (cell free) in enclosed Eppendorf tubes in a water bath at 38, 55, 80 or 120°C (autoclave) for 30, 15 and 10 min respectively.

Salinities and temperature ranges of the algicidal bacterial species were tested using MA broth. Tests on the effect of salinity changes were completed at 22°C

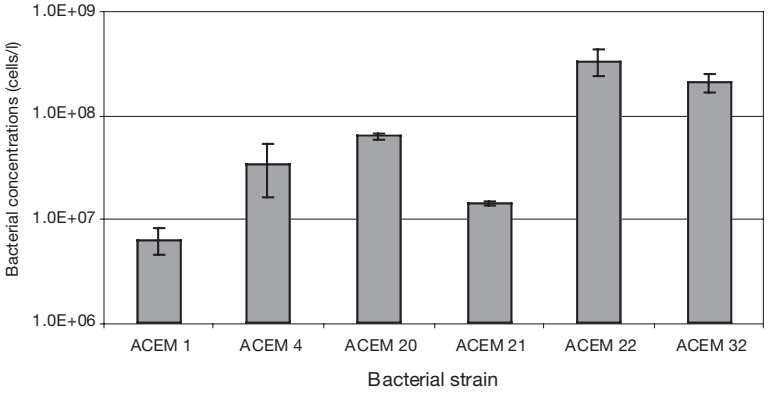

Fig. 1. Bacterial cell concentration (CFU) required to lyse *Gymnodinium catenatum* vegetative cells within 3 h. The same numbers of bacteria were required independent of whether the addition included bacterial cells and filtrates oral bacterial filtrates alone

and tests on the effect of temperature changes were completed at a salinity of 28. Observations of changes in the algicidal activity dependent on storage time and temperature were also observed during the course of the experiments.

Size fractionations of algicidal compounds were determined by placing the bacterial algicidal supernatant in dialysis tubing (5000 and 10 000 amu) and completing biocidal tests after fractionation. Further separation of the algicidal compounds was achieved by HPLC using a Waters Alliance 2690 HPLC, coupled with a photo diode array detector, a reverse phase C_{18} column (Nova-Pak C_{18}, 3.9 × 150 mm) and a Finnigan LCQ with APCI source-vaporizer 450, capillary 170, sheath gas 60, aux gas 15, source current 5 µamp or Finnigan LCQ with Electrospray source, capillary 200, sheath gas 90, aux gas 15, ESI needle 5KV. The scan range was m/z 100 to 1200 (or m/z 100 to 2000 for the Electrospray source). Data-dependent MS-MS scans were collected from the most intense ions. The elution gradients included a gradient of water—2% acetic acid-methanol at 0.8 ml min^{-1} or a 50:50 methanol: water gradient at 0.8 ml min^{-1} finishing with 90% methanol at 25 min. Fractions were collected and tested via assays using *Gymnodinium catenatum*. Controls were run using media blanks. All solutions were evaporated under gaseous nitrogen to dryness and adjusted with artificial sea water to double the equivalent volume added of the bacterial supernatant. These were then added to the algal cultures.

A normal phase cyano column (Nova-Pak CN HP, 3.9 × 150 mm) using a gradient of methanol (A), hexane (C) and water (D) at 0.8 ml min^{-1} (95:5% A:C for 3 min, 100% A for 30 min, 50:50% A:D for 40 min, which was then held for 20 min) was used to separate fractions, which were then collected and tested against *Gymnodinium catenatum*.

All algicidal bacteria, except for ACEM 32 (*Bacillus cereus* strain), were highly pigmented. Extracted fractions containing bacterial pigment were tested for their algicidal activity because of these intense pigments and to eliminate them as a source of algicidal activity. Additionally, in the study by Egan et al. (2002), the bioactive activity of their bacteria varied in relation to pigment composition.

Algicidal bacteria were cultured in 1/10MA in 500 ml conical flasks overnight at 18°C. Pigments were extracted with acetone or methanol/dichloromethane. For acetone extraction, bacterial cells were filtered, the filters placed in 90% acetone, agitated ultrasonically for 5 min, stored overnight at –20°C and then resonicated, centrifuged and evaporated to dryness under gaseous nitrogen. Pigments were then solubilised with artificial seawater (salinity 28) and tested as below. The methanol/dichloromethane extraction used the modified (White et al. 1979) 1-phase $CHCl_3$-MeOH-H_2O Bligh & Dyer (1959) method. After phase separation both the aqueous and organic layers were rotary evaporated at 35°C to dryness and the resultant pigments, dark purple and dark green, respectively, solubilised with sea water.

The resulting pigment extracts were made up to 1 ml and 100 µl aliquots were tested on *Gymnodinium catenatum* as indicated above. Extracts also contained unidentified compounds that co-extracted with the solvents used.

AHL assay. Algicidal bacteria were evaluated with an AHL bioreporter, based on the transcriptional activator TraR of *Agrobacterium tumefaciens*. TraR can respond to 3-oxo-AHL, 3-hydroxy-AHL and alkanoyl-AHL with chain lengths ranging from C_4 to C_{12}. The fusion reporter from the *A. tumefaciens* tumour inducing plasmid has been shown to be one of the most sensitive tests and versatile detectors of all AHL tests (Cha et al. 1998). AB minimal media was used (Clark & Maaløe 1967) and plates were supplemented with 4.5 µl ml^{-1} tetracycline and 50 µg ml^{-1} spectinomycin, streaked with *A. tumefaciens* strain A136 and incubated at 30°C. Fifty ml of AB media was inoculated with *A. tumefaciens* culture and incubated at 30°C for 24 h. Molten AB agar media was cooled to 48°C and the 24 h *A. tumefaciens* culture was then added. Wells were punched in the centre of each agar plate and 50 µl of algicidal supernatant from 24 and 48 h cultures were added to the well. A positive control was included (*Vibrio fischerii*). The induced blue zone around the well was measured at 24 and 48 h.

AI-2 assay. Protocols from Joyce et al. (2000) were used for the AI-2 assay. Confluent growth from 24 h

algicidal bacteria plate cultures were collected by swabbing a plate flooded with 5 ml of 1/10 MA broth. The swab was then inoculated into 1/10 MA broth to a starting optical density of 0.1 at 600 nm, and incubated at 37°C. Cell free culture fluids were prepared by centrifugation followed by filtration (Millipore 0.2 µm).

For the AI-2 assay, 10 µl of each of the cell free cultures fluids were added to a 96 well microtiter dish (IWAKI) and assayed for AI-2 activity with strain *Vibrio harveyi* BB170 as described in Surette & Bassler (1998, 1999). For each preparation, 10 µl of the corresponding sterile medium was added to the wells as a negative control and 10 µl of *V. harveyi* cell-free media was used as a positive control for AI-2 activity. *V. harveyi* BB170 was grown overnight with aeration at 30°C in AB medium then diluted 1:5000 in fresh AB medium, and 90 µl of the diluted culture was added to the wells containing the cell-free media or media controls. The microtiter dishes were shaken at 200 rpm in a rotary shaker at 27°C. Light production was quantified every hour with a Wallace Model 1450 Microbeta Plus liquid scintillation counter. Data are reported as the fold stimulation of light emission by *V. harveyi* BB170 over that obtained for the corresponding growth medium alone. Assays were performed in triplicate.

Algicidal activity of field bacteria. Seawater samples were taken from a site in the lower middle reaches of the estuary during the broader biological, physical and chemical study (CSIRO Huon Estuary Study Team 2000). The site chosen for this experiment was known for having high cell counts of *Gymnodinium catenatum* when this alga had bloomed in the previous 2 yr (Site F3: CSIRO Huon Estuary Study Team 2000). Water samples for the algicidal experiment were taken from the pycnocline (typically 2 m) by means of a 5 l Niskin bottle. These samples were plated onto MA and incubated at 22°C for 1 wk to attain between 30 and 300 colonies for each sampling date. Colonies that were similar morphologically to ACEM 4, ACEM 1, ACEM 20, ACEM 21 and ACEM 22 were treated as bacteria with algicidal potential. After 1 wk incubation at 22°C, colonies were transferred to an Eppendorf tube containing 1/10MA. The cultures were incubated at 22°C for 2 d. The bacterial suspensions were then centrifuged and a 100 µl subsample of each of the supernatants was tested against *G. catenatum* using the previously mentioned algicidal assay.

Although identification of bacterial samples using morphological characteristics introduces bias with respect to the representation of the bacterial population, all algicidal colonies grew rapidly and well on MA and all had highly distinct morphologies as mentioned in 'Results'. Field samples had already been plated and collected every 2 wk for 1 yr prior to this experiment. A range of 16 to 89 (average 30) isolates were tested for algicidal activity for each field date. Although bacterial concentrations varied between sample dates similar bacterial types would reappear that were easily recognisable each week or each season. Algicidal activity was registered if cell lysis occurred within 3 h.

RESULTS

Characterisation of algicidal bacteria

Five of the original 75 bacteria isolated from the Huon and deposited in the ACEM collection were algicidal and were phylogenetically identified (Table 1). The GenBank accession numbers of the 3 novel isolates investigated in this study were ACEM 4 (AF295592), ACEM 20 (AY035869) and ACEM 22 (AYO35870).

ACEM 4 was a strain or close relative of *Pseudoalteromonas tunicata.* ACEM 1 was closely related to *Pseudomonas piscicida*. ACEM 21 was a strain of *Cellulophaga lytica*, ACEM 32 was a strain of *Bacillus cereus*. ACEM 22 (*Planomicrobium* sp.) and ACEM 20 (*Zobellia* sp.) were 2 novel species whose closest relatives were *Planococcus kocurii* and *Zobellia uliginosa*, respectively. The genus *Zobellia* is very closely related to the genus *Cellulophaga*.

All 5 algicidal isolates, except ACEM 32, were highly pigmented and had distinct morphologies on solid media: ACEM 4 (*Pseudoalteromonas* sp.), dark green colonies and black media pigmentation; ACEM 1 (*Pseudoalteromonas* sp.), yellow colonies with white edges; ACEM 21 (*Cellulophaga lytica*), iridescent spreading orange colonies; ACEM 20 (*Zobellia* sp.), yellow-orange undulate colonies; and ACEM 22 (*Planomicrobium* sp.), round orange colonies and formed rods in logarithmic phase and cocci in stationary phase. All grew between 2 and 37°C, except ACEM 4 which did not grow above 25°C and ACEM 22 which grew at 55°C. All 5 algicidal isolates grew at salinities of 66 and only the 2 Firmicutes, ACEM 22 and ACEM 32, grew at salinities less than 5.

Table 2. Generation time and specific growth rate of algicidal isolates (OD 660 nm and 22°C)

Algicidal isolate	Generation time (min)	Specific growth rate (µ)	r^2
ACEM 1	7.49	7.72	0.931
ACEM 4	7.64	7.56	0.975
ACEM 20	9.15	6.31	0.996
ACEM 21	10.21	5.66	0.997
ACEM 22	7.39	7.82	0.996

Table 3. Comparison of bacterial growth stage and the effect of algicidal activity on *Gymnodinium catenatum* vegetative cells

Growth phase	Algicidal isolate					
	ACEM 1	ACEM 4	ACEM 20	ACEM 21	ACEM 22	ACEM 32
Latent and early log	–	–	–	–	–	–
Mid-log	50% cell lysis and cell rounding	10 to 30% cell lysis and cell rounding	<10% cell lysis and cell rounding	10 to 30% cell lysis and cell rounding	10 to 30% cell lysis and cell rounding	–
Stationary	100% cell lysis	100% cell lysis	≥80% lysis and cell rounding	≥90% lysis and cell rounding	≥80% lysis and cell rounding	≥80% lysis and cell rounding

Rapid growth rates and generation times were observed for all algicidal bacteria (Table 2). Specific growth rates were high (Table 2) and generation times were 7 to 10 min. The growth rate of ACEM 32 (*Bacillus* sp.) is not presented as colloidal balls formed, making it difficult to accurately measure optical density.

No bacteria were algicidal in latent or early log phase (Table 3). All bacteria, except ACEM 32, were algicidal during mid-log phase and early and late stationary phase. ACEM 32 reached late log phase at 10 to 14 h; production of algicidal components in ACEM 32 occurred after 20 h. Therefore, ACEM 32 was only algicidal in stationary phase.

Specificity of algicidal isolates

Algicidal supernatants of the bacteria were tested on a range of algal and heterotrophic species previously used in studies of algicidal bacteria (Table 4). The main algal species of interest was *Gymnodinium catenatum* as this was the toxic alga that commonly bloomed in the Huon Estuary.

Previously the methods of bacterial algicidal attack have been described as 'direct' and 'indirect' (Doucette et al. 1998). 'Direct' indicates that physical contact is required between the bacteria and the algal cell before the algal cell lyses and 'indirect' indicates that the bacteria exudes the lysing compound into the surrounding media or water column (Doucette et al. 1998). Hence, detection of the direct attack required the presence of bacterial cells and indirect attack was ascertained by adding only the filtrate or exudate from the bacterial medium.

Algicidal components produced by all 5 bacterial strains were extracellular and therefore the strains primarily used indirect attack. Algicidal lysis started within 15 min of adding the filtered supernatant to the dinoflagellate culture and total lysis usually occurred within 2 h. Bacterial numbers required for algal lysis were generally between 10^6 and 10^{10} cells l^{-1} (Fig. 1). Numbers were the same whether filtrate or filtrate plus bacterial cells were added.

The mode of lysis using extracellular compounds was similar for all algicidal bacteria and to published data for strain ACEM 1 (Lovejoy et al. 1998). After 2 to 15 min, chain-forming species such as *Gymnodinium catenatum* (Fig. 2a) would separate into single cells (Fig. 2b). Fig. 2c shows a single unrounded cell of *G. catenatum* still unaffected by exposure to the algicidal component surrounded by other rounded algal cells. During the period of cell rounding, some cells would not become rounded for up to 1 h, whereas others would start to lyse. Occasionally large temporary cysts would form in cultures that contained algicidal bacteria and these would also eventually lyse. Within 30 to 45 min, single cells would start to show cell rounding and thinning of the cell wall (Fig. 2c) followed by lysis of the cell wall after 1.5 to 4 h (Fig. 2d). When bacterial cells from ACEM 1 and ACEM 21 were added with their algicidal supernatant, vigorous bacterial swarming was observed around the algal cell once the alga had began to lyse (Fig. 2d).

All bacterial supernatants, except ACEM 20 and ACEM 22, lysed the vegetative forms of the toxic algae *Gymnodinium catenatum*, *Chattonella marina* and *Alexandrium minutum*. ACEM 20 and ACEM 22 did not lyse *A. minutum* but lysed *G. catenatum* and *C. marina*. No effect of algicidal activity was observed on any of the diatom, rotifer or thraustochytrid species tested.

Algicidal activity against 4 Tasmanian dinoflagellates was tested (Tables 1 & 4). One armoured dinoflagellate species, *Protoceratium reticulatum*, was unaffected by any of the algicidal bacteria and no cells lysed or rounded. The algicidal components of both *Pseudoalteromonas* species were detrimental towards 3 dinoflagellate species, however, one of these species (*Gyrodinium* sp.) recovered and survived after treatment. Some cells lysed or rounded; however, the unlysed *Gyrodinium* cells eventually recovered normal movement and further lysis did not occur. The other algicidal strains were not as effective at lysing the dinoflagellates as ACEM 1 and ACEM 4 (*Pseudoalteromonas* sp.), and had no effect on the *Dinophyta* species. They also had no effect on *Gyrodinium* except

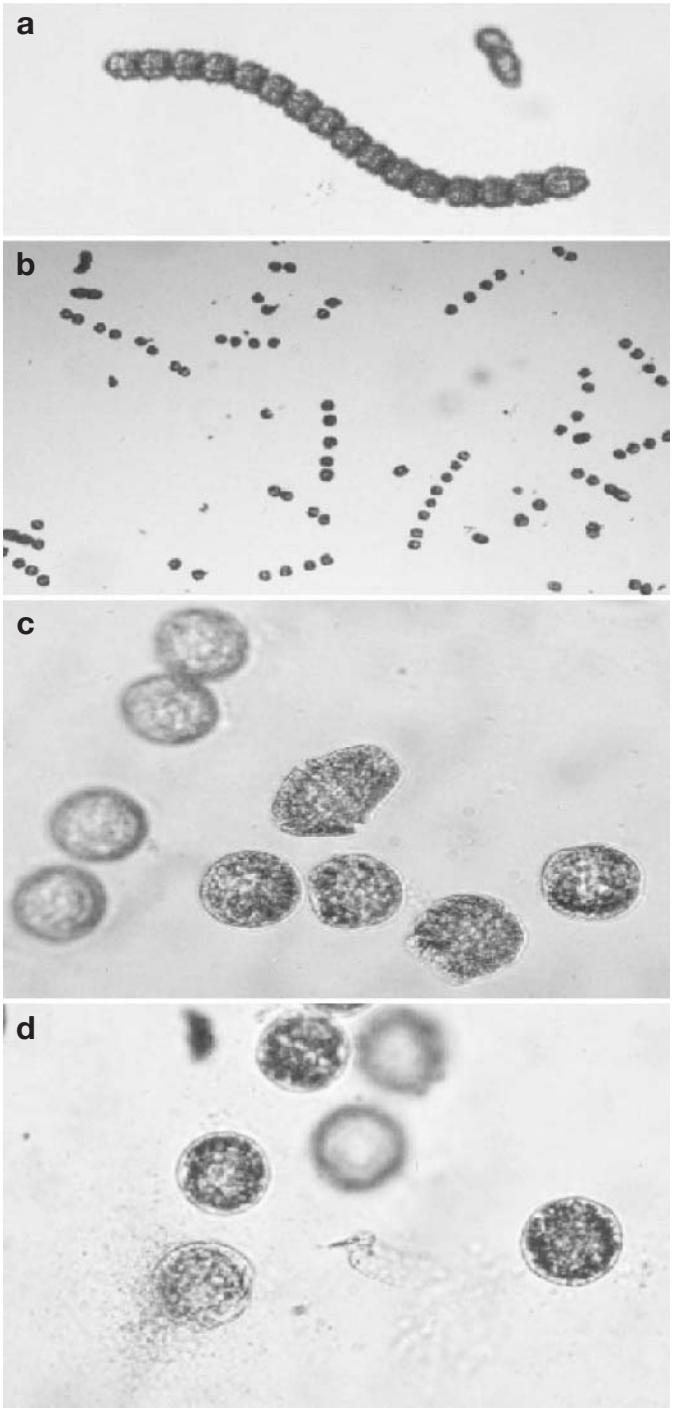

Fig. 2. *Gymnodinium catenatum*. Images showing time series of vegetative cells after addition of algicidal supernatant from ACEM 1 (a,b,c). The addition of algicidal bacteria (ACEM 1) is shown in (d) to demonstrate swarming affect of this bacteria. Similar lysis of the cell wall is noted with the addition of supernatant alone

ACEM 21 (*Cellulophaga lytica*). ACEM 21 caused some lysis and cell rounding but the *Gyrodinium* species survived.

Of the additional *Pseudoalteromonas* species tested for algicidal activity against *Chattonella marina* and *Gymnodinium catenatum*, only *Pseudoalteromonas rubra*, *P. tunicata* and *P. ulvae* lysed the 2 toxic algal species.

ACEM 21 produced extracellular exudates that were capable of lysing the algae, but the bacteria also demonstrated the ability to attack the algal cells directly. We also confirmed previous descriptions of the ability of ACEM 1 to directly attack algal cells (Lovejoy et al. 1998) although this was most apparent once the algal cells had started leaking (20 to 60 min), and bacterial cells were highly concentrated and physically active around these leakage areas. This swarming behaviour was apparent to the degree that the algal cells were physically moved around the field of view by the bacterial swarm. The swarming effect was not apparent if the supernatant was added alone, but cell lysis occurred within a similar time frame. If bacteria were added with the supernatant, faster mechanical disintegration of cells was observed in all cultures apart from those with ACEM 22 added. The physical breakdown of the algal cells appeared to allow the bacterial swarm to become more effective once the integrity of the algal cell wall was broken.

Stability and loss of algicidal activity

The algicidal activity of the filtered supernatant for the *Pseudoalteromonas* species ACEM 1 and ACEM 4 remained unchanged after a week at room temperature (23°C), 1 to 3 mo at 4°C or 6 mo at –20°C. Noticeable deterioration was observed for longer periods. After 12 mo, algicidal activity had disappeared for samples at 4°C and was very poor (<10% lysis) at –20°C.

The ability to retain algicidal activity after heat treatment was also tested. All supernatants were algicidal after 30 min at 38°C, 15 min at 55°C, 10 min at 80 or 120°C (autoclave). The mode of algal lysis appeared to be the same as before heating, i.e. it did not appear to be a toxic artefact caused by heating. Overall, this indicates that the algicidal mechanism in each of the bacteria was not enzymatic.

Algicidal bacteria would occasionally lose their ability to lyse algal cells completely. This occurred sporadically for all bacteria except ACEM 21 (*Cellulophaga lytica*). The loss of algicidal activity was often restored by isolation of a healthy bacterial colony into seawater for 1 or 2 mo at 20°C. ACEM 22 (*Planomicrobium* sp.) often lost activity after being cryo-

genically stored. The algicidal revival method mentioned was used twice successfully for ACEM 22. However, for a period of over 1 yr, it would not recover any algicidal activity. The addition of the spent, filtered supernatant of another algicidal bacteria to ACEM 22 (A. Franks pers. comm.) was eventually used to reinitiate algicidal activity in ACEM 22. This method was also tested for *Escherichia coli* and other environmental strains to identify the possibility of initiating activity in species that were not algicidal, but this did not occur.

Algicidal activity could be lost in ACEM 1 once the culture had been continuously agitated for 3 to 4 d. After this period, the media would appear clear, as though no growth had occurred. Autolysis was a possible cause of this phenomenon. The algicidal activity of this broth was severely affected and became equivalent to the culture in early log phase. The other algicidal isolates did not exhibit autolysis when grown under similar conditions.

Algicidal activity varied dramatically for bacterial isolates taken from the estuary at different sampling dates that were identified as strains of algicidal species (algicidal-like). Up to 90 % of the algicidal-like isolates were algicidal for 1 date, yet similar isolates in the following weeks showed no such activity (Fig. 3). Interestingly, many algicidal-like isolates were not 'algicidal' at the peak of a *Gymnodinium catenatum* bloom even though they were present before and after the bloom declined.

Algicidal-like isolates were present on each of the sampling dates and, although the proportions of the isolates changed, similar species were observed in

Table 4. Mode of attack of algicidal bacteria against host species. Bold text indicates results for this study, other results are previously published; *indigenous Tasmanian algal species. D: direct attack or attachment onto algal cell causing lysis of host; I: indirect attack (bacterial algicidal exudate) causing lysis of host; IR: indirect attack with some cells lysed but eventual recovery of unlysed host cells. ne: no effect. Table adapted from Doucette et al. (1998)

	Algicidal bacterial genera, strain[a]														
	Cellulophaga and *Zobellia*					*Pseudo-alteromonas*					*Bacillus*	Flavobacteria		*Vibrio*	*Planomicrobium*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Diatoms															
Skeletonema costatum	D	D		**ne**	**ne**		I	ne	ne	**ne**	**ne**	ne	ne	ne	**ne**
Chaetoceros didymum	D			**ne**	**ne**	I			ne	**ne**	**ne**				**ne**
Mixed Antarctic diatoms				**ne**	**ne**				**ne**	**ne**	**ne**				**ne**
Thalassiosira sp.	D	D					I						ne	ne	
Ditylum brightwellii	ne	D				ne							ne	ne	
Eucampia zodiacus	D						I								
Raphidophytes															
Chattonella antiqua	ne	D	D			I		ne					ne		
Chattonellamarina		D	ne	**I**	**D/I**	I	I	ne	D/I	**I**	**I**	ne			**I**
Heterosigma akashiwo		D	ne			ne		ne	D/I			ne	ne	I	
Fibrocapsa japonica		D	ne												
Dinoflagellates															
Gymnodinium catenatum	ne	D	ne	**I**	**D/I**	I		I	D/I	**I**	**I**	I	I	I	**I**
*Protoceratium reticulatum**				**ne**	**ne**				ne	**ne**	**ne**			**ne**	
Polarellaglaciola (polar dinoflagellate)				**I**	**D/I**				**I**	**I**	**I**				**I**
Gymnodinium sanguineum									D/I						
Gyrodinium sp.*				**ne**	**IR**				**IR**	**IR**	**ne**				**ne**
*Dinophyta**				**ne**	**ne**				**I**	**I**	**ne**				**ne**
*Gymnodinioid**				**I**	**I**				**I**	**I**	**I**				**I**
Alexandrium minutum				**ne**	**I**				IR/**I**	**I**	**I**				**ne**
Thraustochytrida, Rotifers				**ne**	**ne**				**ne**	**ne**	**ne**				**ne**
Prasinophyte, Cryptophyte, Cyanobacteria									ne						

[a]1: Mitsutani et al. (1992); 2: Imai et al. (1993); 3: Furuki & Kobayashi (1991); 4, 5, 10, 11, 15: ACEM 20, ACEM21, ACEM 4, ACEM 32, ACEM 22, this paper; 6: Imai et al. (1995); 7: Lee et al. (2000); 8, 13, 14: Yoshinaga et al. (1995); 9: Lovejoy et al. (1998); 12: Fukami et al. (1992)

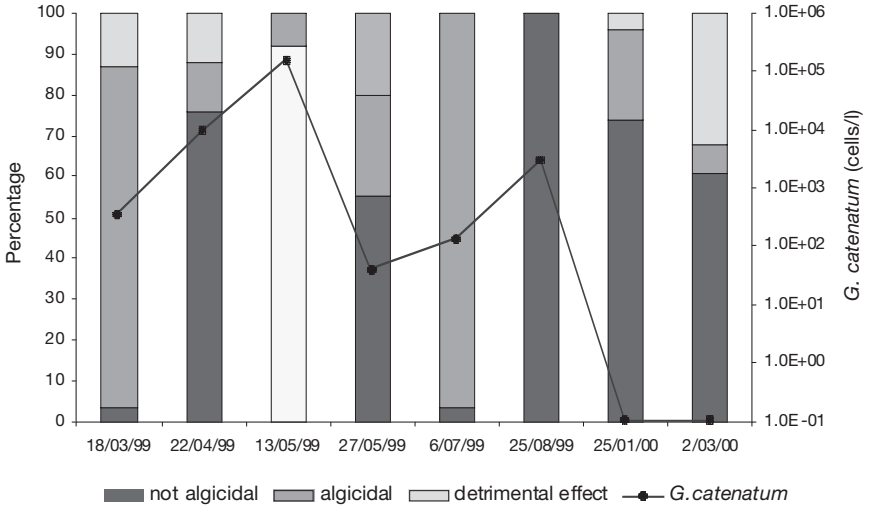

Fig. 3. Algicidal-like bacteria isolated from the Huon River, Tasmania, over 1999-2000 season and their algicidal activity for particular dates compared with *Gymnodinium catenatum* vegetative cell numbers (*G. catenatum* counts; Parker 2002). Detrimental effect was algal cell rounding but later recovery

consecutive weeks. These species also varied in their algicidal activity. For example, the spreading iridescent orange ACEM 21-like isolate was algicidal whenever present. In comparison, Gram-positive ACEM 22-like orange isolates that occurred at the peak of the *Gymnodinium catenatum* bloom were not algicidal at this time, although similar isolates in the weeks before and after were algicidal.

Characterisation of algicidal components

The pigment-containing fractions of algicidal strains did not possess algicidal activity. Colony pigmentation of both *Pseudoalteromonas* species ACEM 1 and ACEM 4 could be altered using different media, a typical characteristic of *Pseudoalteromonas*. Occasionally, ACEM 1 and ACEM 4 produced non-pigmented variants that co-occurred with their respective yellow or green colonies. The non-pigmented variants were not a contaminant as they reverted back to yellow and green colonies, respectively, if replated. If the non-pigmented variants were repeatedly replated for a number of generations (n = 4 to 10), algicidal activity in ACEM 1 and ACEM 4 continued to occur. Changes in pigmentation did not appear to affect the algicidal activity. Pigment formation in these isolates was therefore not essential to the algicidal activity of the isolates, but all isolates had the ability to produce pigment as part of their cell biochemistry.

Algicidal components of ACEM 1 and ACEM 4 were less than 5000 amu. Analysis by HPLC-MS-MS established that the compounds were highly polar and of low molecular weight. The algicidal compounds eluted in the first 2 min of the elution gradient (water) using the C_{18} reverse phase column. HPLC analysis with the normal phase column resulted in the compounds being retained on the column. Use of ion exchange chromatography, Sephadex columns and XAD columns were unsuccessful in isolating and/or concentrating these fractions.

Quorum sensing

The tests to determine the presence of AHL were negative for all algicidal species. The *Agrobacterium tumefaciens* TraR test is the most sensitive for detection of AHL and the result suggests that the typical pathway for gene regulation via AHL does not induce the algicidal or other bioactive mechanisms in these species.

The second form of regulatory expression tested was AI-2. AI-2 uses similar regulatory expression as for the production of AHL. *Vibrio harveyi* BB170 was used to test for this second mechanism of quorum sensing control. The algicidal strains all showed some activation of lux genes in comparison to the negative control (Fig. 4). In particular, strong signals were observed for Firmicute isolates ACEM 22 and ACEM 32, respectively; ACEM 22 at late and mid-log phase, and ACEM 32 at late log phase. ACEM 20 and ACEM 1 demonstrated activity for all growth phases. The results indicate that for the algicidal Gram-negative species the bacteria appear to use the AI-2 mechanism at mid- to late stage of log phase.

DISCUSSION

All algicidal species investigated in this study employed indirect or extracellular methods of lysing algal cells. Two isolates (1 previously described in Lovejoy et al. 1998) also used direct attack or attachment to the algal cell. To date, only a few algicidal bacteria have been isolated that attack diatom species (Table 4; Mitsutani et al. 1992, Imai et al. 1993, Lee et al. 2000). Of these 3 studies, 2 were direct attacks by *Cellulophaga* species and one involved a protease produced by a *Pseudoalteromonas* strain. Proteases are usually not heat tolerant, so it is unlikely that the

Pseudoalteromonas from the Huon Estuary used this method to lyse algal cells.

In contrast to species algicidal towards diatoms, dinoflagellates and flagellates have demonstrated particular sensitivity to the algicidal components of bacteria (Table 4). Interestingly the native Tasmanian dinoflagellates in this study appeared less affected overall by the bacterial exudates. Unlike *Gymnodinium catenatum*, which is an introduced species, it is possible that these native algal species may have some resistance to the resident algicidal bacteria. However, further research is necessary to determine if this proposition is of significance.

To our knowledge, Lovejoy et al. (1998) is the only previously published work to mention the effect of algicidal species against higher order organisms (Table 4). The 5 algicidal isolates in this study also demonstrated no effect on Thraustochytrida or rotifers indicating low level class-specific effects.

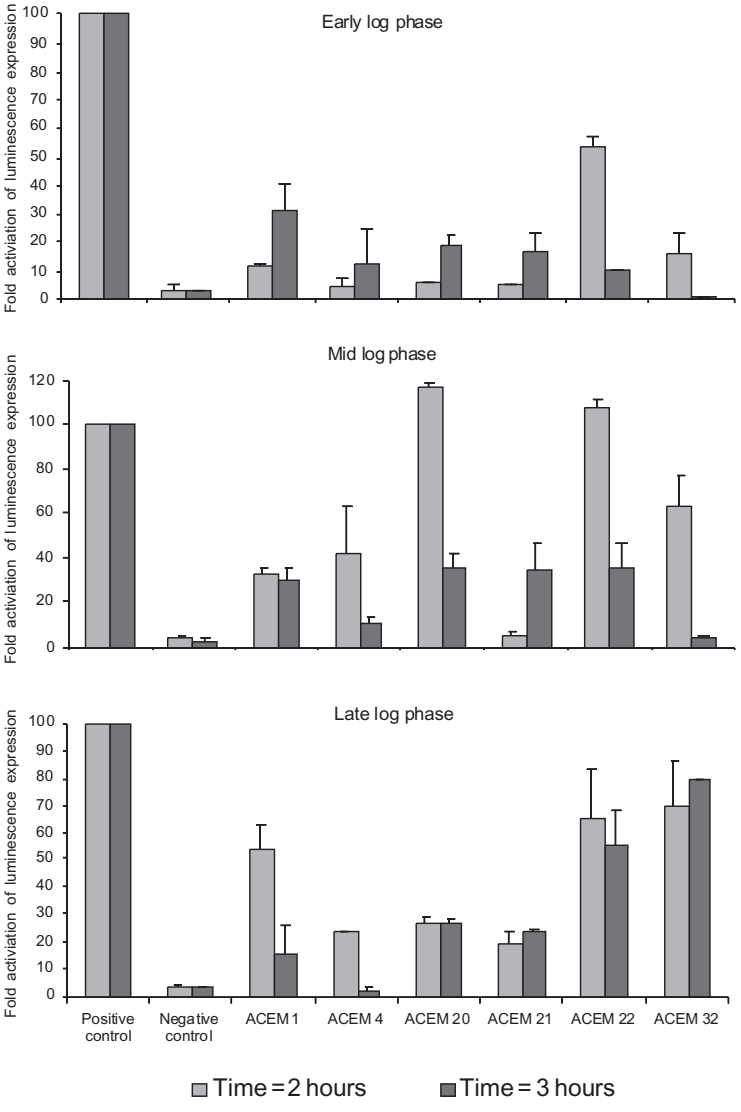

Fig. 4. Time series of algicidal strains at different growth phases, showing induction of the luminescence gene via the autoinducer mechanism AI-2. *y*-axis shows activity, reported as fold activation of luminescence of the algicidal strains over the level of luminescence when sterile media was added. Luminescence (n = 3) is reported with respect to the positive control. Positive control: luminous bacterium *Vibrio harveyi*. At and after 4 h, the negative control becomes positive; therefore, results after 3 h are not shown

Flavobacteria

Cellulophaga are one of the most prolific and widely studied algicidal genera (Stewart & Brown 1969, Imai et al. 1991, 1993, Mitsutani et al. 1992, Yoshinaga et al. 1998, Kondo et al. 1999, Toncheva-Panova & Ivanova 2000). The genus *Cellulophaga* and the Class Flavobacteria can dominate many marine systems and are often associated with marine snow and the decay of algal blooms (Delong et al. 1993, Crump et al. 1999, Riemann et al. 2000). They grow easily in oligotrophic conditions as they can utilise a variety of carbon sources for growth, but can also thrive on nutrient-rich media such as marine snow or enriched environmental conditions. They are found throughout the water column of temperate and polar marine environments (Glöckner et al. 1999, Cottrell & Kirchman 2000, Pinhassi & Hagström 2000, Fandino et al. 2001) and are common in fresh water where they have also demonstrated swarming and predatory algicidal abilities (Stewart & Brown 1969). Pinhassi & Hagström (2000) reported the domination of a coastal community by Flavobacteria during a coastal phytoplankton decline (43% of intact cells). A study by Glöckner et al. (1999) demonstrated that members of the Class Flavobacteria were the most abundant group detected in a number of different marine systems, accounting for an average of 18% (2 to 72%) of the DAPI stained cells.

ACEM 20 and ACEM 21 are both members of the Flavobacteria and their algicidal activity differed to that previously described for most other members of this class (Imai et al. 1993, 2001). Gliding bacteria typically attach directly to a cell (direct attack) rather than useing extracellular compounds (indirect attack). In other studies of algicidal *Cel-*

lulophaga-like species, all but one have shown predatory mechanisms only (Table 4). Recently, another *Cellulophaga*-like species unrelated to the 2 described in this study (89% 16S rDNA sequence similarity to ACEM 20, G. Doucette pers. comm.) was reported as using extracellular mechanisms and indirect attack. Both Flavobacteria in this study produced an algicidal exudate. In the case of ACEM 21, this was in addition to a predatory mechanism of physically attacking the cells.

Pseudoalteromonas

Pseudoalteromonas is the second most abundant and commonly reported genus of algicidal bacteria after *Cellulophaga* (Baker & Herson 1978, Imai et al. 1995, Lovejoy et al. 1998, Lee et al. 2000). Lysing occurs through the indirect method of extracellular exudation, and they have previously shown species-specific algicidal activity (Lovejoy et al. 1998). They also have the ability to lyse cells and swarm using their flagella once the cells are leaking (Imai et al. 1995, Doucette et al. 1998, 1999). The most highly studied species of this genus to date is *Pseudoalteromonas tunicata* which produces at least 3 different bioactive compounds that act independently: antifungal, inhibition of algal spore germination and antifouling (Holmström & Kjelleberg 1999, Egan et al. 2000). *P. tunicata* is closely related to ACEM 4.

Pseudoalteromonas rubra, *P. ulvae* and *P. tunicata* were the only other *Pseudoalteromonas* species of 8 tested that possessed the same algicidal activity against *Gymnodinium catenatum* and *Chattonella marina* as the 2 *Pseudoalteromonas* in this study (ACEM 1 and ACEM 4). *Pseudoalteromonas* are known for their many bioactive compounds (Gauthier 1976, Mikhailov & Ivanova 1994). Other *Pseudoalteromonas* species tested such as *P. luteoviolacea* (McCarthy et al. 1994), *P. aurantia* (Gauthier & Breittmayer 1979) and *P. piscicida* (Buck et al. 1963) have all produced antibacterial or bioactive compounds. In our study, these species did not demonstrate any algicidal activity. Given the nature of these algicidal components our results suggest some of the bacterial strains stored in culture collections may have lost activity over time. Other workers examining this genus have also found a lack of any bioactivity in many of the species (C. Holmström pers. comm.).

Lysis of *Alexandrium minutum* by ACEM 1 (*Pseudoalteromonas* sp.) in this study was in contrast to the study by Lovejoy et al. (1998) in which ACEM 1 caused *A. minutum* cells to round but recover after 24 h. The variation between the 2 studies may be due to the maintenance of bacterial cultures in this study on MA before completion of the biocidal assays on 1/10MA. In the experiment by Lovejoy et al. (1998), ACEM 1 was maintained at 1/10MA.

Firmicutes

ACEM 22 (*Planomicrobium* sp.) and ACEM 32 (*Bacillus cereus*) represent the first description of Gram-positive species that produce algicidal components. The *B. cereus* group produces many extracellular compounds that include haemolysin, a soluble toxin lethal for mice, insecticidal toxin, phospholipase C and bacterial-lytic and proteolytic enzymes (Claus & Berkeley 1989, Lereclus 2000). *B. mycoides*, a closely related species to ACEM 32, has shown bioactive properties that have been used in the agriculture and biomedical industries (Hammad & El-Mohandes 1999, Pruss et al. 1999), and can be the causative agent in agricultural infections (Grodnitskaya & Gukasyan 1999). Most of the toxins and bioactive compounds in the *B. cereus* group are only produced in stationary phase (Lereclus 2000). In this study, ACEM 32 only produced algicidal exudates in stationary phase and not in log phase.

The genus *Planomicrobium* is closely related to the *Bacillus cereus* group, but is essentially a marine genus with no previously reported pathogenicity or algicidal abilities. The *Planomicrobium* isolate, ACEM 22, was the only species that did not show swarming activity. Although fast growing, the algicidal ability of ACEM 22 after being kept in culture for long periods of time was highly intermittent when compared with the other algicidal isolates.

The 2 Firmicutes were also unusual because most algicidal bacteria are of Class Flavobacteria or the genus *Pseudoalteromonas*. It is possible that to date descriptions of algicidal species have been influenced by bacterial culturability.

Quorum sensing

The association with and potential for control of toxic algal blooms has directed most algicidal bacteria research (Stewart & Brown 1969, Imai et al. 1993, Doucette et al. 1998, 1999, Lovejoy et al. 1998, Kim et al. 1998, Holmström & Kjelleberg 1999, Yoshinaga et al. 1999, Nagasaki et al. 2000). Very little is known about algicidal bacteria that affect non-toxic algal blooms. All algicidal species apart from ACEM 32, produced algicidal compounds in both late log phase and stationary phase. Many bacterial secondary metabolites, bioactive and algicidal compounds, are only produced during stationary or late log phase (Fukami et al. 1992, Mikhailov & Ivanova 1994, Lereclus 2000).

There is also the question of whether the algicidal activity of these species occurs continuously or episodically in the marine environment. There has been a general assumption that bacteria continuously and simultaneously release their bioactive components in the natural ecosystem. We suggest that some algicidal bacteria may not always exude algicidal components, and may have periods in the natural environment in which they are not producing one or more of their bioactive compounds. The reason for this variation may be environmental or be caused by strain variation. Although no algicidal bacteria in this study produced the quorum sensing homoserine lactones (AHL) according to the *Agrobacterium* assay, many bioactive environmental bacteria do not have AHL mechanisms; instead, they appear to use an alternative induction pathway (AI-2) which can activate at lower cell concentrations than required for AHL (Bassler 1999, Schauder et al. 2001). This alternative AI-2 mechanism may play a role in switching on algicidal activity for the Gram-negative bacteria once the bacteria reach a specific cell density. The results for the algicidal bacteria indicate moderate to good induction of this second mechanism when compared to the negative control. ACEM 1 demonstrated induction for early log phase and ACEM 21 for mid-log phase. The AI-2 mechanism has been thought to be for bacteria communicating between species rather than AHL. This type of interspecies communication in the marine environment would be a more realistic mechanism of action (Surette & Bassler 1999). In the marine environment, there is likely to be a greater metabolic benefit for these different bioactive mechanisms to be activated individually or as the bacteria requires, rather than simultaneously and continually.

Gram-positive bacteria do not generally employ AHL as signals. They secrete processed peptide signalling molecules via a dedicated exporter protein (Bassler 1999). For example, in *Bacillus subtilis*, 2 peptide signals allow the bacteria to control DNA uptake and sporulation. The secretion mechanics of the export of the peptides have not been identified (Bassler 1999). The positive results in the autoinduction experiment for ACEM 32 (*B. cereus*) and ACEM 22 (*Planomicrobium* sp.) were likely to be the result of this Gram-positive quorum signalling mechanism which may play a role in the bioactive mechanisms in these species.

Algicidal activity

The bacteria in this study exhibited predatory and species specific algicidal abilities. They also grew well on nutrient-rich media, had very short generation times and high specific growth rates. Because of these particular characteristics, the algicidal isolates, particularly the genera *Cellulophaga* and *Pseudoalteromonas*, could be grouped as opportunistic or *r*-strategists (Weinbauer & Hofle 1998). For example, the growth rates for the algicidal isolates indicated that they could live as attached or free-living bacteria as they would be able to take advantage of a sudden increase in nutrients. Therefore, all algicidal strains have the ability to take advantage of the high summer water temperatures (19°C) and/or increased nutrients that occur during dinoflagellate blooms at temperatures and salinity conditions typically found in the estuary. In addition, related results from Skerratt (2001) using fluorescent *in situ* hybridization and the genus and cluster specific probes for *Pseudoalteromonas* (Pukall et al. 1999) and Class Flavobacteria (Manz et al. 1996), demonstrated that this genus and class were both major contributors to the bacterial community in the estuary during the algal bloom periods.

Achieving a bacterial density high enough to induce lysis of dinoflagellate vegetative cells would be possible if algicidal bacteria were to dominate the bacterial population in the Huon Estuary. Attachment of bacteria to marine snow or algal cells also creates a zone of high bacterial numbers and thus, a greater likelihood of more effective algicidal activity. Highly diffusible compounds such as the algicidal components could become rapidly diluted out. However, in the context of a bloom situation in the estuary this is not as significant when algae and bacteria are closely confined. As *Gymnodinium catenatum* blooms generally occur in a stable water column in the Estuary (CSIRO Huon Study Team 2000), this lowers the rate of bacterial cells being washed away. The tidal pull of the lower marine water and the flow of the river effectively recirculates the water within the estuary during summer and autumn, and this in turn keeps high levels of dinoflagellates within the estuary (CSIRO Huon Study Team 2000). *G. catenatum* in particular generally only blooms when the estuary is well stratified and highly stable (CSIRO Huon Study Team 2000). Therefore, it is realistic that the bacteria could stay in the vicinity of the bloom despite the diurnal migration of *G. catenatum* up and down through the water column.

The swarming capacity and attack of algal cells by ACEM 1 was first observed by Lovejoy et al. (1998) and although not an unusual bacterial ability, the swarming attacks of ACEM 21 and ACEM 1 observed in this study were physically aggressive in comparison with those by ACEM 20, ACEM 4 and ACEM 32. Rapid movement in isolates such as ACEM 21 may also enable the bacteria to more effectively access nutrients in the marine environment and capitalise on patchy nutrient availability.

Algicidal compounds

Structural elucidation of algal lytic compounds has remained elusive for over 20 yr, despite researchers attempting their isolation, purification and characterisation (Baker & Herson 1978, Dakhama et al. 1993, Doucette et al. 1998). Part of the difficulty in isolation was the highly polar nature of the compounds and the loss or degradation of the bioactive components when trying to concentrate them or form derivatives. Algicidal compounds and their characteristics vary markedly across the many species of algicidal bacteria (Doucette et al. 1998). The one similarity was that all were highly polar, making them readily diffusible in the marine environment. The compounds can be heat labile (Baker & Herson 1978) or heat tolerant (Dakhama et al. 1993), of a large (Lee et al. 2000) or small molecular weight (Fukami et al. 1992, Dakhama et al. 1993), made up of a number of compounds or only one, as well as being species specific or non-specific in their mode of action (Doucette et al. 1998). The algicidal compounds in ACEM 1 were small molecular weight (<300 amu), highly polar and heat stable. Algicidal compounds in the other 5 species in this study were also heat tolerant, although all lost activity if left for an extended period of time.

The extracted pigments of the algicidal bacteria were not the algicidal component. Non-pigmented colonies in this study exhibited algicidal activity; however, they were capable of reverting back to their original pigmented state. By contrast, the unpigmented mutants in the study of Egan et al. (2002) were not algicidal but they remained unpigmented. Pigment formation may therefore be associated with algicidal activity, but it does not have to be expressed for the algicidal activity to exist.

CONCLUSION

For control of algal blooms such as *Gymnodinium catenatum*, the vegetative cells of this species could be affected in the natural environment by high numbers of algicidal bacteria either by natural means or via human intervention. In practice, many toxic marine algal blooms, unlike freshwater cyanobacterial blooms, occur in open ocean areas and estuaries which are not easily confined. However, the discovery of algicidal bacteria in the marine environment supports increasing recognition that marine bacteria can produce complex secondary metabolites and/or their derivatives. As research continues, it is possible that algicidal species will be found to be more widespread and common in other bacterial genera. The isolation of 5 algicidal species, 3 of which were novel, is beneficial for increasing knowledge of bacteria with bioactive potential in the marine environment. Future studies on understanding whether algicidal or bioactive mechanisms switch on and off with different environmental triggers, would be a valuable contribution to knowledge of the ecology of microbial systems.

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