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Phylogenetic and functional diversity of the cultivable bacterial community associated with the paralytic shellfish poisoning dinoflagellate *Gymnodinium catenatum*

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Abstract

Gymnodinium catenatum is one of several dinoflagellates that produce a suite of neurotoxins called the paralytic shellfish toxins (PST), responsible for outbreaks of paralytic shellfish poisoning in temperate and tropical waters. Previous research suggested that the bacteria associated with the surface of the sexual resting stages (cyst) were important to the production of PST by *G. catenatum*. This study sought to characterise the cultivable bacterial diversity of seven different strains of *G. catenatum* that produce both high and abnormally low amounts of PST, with the long-term aim of understanding the role the bacterial flora has in bloom development and toxicity of this alga. Sixty-one bacterial isolates were cultured and phylogenetically identified as belonging to the Proteobacteria (70%), Bacteroidetes (26%) or Actinobacteria (3%). The Alphaproteobacteria were the most numerous both in terms of the number of isolates cultured (49%) and were also the most abundant type of bacteria in each *G. catenatum* culture. Two phenotypic (functional) traits inferred from the phylogenetic data were shown to be a common feature of the bacteria present in each *G. catenatum* culture: firstly, Alphaproteobacteria capable of aerobic anoxygenic photosynthesis, and secondly, Gammaproteobacteria capable of hydrocarbon utilisation and oligotrophic growth. In relation to reports of autonomous production of PST by dinoflagellate-associated bacteria, PST production by bacterial isolates was investigated, but none were shown to produce any PST-like toxins. Overall, this study has identified a number of emergent trends in the bacterial community of *G. catenatum* which are mirrored in the bacterial flora of other dinoflagellates, and that are likely to be of especial relevance to the population dynamics of natural and harmful algal blooms.
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1. Introduction

Gymnodinium catenatum (Graham) is an unarmoured (naked) marine dinoflagellate responsible for paralytic shellfish poisoning (PSP) in temperate and tropical waters off all major continents [1]. It is the only naked dinoflagellate known to produce a suite of potent neurotoxins, the saxitoxins (STX) and related derivatives, collectively termed paralytic shellfish toxins (PSTs). Outbreaks of PSP in the human population result from blooms of *G. catenatum* being ingested by filter-feeding bivalves that accumulate the PSTs. Human consumers of the PST-contaminated shellfish suffer symptoms ranging from tingling of the lips through to respiratory failure in extreme cases. The worldwide distribution and incidence of *G. catenatum* and other harmful algal blooms (HABs) has increased markedly in the past few decades [2,3] fuelling significant interest in the factors that influence the growth and toxicity of HAB species.

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There is considerable debate surrounding the 'true' source of the PST produced by *G. catenatum*, *Alexandrium* spp. and *Pyrodinium bahamense* var. *compressum*, particularly the role that bacteria may play in bloom toxicity. A bacterial source of PST production was first put forward by Silva et al. [4], who described the presence of intracellular bacteria in PST-producing dinoflagellates. Later, bacteria associated with these PST-producing dinoflagellates were also shown to produce PST-like toxins [5]. Subsequent studies indicate that bacteria may potentially influence PST content or production of the algal culture in several ways: autonomous production of PST-like toxins (e.g. [6]); modulating the toxicity of their host [7–9]; biotransformation of the PST derivatives [10]; or possibly a combination of these factors. The specific mechanisms of interaction are currently unknown, although the relationship between toxin production and the bacterial flora may well be manifested at a much broader community level, through the production of stimulatory or inhibitory factors that may induce or repress toxin production [11].

Bacteria have a potentially more profound role in the development of HAB events, beyond just their potential effects on toxin production (for review see [12]). They are able to exert considerable influence on bloom population dynamics through 'bacterial–algal' interactions, for example positive stimulation of growth [13–15], promotion of sexuality [16], antagonism mediated via the production of algicidal factors (e.g. [17]) and inhibition of cyst formation [18], and protective effects associated with an existing microflora insulating the algal host from antagonistic bacterial activity [19].

Our interest in the bacteria associated with *G. catenatum* stems from the observation that as part of its sexual cycle, *G. catenatum* produces sexual resting cysts [20] that, when germinated in the laboratory setting, often produce 'atypical' cultures which express abnormally low amounts of PST per cell (Table 1). We postulated that the process of sonicating and sterile washing during isolation was likely to remove much of the cysts' surface-associated bacteria, and that after laboratory germination the reduced bacterial diversity may affect the cells ability to produce 'normal' levels of PSTs [21]. Preliminary evidence showed that these atypical PST-producing cultures had a reduced bacterial diversity compared to their toxic counterparts [21].

To examine the potential link between the bacterial flora on the cyst surface and resulting *G. catenatum* culture toxicity, we compared the cultivable bacterial flora from a range of *G. catenatum* strains – representative of both typical and atypical PST-producing cultures from a variety of geographic populations (Table 1). Our main objectives were to: (1) document the typical bacterial flora of this dinoflagellate; (2) assess the phylogenetic, and by inference from this, the functional diversity of the bacteria associated with typical and atypical PST-producing cultures; and (3) examine the function that specific bacteria might have in both the lifecycle and toxicity of *G. catenatum*. To address these questions, small subunit rDNA (SSU rDNA) sequencing was used to establish the phylogenetic affiliations of all cultivable bacteria, and subsequently, we examined the ability of bacteria to grow photoheterotrophically, to utilise hydrocarbons and to autonomously produce PST.

2. Materials and methods

2.1. Algal culture

The *G. catenatum* strains used in this study (Table 1) were grown at 18°C in 25 cm^2 tissue culture flasks (Nunc, Norway) in GSe medium [22] with a photon flux density of 50–70 μmol m^{-2} s^{-1} from cool-white fluorescent lighting (Phillips, The Netherlands) with a 12:12 h light:dark photoperiod. All cultures were handled aseptically to prevent bacterial contamination and cross-contamination between cultures.

2.2. Bacterial culture

Bacteria were isolated and maintained on a modified marine agar (ZM/10) prepared with 75% aged filtered (1.0 μm pore size) natural seawater, 0.05% bacto-peptone (Difco, USA), 0.01% yeast extract (Difco) and 1.5% bacto-agar (Difco), supplemented following autoclaving with sterile trace elements and vitamins at the same concentrations as used in GSe medium [22]. Zobells 2216E marine agar and broth supplemented with GSe trace elements and vitamins (ZM/1) were used as necessary.

Bacterial isolation was performed by harvesting 1 ml of a late-logarithmic phase *G. catenatum* culture by brief centrifugation (12 000 × *g* for 10 s). The spent medium was removed and the algal cell pellet resuspended in 100 μl of sterile seawater and vortexed. The cell suspension was diluted 10-fold and 100 μl of each dilution spread onto ZM/10 agar plates and incubated in the dark at 18°C for 3 weeks. Bacterial colonies with distinct colony morphology were picked (and the number of that colony morphology counted) and serially passaged on ZM/10 agar until the purity of the isolate was assured. Bacterial isolates were grown in ZM/10 or ZM/1 broth, glycerol was added (20% v/v), and the cells were stored at −80°C.

The ability of bacterial strains to utilise aliphatic hydrocarbons as a sole carbon source was demonstrated following the growth of bacteria on a synthetic seawater agar SM1 [23] prepared with deionised distilled water and supplemented with sodium nitrate or ammonium nitrate as the nitrogen source. Hydrocarbons, *n*-tetradecane or *n*-hexadecane (Sigma-Aldrich, UK), were supplied in the vapour phase by adding 200 μl to a sterile filter pad in the lid of the Petri dish. Growth was examined after 3 weeks of incubation at 25°C.

Table 1
The geographic origin and the PST content of the *G. catenatum* strains examined in this study

Strain name	Total PST content (fmol cell^{-1})	Isolation source	Culture collection[a]
Typical PST content:			
CAWD101	n.d.[b]	Kaitaia, New Zealand (2000)	Cawthron Institute (NZ)
GC21V	244	Ria de Vigo, Spain (1986)	CCMP (USA)
GCDE08	189	Derwent Estuary, Tasmania (1987)	CSIRO (Australia)
YC499B15	316	Yellow Sea, Korea (1998)	T.G. Park (Korea)
Atypical PST content:			
GCHU11	4	Huon Estuary, Tasmania (1988)	CSIRO (Australia)
GCJP01	b.d.[c]	Seto Inland Sea, Japan (1985)	CSIRO (Australia)
GCTRA14	3	Spring Bay, Tasmania (1993)	CSIRO (Australia)

[a]Algal culture collection from which strains analysed in this study were acquired.
[b]Not determined.
[c]Below detection limit.

2.3. Toxin analysis

Analysis of the ability of bacterial strains to autonomously produce STX and PST-like toxins was conducted on bacterial cell pellets harvested from 100 ml of ZM/1 broth grown in the dark for 48 h at 25°C on an orbital shaker (160 rpm). Cell pellets were resuspended in 0.05 N acetic acid, sonicated, and cell debris removed by centrifugation and 0.2 μm membrane filtration prior to being assayed with the saxiphilin and rat brain sodium channel assays [24].

Analysis of the PST content of *G. catenatum* cultures was by liquid chromatography fluorescence detection of the C-toxins, gonyautoxins and STX as described by Negri and Llewellyn [25], and detection of the GC-toxins as described by Negri et al. [26].

2.4. DNA manipulation

Bacterial genomic DNA was extracted using a method based on cetyltrimethylammonium bromide purification (CTAB) [27]. The polymerase chain reaction (PCR) was used to amplify the SSU rDNA gene from chromosomal DNA using the primer pair 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT) [28]. PCR was carried out on an MJ Research PTC200 DNA Engine thermocycler and used 1 U of *Taq* polymerase (ABgene, UK) in a 50 μl reaction containing a final concentration of 1.8 mM Mg^{2+}, 20 mM NH_4SO_4, 75 mM Tris–HCl (pH 8.8) and 0.01% Tween 20. Cycling parameters were as follows: 94°C for 2 min; 26 cycles of 55°C for 30 s, 72°C for 2.5 min and 94°C for 10 s; followed by 72°C for 10 min. The PCR products were purified through Centricon-PCR ultrafilters (Millipore, UK) according to the manufacturer's instructions and sequenced in both directions using 27F and 1492R primers and ABI-Prism 'Big-Dye' terminator chemistry (Applied Biosystems, USA) according to the standard protocols. Sequence reactions were electrophoresed on an ABI 377 DNA sequencer (Applied Biosystems), and resulting sequences aligned and manually checked for consistent base-calling, using Sequence Navigator (Ver. 1.0.1, Applied Biosystems, USA).

Detection of *pufLM* photosynthetic reaction centre genes in bacteria was carried out by PCR amplification with the forward (5′-CTKTTCGACTTCTGGGTSGG-3′) and reverse (5′-CCATSGTCCAGCGCCAGAA-3′) primers as described by Béja et al. [29]. PCR reactions contained 1.0 U/50 μl of a 7:1 (U/U) mixture of *Taq* and *Pfu* polymerase (Promega, UK) in a reaction containing a final concentration of 2 mM Mg^{2+}, 20 mM NH_4SO_4, 75 mM Tris–HCl (pH 8.8) and 0.01% Tween 20. Cycling was carried out using an MJ PTC200 DNA Engine thermocycler as follows: 94°C for 2 min; 30 cycles of annealing between 48 and 58°C for 30 s, 72°C for 2.5 min, 94°C for 10 s; followed by 72°C for 10 min. PCR products of ca. 1500 bp were purified through Centricon-PCR filters, A-tailed, ligated into pGEM-T Easy (Promega, UK) and transformed into *Escherichia coli* XL1-Blue (Stratagene, The Netherlands). Selected clones were sequenced in both directions using the primers ARD-F (GCCATGGCGGCCGCGGGAATT) and ARD-R (AGGCGGCCGCGAATTCACTAG) that bind immediately adjacent to the pGEM-T Easy cloning site. DNA sequencing and sequence analysis were carried out as outlined above.

2.5. Genetic identity and phylogenetic inference

SSU rDNA sequences were aligned with representative SSU rDNA sequences of other bacteria using the software programme Clustal X [30] and were again checked for any potential sequencing errors. Genetic identity was determined using Ribosomal Database Project II (RDP II) Sequence Match [31]. Genetic identity was in most instances assigned to the closest taxonomically described bacterial sequence in the RDP II and listed in Table 2 according to Bergey's taxonomic outline of the prokaryotes [32].

Phylogenetic inference was performed on visually corrected alignments using PAUP 4.0* [33]. All ambiguous alignment positions were masked from the analysis. The method of maximum likelihood (ML) using the estimated rates of transition/transversion and α-shape parameters

Table 2
Phylogenetic affiliation of the bacteria isolated from *G. catenatum* and their percentage dominance in the culture from which they were isolated

Strain no. and taxonomic affiliation	Accession no.	CFU[a] (%)	Closest relative in the RDP II[b] Species	Accession no.	ID (%)	*G. catenatum* strain
Proteobacteria (Alpha)						
Rhizobiales						
Phyllobacteriaceae						
DG943	AY258089	7.2	*Mesorhizobium amorphae*	AF041442	96.4	GCJP01
DG1023	AY258096	20.7	*Mesorhizobium chacoense*	AJ278249	94.2	CAWD101
Rhodobiaceae						
DG948	AY258091	3.6	*Rhodobium orientis*	D30792	90.8	GCJP01
Rhodobacterales						
Rhodobacteraceae						
DG869	AY258074	61.2	*Roseobacter gallaeciensis*	Y13244	93.8	GCHU11
DG874	AY258075	2.0	*Roseobacter* sp.	AF098495	92.6	GCDE08
DG877	AY258076	32.9	unidentified alphaproteobacterium	AB018689	99.8	GCDE08
DG878	AY258077	4.6	*Roseobacter* sp.	AF098495	92.5	GCDE08
DG882	AY258078	39.4	*Ruegeria gelatinovorans*	D88523	95.0	GCDE08
DG885	AY258079	0.8	*Sulfitobacter mediterraneus*	Y17387	94.5	GCDE08
DG888	AY258080	65.1	*Roseobacter* sp.	AF098495	92.0	YC499B15
DG889	AY258081	13.1	*Roseobacter litoralis*	X78312	94.4	YC499B15
DG891	AY258082	0.7	*Stappia aggregata*	D88520	98.1	YC499B15
DG895	AY258084	0.1	*Hyphomonas johnsonii*	AF082791	98.4	YC499B15
DG897	AY258085	6.5	*Roseobacter* sp.	AF098495	92.4	YC499B15
DG898	AY258086	0.4	*Ruegeria algicola*	X78315	97.8	YC499B15
DG941	AY258087	7.2	*Roseobacter* sp.	AF098495	92.6	GCJP01
DG942	AY258088	46.9	*R. litoralis*	X78312	94.5	GCJP01
DG944	AY258090	18.1	*Roseovarius tolerans*	Y11551	94.2	GCJP01
DG981	AY258094	39.3	unidentified alphaproteobacterium	AJ002565	94.2	GCTRA14
DG1020	AY258095	41.4	*S. mediterraneus*	Y17387	98.8	CAWD101
DG1127	AY258099	32.2	*Roseobacter* sp.	AF098495	96.0	GC21V
DG1128	AY258100	48.3	*Ruegeria atlantica*	AF124521	92.4	GC21V
DG1132	AY258102	5.6	*R. litoralis*	X78312	93.1	GC21V
DG1133	AY258103	0.8	unidentified alphaproteobacterium	AB018689	99.8	GC21V
Rhodospirillales						
Rhodospirillaceae						
DG949	AY258092	10.8	*Azospirillum doebereinerae*	AJ238567	86.3	GCJP01
DG1026	AY258098	4.1	unclassified			CAWD101
DG1131	AY258101	2.4	*Thalassospira lucentensis*	AF358664	89.0	GC21V
Sphingomonadales						
Sphingomonadaceae						
DG892	AY258083	0.1	*Sphingopyxis alaskensis*	AF145754	98.4	YC499B15
DG978	AY258093	32.7	*Blastomonas ursincola*	AB024289	92.3	GCTRA14
DG1024	AY258097	8.3	*B. ursincola*	AB024289	92.9	CAWD101
Proteobacteria (Gamma)						
Alteromonadales						
Alteromonadaceae						
DG870	AY258106	20.4	*M. hydrocarbonoclasticus*	AB019148	95.5	GCHU11
DG879	AY258107	13.1	*M. hydrocarbonoclasticus*	AB019148	95.5	GCDE08
DG893	AY258110	0.1	*M. hydrocarbonoclasticus*	AB019148	95.7	YC499B15
DG979	AY258112	14.7	*M. hydrocarbonoclasticus*	AB019148	95.1	GCTRA14
DG980	AY258113	8.2	*M. hydrocarbonoclasticus*	AB019148	95.2	GCTRA14
DG1022	AY258114	8.3	*Alteromonas macleodii*	Y18228	92.2	CAWD101
DG1135	AY258115	0.8	*Pseudoalteromonas luteoviolacea*	X82144	96.3	GC21V
DG1136	AY258116	0.1	*M. hydrocarbonoclasticus*	AB019148	95.2	GC21V
Oceanospirillales						
Oceanospirillaceae						
DG940	AY258111	3.6	*Oceanobacter kriegii*	AB006767	90.4	GCJP01
Alcanivoraxaceae						
DG812	AY258104	0.2	*Alcanivorax borkumensis*	Y12579	99.4	CAWD101
DG813	AY258105	0.3	*A. borkumensis*	Y12579	99.4	YC499B15
DG881	AY258109	0.7	*A. borkumensis*	Y12579	99.5	GCDE08
Pseudomonadales						
Moraxellaceae						
DG880	AY258108	0.7	*Acinetobacter lwoffii*	X81665	99.3	GCDE08

Table 2 (*Continued*).

Strain no. and taxonomic affiliation	Accession no.	CFU[a] (%)	Closest relative in the RDP II[b] Species	Accession no.	ID (%)	*G. catenatum* strain
Bacteroidetes						
Flavobacteriales						
Flavobacteriaceae						
DG868	AY258117	7.1	*Zobellia* sp.	AF530137	96.3	GCHU11
DG886	AY258120	0.7	*Zobellia* sp.	AF530137	96.3	GCDE08
DG945	AY258123	1.1	*Aequorivita antarctica*	AY027804	89.1	GCJP01
DG975	AY258125	1.6	*Zobellia* sp.	AF530137	93.7	GCTRA14
DG976	AY258126	0.2	marine psychrophile	U85882	96.8	GCTRA14
DG977	AY258127	3.3	*Zobellia uliginosa*	M62799	85.8	GCTRA14
DG1025	AY258129	4.1	*Polaribacter franzmannii*	U14586	92.3	CAWD101
DG1027	AY258130	8.3	*Arenibacter latericius*	AF052742	91.8	CAWD101
DG1030	AY258132	0.2	*Zobellia* sp.	AF530137	92.3	CAWD101
DG1134	AY258134	1.6	*Cellulophaga fucicola*	AJ005973	93.2	GC21V
Sphingobacteriales						
Flexibacteraceae						
DG873	AY258118	11.2	*Microscilla furvescens*	AB078079	91.3	GCHU11
DG887	AY258121	0.7	*Cyclobacterium* sp.	AJ244689	91.8	YC499B15
DG890	AY258122	13.1	unidentified bacterium	AJ224942	84.7	YC499B15
DG946	AY258124	1.4	*M. furvescens*	AB078079	96.5	GCJP01
DG1021	AY258128	4.1	unidentified bacterium	AF087043	85.7	CAWD101
DG1129	AY258133	8.1	*Flexibacter aggregans*	AB078038	88.0	GC21V
Actinobacteria						
Actinomycetales						
Micrococcaceae						
DG876	AY258119	5.3	*Micrococcus luteus*	AF057289	99.2	GCDE08
Nocardioidaceae						
DG1029	AY258131	0.4	*Nocardioides* sp.	U61298	91.4	CAWD101

[a]Relative abundance (percentage) of each bacterial isolate in the *G. catenatum* culture from which it was isolated.
[b]Named species are based on the most similar named SSU rDNA sequence listed on the RDP II [31]. The percentage identity and accession number of the closest species is shown alongside.

(estimated from the data set following heuristic searching) were used to infer each phylogeny. Bootstrap support for each inferred tree was established following resampling of 1000 data sets based on neighbour-joining analysis [34].

3. Results

3.1. Strain characterisation

A total of 61 distinct bacteria spanning three phyla were cultured from the seven strains of *G. catenatum* (Tables 2 and 3). Thirty (49%) of the bacterial strains were affiliated with the Alphaproteobacteria, of which 21 (34%) were affiliated within the Rhodobacteraceae, with one or more isolates of this family identified in all seven *G. catenatum* cultures. Thirteen (21%) isolates were affiliated with the Gammaproteobacteria, with each *G. catenatum* culture having one or more Gammaproteobacteria. Of this group, eight isolates were affiliated to the Alteromonadaceae, cultured from six of the seven *G. catenatum* cultures. The remaining isolates came from two phyla, the Bacteroidetes (26%) and the high G+C% Gram-positive Actinobacteria (3%) (Table 3).

From cross-referencing of colony counts and SSU rDNA data, the cultivable bacterial flora of the *G. catenatum* cultures examined was shown to be dominated by the Alphaproteobacteria (Table 2). In all *G. catenatum* cultures examined, a single member of the Rhodobacteraceae was the most abundant bacterium, accounting for between 39 and 65% of the cultivable flora. The relative abundance of any one individual Alphaproteobacteria ranged considerably (0.1–65%), averaging 19%, while the average culture abundance of the Rhodobacteraceae was 22%. The abundance of Gammaproteobacteria and Bacteroidetes in *G. catenatum* cultures averaged 5 and 4% respectively.

The phylogenetic relationship of the cultivable strains was compared to their closest SSU rDNA affiliates and selected bacterial type species (Figs. 1, 2 and 3). Bootstrap support for higher order branching between Alphaproteobacteria lineages (Fig. 1) was typically low. While this implies uncertainty, maximum parsimony analysis did nevertheless infer a similar tree to the ML analysis. Bootstrap support for the branching order of both the Gammaproteobacteria and Bacteroidetes/Actinobacteria ML was high. A significant number of the bacterial strains (39%) had less than 93% SSU rDNA identity and only a

relative few (18%) exceeded an identity of 97% to their closest relative. Phenotypic characteristics such as cell morphology, pigmentation, motility, catalase and oxidase activity were recorded (data not shown) and, for most novel genera and species, these tests were consistent with the family or genus to which they showed highest phylogenetic affiliation.

A number of the bacterial strains isolated were phylogenetically closely related to one another, while having originated from *G. catenatum* cultures from different parts of the world (see Figs. 1 and 2). For example, strains of *Alcanivorax* sp. were isolated from *G. catenatum* cultures originating from New Zealand, Australia and Korea (Table 2). All three of these *G. catenatum* cultures had been isolated and maintained in exclusion of each other (Table 1) up to the point of this study. Another distinct group was a specific clade of *Roseobacter–Roseovarius*-like strains (DG874, 878, 888, 897, 941, 944 and 1127; Fig. 1) that originated from *G. catenatum* cultures isolated from the sea areas as separate as Australia, Korea, Japan and Spain. In addition, many strains were also closely related to bacteria identified in association with other dinoflagellates (denoted with an asterisk; Figs. 1, 2 and 3) such as the PST-producing *Alexandrium tamarense*, *Alexandrium lusitanicum* and *Alexandrium affine*, the diarrhetic shellfish poisoning (DSP) *Prorocentrum lima*, and the non-toxic dinoflagellate *Scrippsiella trochoidea*. These similarities were especially evident among the dinoflagellate-derived strains belonging to the Rhodobacteraceae (Fig. 1) and Alteromonadaceae (Fig. 2) families.

3.2. Bacterial diversity of atypical PST-producing G. catenatum

One of the specific aims of this work was to understand if atypical PST-producing *G. catenatum* cultures had a microbial flora distinct from that of toxic *G. catenatum* cultures. The data presented in Table 3 show that of the three atypical PST-producing *G. catenatum* cultures analysed here, there were in two instances a reduction in the number of strains cultivable. Bacterial isolation from other atypical PST-producing *G. catenatum* cultures showed that lower numbers of cultivable bacteria were typical (data not shown). The number of bacteria isolated from two of the three atypical PST-producing *G. catenatum* cultures showed a reduction in the Alpha- and Gammaproteobacteria diversity of these cultures, as compared to typical PST-producing *G. catenatum* (Table 3). The numbers of Bacteroidetes isolated remained approximately the same (Table 3), but while this appeared to make them a relatively more dominant feature of these cultures, their relative abundance (colony-forming units (CFU)) in the atypical PST-producing *G. catenatum* cultures (5%) was not obviously different to that in the typical PST-producing cultures (3%).

3.3. Bacterial phenotypes

Extrapolation from the phylogenetic data showed that a number of the strains isolated may have belonged to one or two phenotypic groups: the aerobic anoxygenic photosynthetic (AAP) Alphaproteobacteria, and Gammaproteobacteria capable of hydrocarbon utilisation. Phenotypic characterisation showed that the 13 (67%) of the 21 Rhodobacteraceae isolates were potentially capable of AAP as evidenced by the presence of the conserved photosynthetic reaction centre genes *pufLM*, and in most cases pink-red colony pigmentation following aerobic incubation in the dark, which is indicative of the production of bacteriochlorophyll *a* (Fig. 1). Atypical responses were demonstrated by two isolates (DG874 and 878) that only produced a pink-red pigmentation when grown for at least 4 weeks on a rich medium in the dark. Three isolates were not observed to have any pigmentation (DG869, 882 and 1128), but had *pufLM* genes that could be detected by the PCR. The bacterium DG943 (*Mesorhizobium* sp.) was the only isolate not phylogenetically affiliated to the Rhodobacteraceae, that was capable of AAP, as shown by its pink colony pigmentation following dark, aerobic incubation, and PCR detection of *pufLM* (Fig. 1). Although no AAP Alphaproteobacteria was cultured

Table 3
Summary of the cultivable bacteria identified in each *G. catenatum* culture

G. catenatum strain	Total (*n*)	Number of isolates per lineage			
		Alphaproteobacteria	Gammaproteobacteria	Bacteroidetes	Actinobacteria
Typical PST content:					
CAWD101	11	4	2	4	1
GC21V	9	5	2	2	–
GCDE08	10	5	3	1	1
YC499B15	11	7	2	2	–
Atypical PST content:					
GCHU11	4	1	1	2	–
GCJP01	9	6	1	2	–
GCTRA14	7	2	2	3	–
Total (*n*)	61	30	13	16	2

from *G. catenatum* CAWD101, the PCR was able to detect the *pufLM* genes from the total DNA extracted from *G. catenatum* CAWD101 culture (data not shown). The PCR was used to screen all Gammaproteobacteria isolates for the gene coding for the light-harvesting proteorhodopsin protein [35]. No PCR product was detected in any of the isolates tested (data not shown).

Bacteria with the potential to utilise aliphatic hydrocarbons as a carbon source were identified in the majority of the *G. catenatum* strains examined. Members of the Gammaproteobacteria, with close phylogenetic affiliations to either *Alcanivorax borkumensis* SK2 or *Marinobacter hydrocarbonoclasticus* ATCC 27132, were isolated from six of the seven *G. catenatum* cultures. All three *Alcanivorax* isolates and four of the six *Marinobacter* isolates were shown to be able to utilise *n*-tetradecane and *n*-hexadecane as the sole carbon source. While the remaining *Marinobacter* isolates grew poorly or insufficiently different to the control (no carbon source added) to enable accurate assessment of their growth potential (Fig. 2). All of the Alphaproteobacteria were unable to utilise hydrocarbon as a sole carbon source (data not shown). Unexpectedly, all Alteromonadaceae and *Alcanivorax* were capable of growth on the synthetic seawater agar without any additional carbon source (Fig. 2). Agarolytic activity (colony-sized, shallow depressions ≤ 1 mm) and subsurface growth were observed in all of *Alcanivorax* and *Marinobacter* isolates. Both *Alteromonas* sp. DG1022 and *Pseudoalteromonas* sp. DG1135 grew in the absence of a carbon source but with no evidence of agarolytic activity or subsurface growth.

The bacteria cultured from two strains of *G. catenatum* (GCDE08 and GCHU11) were analysed for the ability to autonomously produce PST-like toxins. All of the nine bacterial strains (DG869, 870, 874, 877, 878, 880, 881, 882 and 885) tested for STX/PST showed no evidence for sodium channel-blocking activity in any of the cell pellets examined (data not shown).

4. Discussion

This study aimed to comprehensively document the bacterial flora associated with the PST-producing marine dinoflagellate *G. catenatum* as a first step to understanding the influence of bacteria on algal growth, physiology and toxicity, and their role in the development of HABs. We have initially focussed on the cultivable bacterial flora so that future research investigating the biochemistry, physiology and genetics of the bacterial–algal interactions can utilise characterised, cultivable organisms [36]. Our previous work had suggested that the bacterial flora may influence the expression of PST production in *G. catenatum*, we therefore chose to examine the bacterial diversity of *G. catenatum* cultures that produce abnormally low amounts of PST.

4.1. Bacterial community composition

The phylogenetic characterisation of the cultivable bacteria isolated from the seven *G. catenatum* cultures demonstrated that there were a number of emergent trends across the *G. catenatum* examined. In summary, the Alphaproteobacteria dominated the strains isolated, and an individual Alphaproteobacteria (Rhodobacteraceae) was always the most numerically abundant bacterium present in each culture (Table 2). Half of all of the Alphaproteobacteria isolated were capable of a mode of photosynthetic growth, termed AAP ([37] and references therein). The second trend was for there to be cultivable oligotrophic and/or hydrocarbon-degrading Gammaproteobacteria present in almost all of the cultures. And thirdly, one or more cultivable isolates belonging within the Flexibacteraceae or Flavobacteriaceae families of the Bacteroidetes were always present in each culture.

The bacterial flora of *G. catenatum* generally mirrors that found associated with other dinoflagellates, being dominated by the Alphaproteobacteria (principally the Rhodobacteraceae – frequently referred to as *Roseobacter* clade; e.g. [38]). For example, 50% of all phylotypes identified in four *Pfiesteria* sp. cultures were affiliated with the Alphaproteobacteria, with the Rhodobacteraceae *Rg. algicola* and *Hyphomonas jannaschiana*-like bacteria among the most numerous of these phylotypes [15]. Rhodobacteraceae were also a dominant feature of the bacterial flora associated with the DSP-producing dinoflagellate *P. lima* [39], and from which the association *Rg. algicola* was originally described [40]. The bacterial flora of *Alexandrium* spp. and *S. trochoidea* cultures were also dominated by Alphaproteobacteria, with the *Roseobacter* clade dominating both the cultivable species and ribotype clones identified [38]. Like *G. catenatum*, members of the *Alteromonadaceae* (*Marinobacter* and *Alteromonas*) were consistently identified in other dinoflagellate cultures [14,15,38,39,41].

The high incidence of Alphaproteobacteria associated with algae does not appear to be restricted to the dinoflagellates, as Alphaproteobacteria, primarily Rhodobacteraceae, were always identified in association with each of six different species of diatom culture [42]. Bacterial culture from the domoic acid-producing pennate diatoms, *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia seriata* and non-toxic *Pseudo-nitzschia delicatissima*, consistently identified one or more Alphaproteobacteria associated with each of these cultures (D. Green, J. Fehling and S. Bates, unpublished data).

A striking feature of the bacterial flora of *G. catenatum* was the high degree of genetic similarity of members of the Alpha- and Gammaproteobacteria (Rhodobacteraceae and the Alteromonadaceae, respectively) compared to other dinoflagellates, particularly the PST-producing genus *Alexandrium* [38]. For example, *G. catenatum* bacterial isolate DG898 and *R. algicola* from *P. lima* [40] shared a sequence identity of 97.6% (Fig. 1) and DG893/DG1136

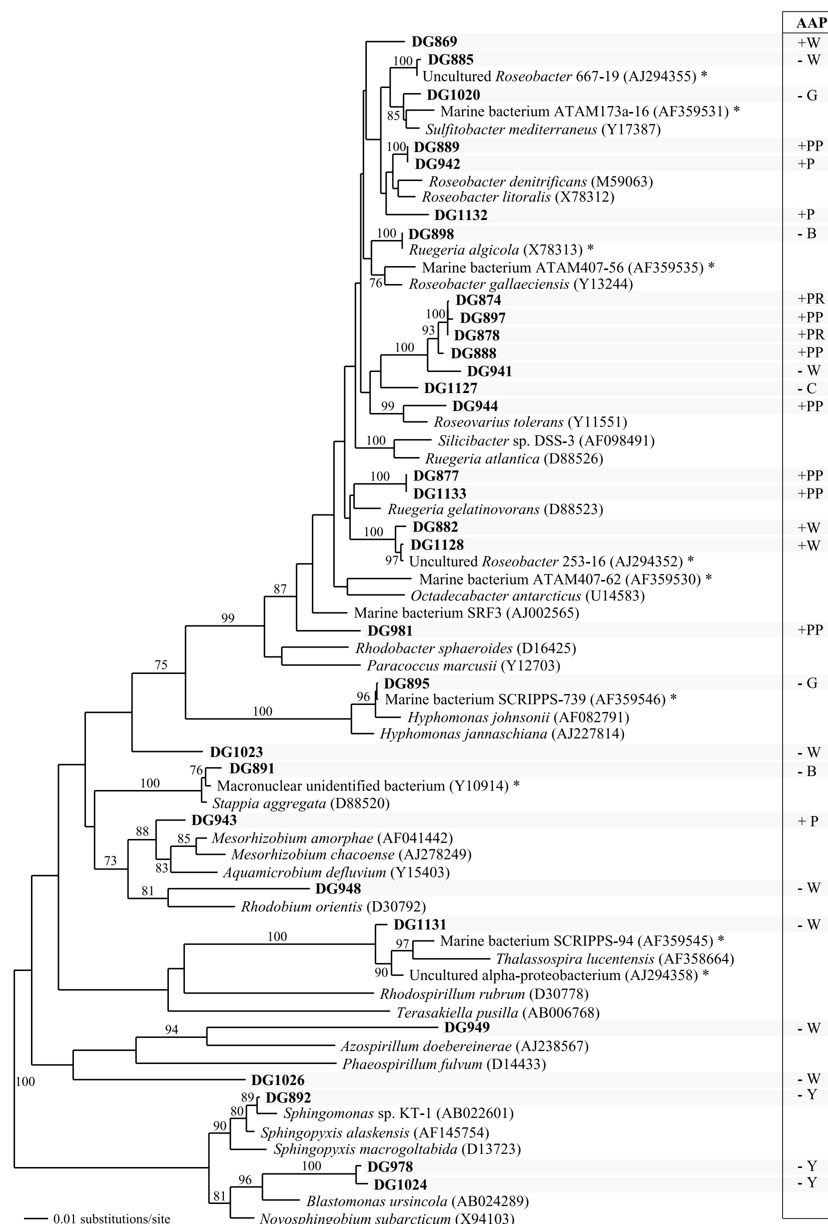

Fig. 1. Phylogenetic inference of the cultivable Alphaproteobacteria and AAP growth potential. ML neighbour-joining analysis of 1254 bp of SSU rDNA was used in the analysis (bootstrap support ≥70% is shown next to its respective branch). The scale bar indicates the number of nucleotide substitutions per site. Asterisks next to accession numbers denote that this bacterial sequence was isolated from a dinoflagellate other than *G. catenatum*. Right of the tree, illustrates which of the Alphaproteobacteria were potentially capable of AAP growth based on colony pigmentation following aerobic, dark growth and the PCR detection of the conserved photosynthetic reaction centre genes, *pufLM*. +, *pufLM* detected; −, *pufLM* not detected; B, beige; C, cream; G, grey; P, pink; PP, pale pink; PR, pale red; W, white; Y, yellow.

and *Marinobacter* sp. 407-13 from *A. tamarense* [38] all shared a sequence identity ≥99.5% (Fig. 2). Similarly, these groups also showed consistently high degree of genetic similarity between the *G. catenatum* cultures examined, even though the *G. catenatum* cultures had originated from separate geographic regions around the world.

The similarities of bacterial flora across different dinoflagellates (compare this study, [15,38,41]) have two potential explanations. Firstly, there are selective mechanisms operating in laboratory cultures [15,42] that favour genera from within the Rhodobacteraceae and Gammaproteobacteria, such as *Marinobacter* and *Alcanivorax*. The selection for specific populations of bacteria adapted to the utilisation of algal extracellular products [43] is one mechanism. Algal extracellular products such as dimethylsulphoniopropionate (DMSP) may well select for specific bacterial taxa such as the *Roseobacter* clade [44], of which the latter are recognised to utilise as a source of carbon and sulphur ([45] and references therein). *G. catenatum* is recognised, like many dinoflagellates, to be rich in fatty acids, sterols, lipids and oils [46], and this may explain the high frequency with which *Marinobacter* and *Alcanivorax* were

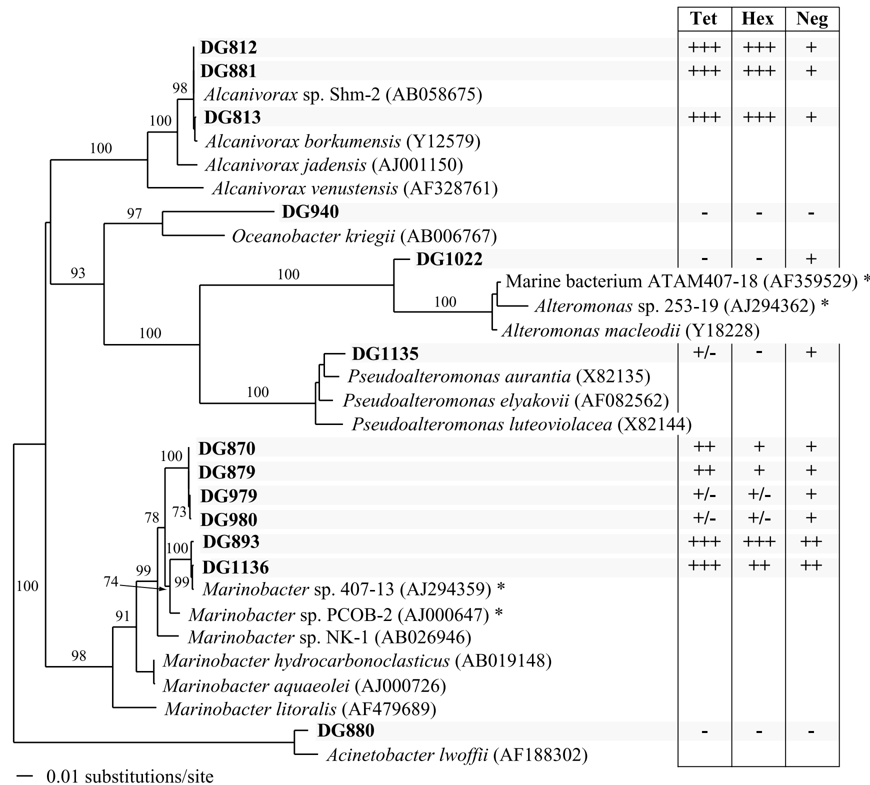

Fig. 2. Phylogenetic inference of the cultivable Gammaproteobacteria and hydrocarbon utilisation. ML neighbour-joining analysis was conducted on 1347 bp of aligned SSU rDNA (bootstrap support $\geq 70\%$ is shown next to its respective branch). Right of the tree, shows qualitative assessment of each bacterial strain's ability to utilise hydrocarbon as the sole carbon source. TET, *n*-tetradecane as carbon source; HEX, *n*-hexadecane as carbon source; NEG, no carbon source; +, ++, +++ indicate the degree of growth; ± possible growth; − no growth.

identified in *G. catenatum* cultures. Isolates of both genera were observed to be capable of utilising complex carbohydrates and hydrocarbons (Fig. 2), and *Alcanivorax* is regarded as a highly fastidious organism that can only utilise aliphatic hydrocarbons and a few fatty acids as a carbon source [23]. The high N and P concentrations used in algal media, or abiotic forces exerted by laboratory glassware and plastics used in the isolation and culture of algae, represent obvious departures from the natural environment, and thus may also contribute to the in vitro development of communities dominated by specific bacterial taxa.

The second explanation for the similarities in the bacterial community across *G. catenatum* cultures and with other dinoflagellates is that the bacteria from these groups may be of specific importance to the growth and physiology of dinoflagellate cells. Bacterial mineralisation of the algal extracellular products and phytodetritus is recognised as being an important part of the 'microbial loop', re-supplying algal cells with readily utilisable forms of C, N and P [47]. The supply of vitamins [48], chelated iron by bacterially produced siderophores [49], or the production of cytokinins [50] are examples where bacterially produced factors have been shown to stimulate algal growth. It may also be that the aerobic photoheterotrophs (AAP) identified in this study, which dominated the cultivable bacterial flora of *G. catenatum* cultures, may have a role in contributing energy to *G. catenatum* growth. This premise extends from the work which has shown that the aerobic photoheterotrophs are an abundant [29] and important component of the marine bacterioplankton community through their ability to contribute to carbon cycling in the oceans [51].

Three reports have identified specific bacteria as key components of the bacterial flora associated with the stimulation of dinoflagellate growth. Sakami et al. [13] demonstrated that several bacteria, one of which was identified as an *Alteromonas* sp., isolated from the cell surface of the ciguatera-producing dinoflagellate *Gambierdiscus toxicus*, stimulated growth of this dinoflagellate. A bacterium related to *Rg. algicola* was identified as especially important to enhancing the growth rate of axenic cultures of *Pfiesteria* sp. [15], and *Alteromonas* spp. were observed to be a fundamental part of the bacterial community responsible for growth enhancement of *Alexandrium fundyense* [14]. Importantly, these bacteria belong to the two bacterial families consistently encountered in *G. catenatum* and other dinoflagellate bacterial communities.

While it is recognised that selective effects may influence the bacterial community of the algal cultures we observed, similar patterns of bacterial association have been noted from field populations. Using fluorescent in situ hybridisation probes, an association was observed between the *Roseobacter* and *Alteromonas* clade's abundance and PST-

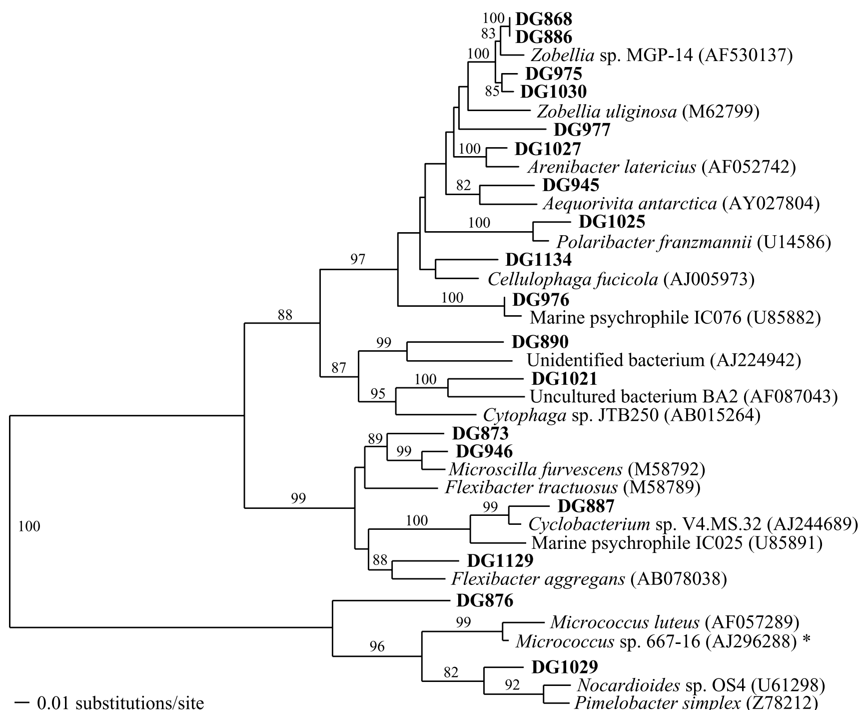

Fig. 3. Phylogenetic inference of the cultivable Bacteroidetes and Actinobacteria. ML neighbour-joining analysis was conducted on 1203 bp of aligned SSU rDNA (bootstrap support ≥70% is shown next to its respective branch).

producing *Alexandrium* spp. cell numbers in natural bloom populations [52]. Analysis of the associated bacterial population with a bloom of the dinoflagellate *Lingulodinium polyedrum* also demonstrated the presence of *Roseobacter* and *Marinobacter* in the free-living fraction and *Roseobacter* in attached bacterial population [53]. Several other field studies have reported the Rhodobacteraceae as a notable feature of the bacterioplankton community associated with mixed phytoplankton blooms [54–56]. These data support the idea that the specific bacterial–algal associations that we and others have observed, are not culture-induced artefacts, but are likely to be relevant and important in natural field populations.

4.2. Role of bacteria in PST production

The role bacteria have in dinoflagellate PST production and bloom toxicity is still an open question. There are several reports of autonomous PST production by bacteria isolated from toxic dinoflagellate cultures (e.g. [5,6,57]) and some have presented convincing chromatographic evidence of their similarity to PST compounds [6], but convincing structural data are still lacking. Recent studies have shown that some suspected PST-like compounds produced by bacteria are structurally unrelated and have been labelled as 'imposter' toxins [58,59].

Bacteria isolated from *G. catenatum* have previously been shown to autonomously produce compounds with activity similar to PSTs [57]. However, our analysis of nine bacterial strains isolated from typical (GCDE08) and atypical (GCHU11) PST-producing *G. catenatum* cultures, demonstrated no bacterium produced any compound with PST-like activity. This has also been confirmed during parallel studies of the bacteria isolated from GCDE08 and another *G. catenatum* strain, GCDE09 (S. Geier and A. Negri, unpublished data). While it is possible that we failed to induce these bacteria to produce PST-like toxins during growth, we did however use growth media and conditions known to elicit autonomous PST production in taxonomically related bacteria isolated from *Alexandrium* spp. [60]. The absence of autonomous PST production by any of our bacterial isolates, together with reported Mendelian inheritance of PST profiles by *Alexandrium* spp. [61,62], and *G. catenatum* ([63]; D. Green, C. Bolch and A. Negri, unpublished data), suggests that PST biosynthesis is associated with the dinoflagellate cell, not its associated bacteria.

While a direct role for bacteria in PST production by *G. catenatum* seems highly unlikely, previous work shows a strong correlation between *G. catenatum* cultures that produce low amounts of PST with cultures generated from cysts germinated under laboratory conditions. We hypothesised that specific bacterial types may be removed during sterile washing of the cysts, and that the resulting low PST production levels were mediated through an altered physiological or nutrient status of the algal cells, in the absence of particular bacterial types [21].

Our observations suggest that the bacterial flora may influence the biosynthesis of the PSTs. A number of explanations are possible, for example: a threshold number or critical consortium of Proteobacteria may be required to induce/promote toxin production by *G. catenatum*,

through for example, the supply of sufficient cofactors or precursors necessary for toxin production [11]; or a specific Proteobacteria may be required to trigger post-germination resumption of 'normal' PST production of *G. catenatum*; and alternatively, a particular bacterium (or community mix) may 'switch-off' PST biosynthesis in some of our *G. catenatum* strains.

4.3. Conclusions

This study has shown that the cultivable bacterial flora of each *G. catenatum* strain is unique at the level of SSU rDNA sequence, yet each of the bacterial communities bares a number of similarities at both the phylogenetic and phenotypic levels, supporting reported studies with other dinoflagellates. Bacteria affiliated to the Rhodobacteraceae and Alteromonadaceae were a consistent feature of *G. catenatum* and other dinoflagellates such as *Alexandrium* spp., and these bacteria may have an important role in the growth of dinoflagellates in laboratory culture and natural bloom populations. The lack of PST/PST-like toxin production by bacteria supports the idea that they do not have a direct role in PST production by *G. catenatum*, however, the reduced bacterial diversity associated with atypical PST-producing *G. catenatum* strains suggests that key bacteria (or consortia of bacteria) are important for the induction of PST production by *G. catenatum* following excystment. Overall, we believe that this study points to a number of bacterial groups that are key to the growth and physiology of *G. catenatum* and that their activity may be fundamental in the development and maintenance of natural blooms. Further work is now underway to characterise their individual and combined influence on the growth and toxicity of *G. catenatum*, and elucidate the mechanisms by with these effects are mediated.

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